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Loss of the Muscle-Specific Chloride Channel in Type 1 Myotonic Dystrophy Due to Misregulated Alternative Splicing

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Summary

Myotonic dystrophy type 1 (DM1) is a dominant multisystemic disorder caused by a CTG expansion in the 3′ untranslated region of the *DMPK* gene. A predominant characteristic of DM1 is myotonia resulting from skeletal muscle membrane hyperexcitability. Here we demonstrate loss of the muscle-specific chloride channel (ClC-1) mRNA and protein in DM1 skeletal muscle tissue due to aberrant splicing of the ClC-1 pre-mRNA. The splicing regulator, CUG binding protein (CUG-BP), which is elevated in DM1 striated muscle, binds to the ClC-1 pre-mRNA, and overexpression of CUG-BP in normal cells reproduces the aberrant pattern of ClC-1 splicing observed in DM1 skeletal muscle. We propose that disruption of alternative splicing regulation causes a predominant pathological feature of DM1.

Introduction

Myotonic dystrophy (DM) is the most common form of adult onset-muscular dystrophy. While skeletal muscle symptoms of hyperexcitability (myotonia) and progressive muscle wasting are characteristic, other common findings include insulin resistance, cardiac conduction defects, ocular cataracts, smooth muscle dysfunction, testicular atrophy, and neuropsychiatric disturbances (Harper, 2001). The most common form of myotonic dystrophy is type 1 (DM1), which is caused by expansion of an unstable CTG triplet repeat in the 3′ untranslated region of the *DMPK* gene located on chromosome 19q13.3 (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992). Unaffected individuals have fewer than 40 repeats while repeat sizes in affected individuals range from ~80 into the thousands. Disease severity and age of onset correlate with repeat length (Harper, 2001).

The expanded *DMPK* allele is transcribed, producing RNA transcripts containing long tracts of CUG repeats ($RNA^{CUG)n}$) that accumulate in nuclear foci (Davis et al., 1997; Taneja et al., 1995). DMPK mRNA from the expanded allele is therefore unavailable for translation, resulting in reduced expression of DMPK protein, at least in adult DM1 (Fu et al., 1993). The close proximity of the CTG expansion to the *SIX5* promoter results in transcriptional repression of *SIX5* as well (Klesert et al., 1997; Thornton et al., 1997). However, several lines of evidence indicate that a gain of function for $RNA^{CUG)n}$, rather than haploinsufficiency of *DMPK* and/or *SIX5*, plays the prominent role in DM1 pathogenesis. First, only CTG expansions, and no DMPK or SIX5 loss-of-function mutations, have been identified among DM1 patients. Furthermore, DMPK- and SIX5-deficient mice display only limited similarities to DM1 patients (Berul et al., 1999; Jansen et al., 1996; Klesert et al., 2000; Reddy et al., 1996; Sarkar et al., 2000). Second, muscle-specific expression of 250 CUG repeats in HSA^{LR} transgenic mice reproduces myotonia and myopathy characteristic of DM1 (Mankodi et al., 2000). Third, DM type 2 (DM2), a disease that is phenotypically remarkably similar to DM1, is caused by a large CCTG expansion in intron 1 of the *ZNF9* gene on chromosome 3q21 (Liquori et al., 2001). As with the expanded allele in DM1, transcripts from the HSA^{LR} transgene and the expanded *ZNF9* allele accumulate in nuclear foci (Liquori et al., 2001; Mankodi et al., 2000). The subtle phenotypic differences between DM1 and DM2 could be due to haploinsufficiency of either *DMPK* and/or *SIX5* (Tapscott and Thornton, 2001). However, the results from DM2 patients and HSA^{LR} mice directly demonstrate that $RNA^{CUG)n}$ and $RNA^{CCUG)n}$ have toxic effects independent of the *DMPK* locus.

The mechanism for an RNA gain of function is thought to involve a *trans*-dominant effect on the function of proteins that bind $RNA^{CUG)n}$, such as CUG binding protein (CUG-BP), elav-like RNA binding protein 3 (ETR-3), muscleblind (MBNL), and PKR (Lu et al., 1999; Miller et al., 2000; Timchenko et al., 1996). CUG-BP and ETR-3 are members of the CELF family of proteins that regulate alternative splicing of specific pre-mRNAs by binding to conserved intronic elements containing U/G-rich motifs (Charlet-B. et al., 2002; Ladd et al., 2001). CUG-BP protein levels are increased in DM1 striated muscle (Savkur et al., 2001; Timchenko et al., 2001). The exact mechanism by which expression of $RNA^{CUG)n}$ induces CUG-BP overexpression is not clear.

Two pre-mRNA targets of CUG-BP regulation, cardiac troponin T (cTNT) and insulin receptor (IR), undergo aberrantly regulated alternative splicing in DM1 striated muscle (Philips et al., 1998; Savkur et al., 2001). Furthermore, the inappropriate splicing patterns of both cTNT and IR are recapitulated in normal cells by overexpression of CUG-BP (Philips et al., 1998; Savkur et al., 2001), strongly suggesting that aberrant regulation of alternative splicing in DM1 is a direct consequence of CUG-BP overexpression. The subsequent inappropriate expression of the nonmuscle IR isoform in adult skeletal muscle directly correlates with decreased insulin responsiveness and the unusual form of insulin resistance observed in DM1 patients.

A predominate and early symptom of DM1 is myotonia, manifested as delayed skeletal muscle relaxation following voluntary contraction (Harper, 2001). Myotonia is due to hyperexcitability of muscle fibers leading to

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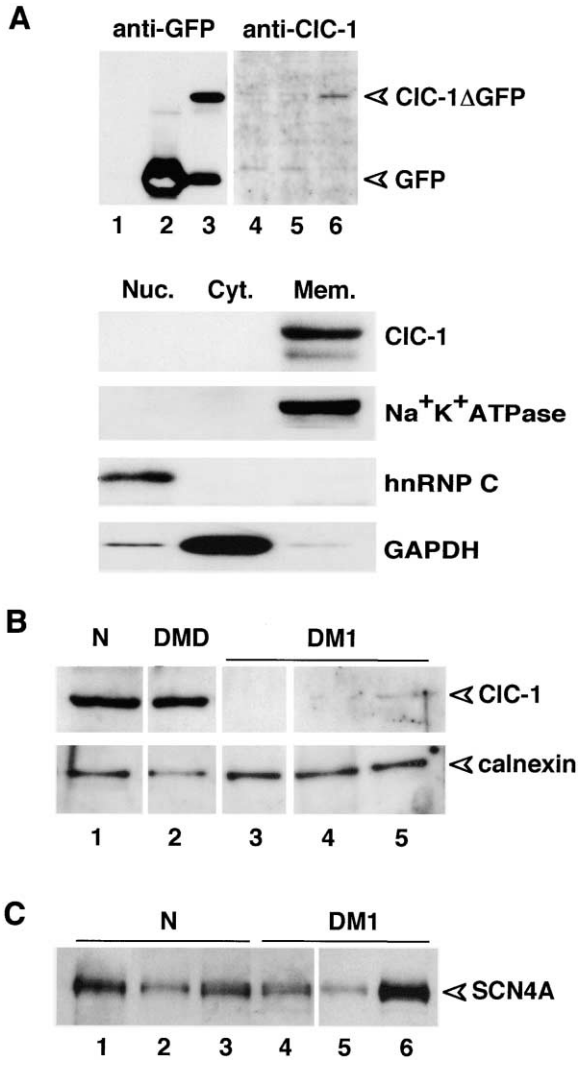

Figure 1. ClC-1 Protein Is Deficient in DM1 Muscle Membranes

(A) Specificity of the anti-ClC-1 antibody. Western blots of whole-cell protein extracts containing transiently expressed ClC-1ΔGFP fusion protein in which the N-terminal 151 amino acids of ClC-1 are fused to the N terminus of GFP (upper panel). Each lane contains 10 μg protein (untransfected, lanes 1 and 4; GFP, lanes 2 and 5; ClC-1ΔGFP, lanes 3 and 6). Anti-GFP antibody (Clontech) was used to demonstrate expression of the ClC-1ΔGFP fusion protein (46 kDa) (lane 3). Detection of the ClC-1ΔGFP fusion protein using the anti-ClC-1 antibody was specific to transfected cultures (lane 6). The anti-ClC-1 antibody detected the endogenous protein (130 kDa) only in membrane fractions prepared from normal human skeletal muscle tissue (lower panel). All lanes contain 10 μg protein. None of the bands was detected by preimmune serum (data not shown). Western blots for hnRNP C (nuclear), GAPDH (cytoplasmic), and Na^+K^+ATPase (membrane) demonstrate the relative purity of subcellular fractions.

(B) ClC-1 protein is reduced or absent in DM1 skeletal muscle membranes. Western blot analysis of ClC-1 in membrane fractions of normal (N), Duchenne's muscular dystrophy (DMD), and myotonic dystrophy (DM1) tissue samples. DMD is characterized by muscle wasting without myotonia. An identical blot was probed in parallel with anti-calnexin antibodies (StressGen).

(C) SCN4A protein levels are unaffected in DM1. Membrane protein extracts were probed with an antibody that recognizes multiple sodium channel protein isoforms (pan sodium channel, SIGMA). SCN4A is the only sodium channel expressed in skeletal muscle (Goldin, 1999). Each lane contains 10 μg protein.

repetitive action potentials producing involuntary after-contractions (Lehmann-Horn and Jurkat-Rott, 1999). Myotonia can be caused by a loss of function of the muscle-specific chloride channel (ClC-1) in humans and animal models. Mutations in the muscle-specific sodium channel (SCN4A) gene also cause less common forms of myotonic syndromes generally associated with potassium sensitivity (Lehmann-Horn and Jurkat-Rott, 1999). Defects in both chloride and sodium conductance in DM1 skeletal muscle have been observed (Franke et al., 1990; Lipicky, 1977). However, studies of ion channel function in DM1 have been inconclusive, and the physiological basis of myotonia in this disease remains controversial.

Here we demonstrate a loss of ClC-1 protein in DM1 skeletal muscle membrane that is sufficient to account for myotonia. The majority of ClC-1 mRNAs detected in DM1 skeletal muscle by RT-PCR contain premature termination codons due to retention of intron 2 or inclusion of two novel exons between exons 6 and 7. These mRNAs are not detected by RNase protection and are presumably degraded by nonsense-mediated decay. In the accompanying paper, Mankodi and colleagues describe loss of ClC-1 protein expression and reduced chloride conductance due to similar patterns of misregulated splicing of ClC-1 mRNAs in the *HSA*^{LR} mouse model for DM1 (Mankodi et al., 2002 [this issue of *Molecular Cell*]). We also show that CUG-BP induces retention of intron 2 by binding to a U/G-rich motif common to other pre-mRNA targets of CUG-BP. We conclude that the primary cause of myotonia in DM1 is loss of ClC-1 due to inappropriate regulation of ClC-1 alternative splicing by increased CUG-BP activity.

Results

ClC-1 Protein Is Reduced in Skeletal Muscle Tissue from DM1 Patients

To determine the level of ClC-1 protein in DM1 muscle tissue, we generated a rabbit polyclonal antibody to an N-terminal peptide from human ClC-1. The specificity of the anti-ClC-1 antibody was demonstrated using a ClC-1-GFP fusion protein (ClC-1ΔGFP) in which the N-terminal 151 amino acids of ClC-1 were fused to the N terminus of GFP. ClC-1ΔGFP was transiently expressed, and expression of the fusion protein was confirmed in whole-cell protein extracts using antibodies to GFP (Figure 1A, lane 3). ClC-1ΔGFP was detected using the anti-ClC-1 antibody (Figure 1A, lane 6). The anti-ClC-1 antibody also detected endogenous ClC-1 protein. Subcellular fractions prepared from normal human skeletal muscle tissue were probed with the ClC-1 antibody, which detected a band of the expected size only in the membrane fraction (Figure 1A). The integrity of all three subcellular fractions was verified by probing blots with antibodies to GAPDH (cytoplasmic), Na^+K^+ATPase (membrane), and hnRNP C (nuclear) (Figure 1A).

Membrane proteins from normal, Duchenne muscular dystrophy (DMD), and DM1 skeletal muscle tissues were assayed for ClC-1 protein on Western blots. ClC-1 protein was detected in normal and DMD skeletal muscle membrane fractions (Figure 1B, lanes 1 and 2), but was undetectable (lanes 3 and 4) or greatly reduced (lane 5)

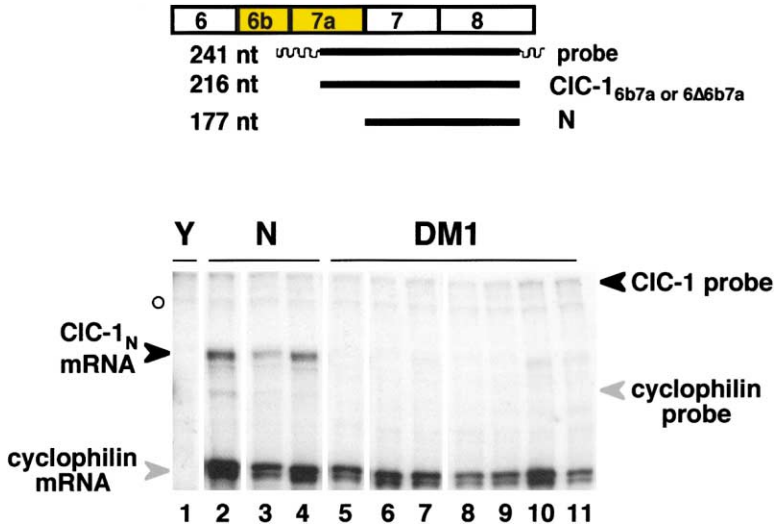

Figure 2. RNase Protection Analysis of ClC-1 mRNA in Normal (N) and DM1 Skeletal Muscle

RNase protection probes for ClC-1 and cyclophilin are included in the same reaction. The ClC-1 probe is 241 nt, and the correctly spliced ClC-1 mRNA (ClC-1_N) protects 177 nt. The position of the expected protection fragment (216 nt) for the novel splice variants ClC-1_{6b7a} or ClC-$1_{6\Delta6b7a}$ (see Figure 3) is indicated by an open circle. The novel splice variants that introduce premature termination codons could not be detected in multiple assays and are probably degraded by nonsense-mediated decay. The cyclophilin probe is 151 nt and it is protected by the first 127 nt of the open reading frame. Both probes are completely digested in the presence of yeast total RNA (Y).

in DM1 skeletal muscle membrane fractions. The membrane-associated protein, calnexin, was detected in all of the same samples run on a parallel blot, demonstrating the integrity of the proteins in the samples. SCN4A was also detected in skeletal muscle membrane fractions of all DM1 patients tested (Figure 1C, and data not shown). Of particular interest, SCN4A was detected in two membrane fraction preparations from DM1 patients in which ClC-1 protein was undetectable, further demonstrating the integrity of these membrane protein preparations (Figure 1B, lanes 3 and 4, and Figure 1C, lanes 5 and 6, respectively). We conclude that ClC-1 protein is absent or greatly reduced in DM1 skeletal muscle tissue, whereas SCN4A protein levels are not reduced in DM1 compared to normal skeletal muscle.

We next quantified the steady-state levels of the ClC-1 mRNA in DM1 skeletal muscle tissue, using an RNase protection assay. A probe for cyclophilin was included as an internal control for RNA integrity. In all of the seven DM1 skeletal muscle tissue samples tested, the ClC-1 mRNA was either undetectable (Figure 2, lanes 5, 6, 8, and 9) or less than 5% of the levels detected in normal tissue (Figure 2, lanes 7, 10, and 11). The signal for ClC-1_N mRNA was 15.3% ($\pm$1.5%) that of cyclophilin in normal skeletal muscle and $\leq$0.7% that of cyclophilin in DM1 skeletal muscle. The ClC-1 probe was designed to detect correctly spliced ClC-1 mRNA as well as the novel splice forms containing premature termination codons described below. Multiple assays failed to detect the novel splice forms in DM1 muscle tissue (Figure 2, and data not shown). The loss of ClC-1 mRNA and protein demonstrated above is comparable to those described for mutations in the *CLCN1* gene shown to cause myotonia in humans, dogs, goats, and mice (Beck et al., 1996; Cannon, 1996; Gurnett et al., 1995; Rhodes et al., 1999; Steinmeyer et al., 1991). We propose that the ClC-1 deficiency demonstrated here is sufficient to explain the myotonia observed in DM1 patients.

Aberrantly Spliced ClC-1 mRNAs Containing Premature Termination Codons Predominate in DM1 Skeletal Muscle Tissue

We hypothesized that the ClC-1 pre-mRNA could be affected by aberrant regulation of alternative splicing as previously demonstrated in DM1 striated muscle for cTNT and the insulin receptor (Philips et al., 1998; Savkur et al., 2001). A search of nucleotide databases for evidence of ClC-1 splice variants identified a mouse EST containing an additional exon between exons 6 and 7 (GenBank AJ011105). To determine whether this region of the human ClC-1 mRNA is aberrantly spliced in DM1, we used RT-PCR to amplify between exons 4 and 8 in RNA from normal and DM1 skeletal muscle tissue. This analysis identified three PCR products in addition to the expected product from correctly spliced mRNA (Figures 3A and 3B). Sequence analysis of the cloned PCR products revealed that two ClC-1 mRNAs contain an insert of 134 nt immediately preceding exon 7 (ClC-1_{6b7a} and ClC-$1_{6\Delta6b7a}$, Figure 3A); one of these RNAs (ClC-$1_{6\Delta6b7a}$) lacks exon 6. The third aberrant mRNA resulted from splicing of exon 5 to exon 8 (ClC-$1_{5\text{-}8}$; Figure 3A). Sequence comparisons of the PCR products to the human *CLCN1* gene (GenBank AC073342) revealed that the 134 nt insert corresponds to two previously unreported exons located between exons 6 and 7. These exons are designated as exons 6b and 7a. The ratio of RT-PCR products suggested that very little of the normal ClC-1 mRNA was produced in DM1 skeletal muscle tissue (Figure 3B). All three of the novel splice products contain premature termination codons that prevent expression of full-length ClC-1 protein. mRNAs containing premature termination codons are typically degraded by nonsense-mediated decay (Hentze and Kulozik, 1999), and this is likely to be the basis for the ClC-1 protein and mRNA deficiencies demonstrated above. Indeed, evidence that ClC-1 mRNA is subject to nonsense-mediated decay comes from a case of familial myotonia in which an extra nucleotide in exon 7 results in a premature termination codon and undetectable mRNA (Nagamitsu et al., 2000).

Results described in the companion paper (Mankodi et al., 2002) indicated that a fraction of ClC-1 mRNA from *HSA*^{LR} skeletal muscle retained intron 2. Based on this information, we performed RT-PCR analysis of the 5′ region of the ClC-1 mRNA and determined that intron 2 was also retained in DM1 patient skeletal muscle (Figure 3C). This aberrant splice was not detected in any of

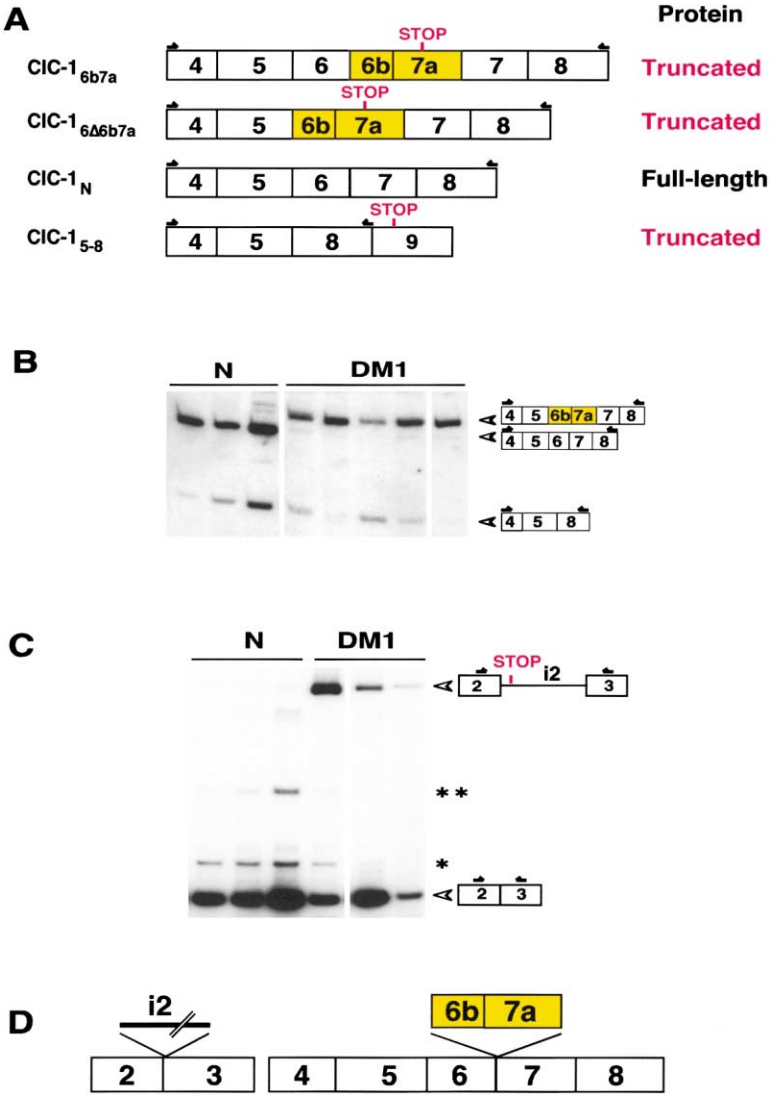

Figure 3. Novel Variant Splice Forms of ClC-1 mRNA Predominate in DM1 Skeletal Muscle

(A) Diagram of ClC-1 mRNA RT-PCR products obtained from a DM1 patient's skeletal muscle tissue. Previously unknown exons are in yellow. Positions of premature termination codons are indicated. ClC-1_{6b7a}, ClC-$1_{6\Delta6b7a}$, and ClC-$1_{5\text{-}8}$ are GenBank accession numbers AY103154, AY103155, and AY103156, respectively.

(B) RT-PCR analysis of total RNA from normal and DM1 skeletal muscle tissue. The ClC-1_{6b7a} PCR product is less than 8% of the total and was not detected in this experiment.

(C) RT-PCR analysis as in (B) using oligonucleotides complementary to exons 2 and 3. Minor splice products are generated by use of a cryptic 5′ splice site (*) and inclusion of an ectopic exon within intron 2 (**). Note that the smaller RT-PCR product from the correctly spliced mRNA is overrepresented due to the preferential amplification.

(D) Summary of aberrant splicing identified in the five intron positions analyzed.

the four normal skeletal muscle samples tested (Figure 3C, and data not shown). Overall, RT-PCR analysis across five intron positions within the ClC-1 mRNA demonstrated two positions of aberrant splicing (Figure 3D). Aberrant splicing at either position results in the introduction of premature termination codons.

Mankodi et al. (2002) report that *HSA*^{LR} mice exhibit aberrant splicing events in ClC-1 mRNA that are strikingly similar to what we find in DM1 patients. Furthermore, transmembrane chloride conductance was reduced to levels sufficient to cause myotonia (Mankodi et al., 2002). These results strongly support the contentions that: (1) loss of ClC-1 function causes the myotonia observed in both the *HSA*^{LR} mice and DM1 skeletal muscle and that (2) RNA$^{(CUG)n}$ induces aberrant splicing of the ClC-1 pre-mRNA and subsequent loss of ClC-1 mRNA. We next addressed the mechanism of the aberrantly regulated ClC-1 alternative splicing.

Aberrant Inclusion of Intron 2 Is Reproduced in Normal Cells by Overexpression of CUG-BP

We have previously demonstrated that the steady-state levels of the splicing regulator CUG-BP are elevated in skeletal muscle tissue from DM1 patients (Savkur et al., 2001). For both cTNT and the IR pre-mRNAs, CUG-BP binds to specific intronic RNA elements in vitro and reproduces the aberrant splicing patterns observed in DM1 patients when overexpressed in normal cells (Philips et al., 1998; Savkur et al., 2001). These results strongly suggest that overexpression of CUG-BP in DM1 muscle induces the aberrant regulation of specific pre-mRNA targets. To investigate the role of CUG-BP in mediating the aberrant splicing of the ClC-1 pre-mRNA, we coexpressed a ClC-1 intron 2 minigene with a CUG-BP expression plasmid. Splicing of intron 2 was quantified by RNase protection. A low basal level of intron 2 retention was observed in cells transfected with the minigene alone (Figure 4A, lane 2). Coexpression of CUG-BP with the minigene strongly induced retention of intron 2 (Figure 4A, lane 3), reproducing the aberrant splicing pattern observed in DM1 patients and in *HSA*^{LR} mice.

To determine whether ClC-1 intron 2 is a direct target for CUG-BP regulation, we searched for CUG-BP binding sites in intron 2. This was done by a UV crosslinking assay using bacterially expressed GST-CUG-BP and 11

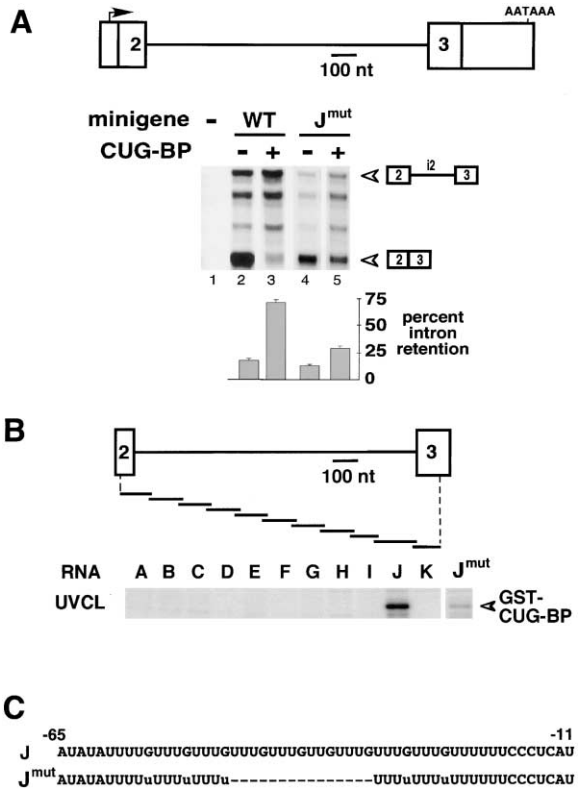

Figure 4. Regulation of ClC-1 Intron 2 by CUG-BP

(A) ClC-1 intron 2 retention is induced by CUG-BP. A ClC-1 intron 2 minigene was expressed with or without CUG-BP in QT35 fibroblasts. Both spliced (159 nt) and unspliced (218 nt) RNAs were detected by an RNase protection assay using a 243 nt uniformly ^{32}P-labeled probe. Additional bands were generated by use of cryptic splice sites. Coexpression of CUG-BP induced intron retention but did not alter the relative ratios of the cryptic splice products. Expression of equivalent levels of CUG-BP in lanes 3 and 5 was confirmed by Western blot analysis (data not shown). The bar graph shows the mean ± standard deviation from three independent transfections.

(B) CUG-BP binds U/G motifs in the 3′ splice site of intron 2. The relative positions of RNAs A through K on ClC-1 intron 2 are indicated on the diagram. UV crosslinking assays included 300 ng of purified recombinant GST-CUG-BP in the presence of 15 μg HeLa nuclear extract to reduce nonspecific binding.

(C) Sequence of wild-type (J) and mutant (J^{mut}) CUG-BP binding sites in intron 2. Deletions are indicated by dashes, and substitutions are indicated in lower case. Numbers indicate positions relative to exon 3.

overlapping uniformly labeled in vitro transcribed RNAs spanning all of intron 2 and portions of exons 2 and 3 (Figure 4B). CUG-BP bound to only one RNA (RNA "J"), which contained the 3′ splice site of intron 2 (Figure 4B). This 3′ splice site is unusual as it contains nine copies of the motif, UGU (Figure 4C), which matches a preferred binding site for CUG-BP (Takahashi et al., 2000) as well as the closely related CELF protein, ETR-3 (Charlet-B. et al., 2002). Mutation of the U/G motifs greatly reduced CUG-BP binding to an RNA that was otherwise identical to RNA "J" (RNA J^{mut}; Figures 4B and 4C).

The J^{mut} mutation had little effect on the basal level of intron 2 retention in the intron 2 minigene (Figure 4A, compare lanes 2 and 4); however, the mutation greatly reduced induction of intron 2 retention by CUG-BP (lane 5). The fact that inclusion of the mutated intron 2 is weakly induced is consistent with the residual binding of CUG-BP to RNA J^{mut} (Figure 4B). Therefore, CUG-BP overexpression induces retention of intron 2 in the ClC-1 pre-mRNA via a U/G-rich binding site in the intron's 3′ splice site. Recombinant CUG-BP also binds to a U/G-rich region downstream of exon 6b (data not shown), and we propose that CUG-BP also regulates splicing of ClC-1 exons 6b and 7a.

We conclude that, like cTNT and IR, ClC-1 alternative splicing is inappropriately altered by the elevated levels of CUG-BP expressed in DM1 skeletal muscle.

Discussion

ClC-1 is the predominant chloride channel of adult skeletal muscle. ClC-1 mutations cause inherited myotonias in humans and other mammals, demonstrating that ClC-1 is essential for electrical stability of the skeletal muscle membrane (Jentsch et al., 1999). Here we demonstrate that ClC-1 mRNA and protein levels are decreased or undetectable in skeletal muscle of individuals with DM1. RT-PCR detected predominantly aberrantly spliced ClC-1 mRNAs containing premature stop codons due to retention of ClC-1 intron 2 and inclusion of two novel exons located between exons 6 and 7. The low abundance of these mRNAs is most likely due to degradation by the nonsense-mediated decay pathway. Retention of intron 2 is reproduced in normal cells by CUG-BP, and sequence-specific binding of CUG-BP to a U/G-rich motif within the 3′ splice site of intron 2 is required for this response. The aberrant splicing pattern of ClC-1 observed in DM1 skeletal muscle tissue can be induced by CUG-BP, consistent with recent reports that CUG-BP is elevated in striated muscle from DM1 patients (Savkur et al., 2001; Timchenko et al., 2001). We conclude from these results that: (1) the primary cause of myotonia in myotonic dystrophy is loss of ClC-1 function, (2) the absence of ClC-1 protein results from aberrant regulation of ClC-1 pre-mRNA alternative splicing, and (3) misregulated splicing is due to the elevated levels of CUG-BP expressed in DM1 skeletal muscle.

The Basis for Myotonia in Myotonic Dystrophy

Myotonia is a characteristic feature of DM1, yet the basis for myotonia has previously remained obscure. A variety of physiological abnormalities have been reported for DM1 muscle membranes. Electrophysiological studies of muscle fibers from DM1 patients have demonstrated variable reduction of chloride conductance as well as increased frequency of sodium channel reopening (Franke et al., 1990; Lipicky, 1977). Both abnormalities could contribute to membrane hyperexcitability in DM1. However, sodium channel late openings like those observed in DM1 patients have also been observed in human and animal myotonias caused by loss-of-function mutations in the *CLCN1* gene (Bryant and DeCoursey, 1980; Franke et al., 1991; Iaizzo et al., 1991; Rudel et al., 1989). These findings suggest that sodium current alterations detected in DM1 are likely to be secondary to reductions in chloride conductance. Furthermore, SCN4A protein levels do not differ between DM1 and

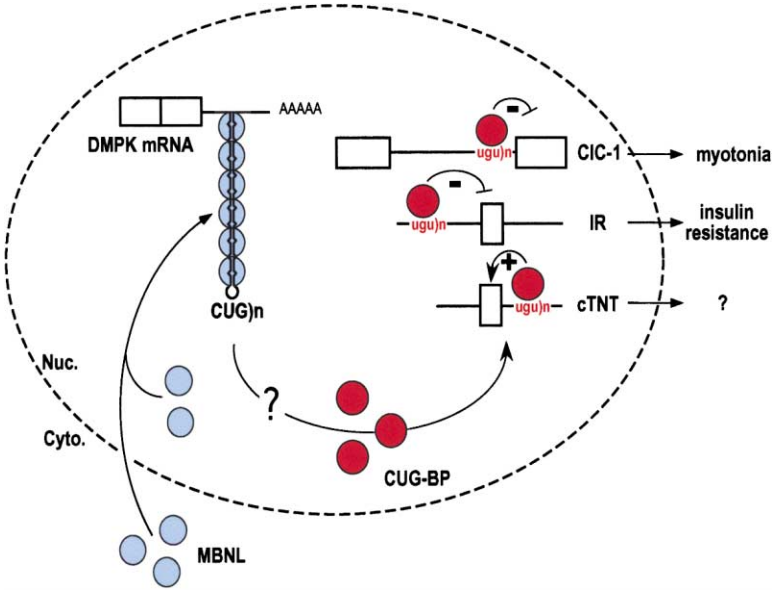

Figure 5. A Model for the RNA Gain of Function in DM1 Striated Muscle

$RNA^{CUG)n}$ accumulates in nuclear foci and recruits double-stranded CUG binding proteins such as muscleblind (MBNL). Nuclear accumulation of $RNA^{CUG)n}$ results in elevated CUG-BP activity by an unknown mechanism. Binding of CUG-BP to U/G motifs within target splicing elements induces splicing patterns that are inappropriate for adult striated muscle. Insertion of premature termination codons in the ClC-1 mRNAs leads to a loss of ClC-1 protein and myotonia in DM1 patients. Skipping of insulin receptor exon 11 results in the expression of the lower signaling isoform, contributing to the insulin resistance observed in DM1 patients. Deleterious effects due to aberrant inclusion of cTNT exon 5 remain to be determined.

normal skeletal muscle membrane preparations (Figure 1C).

Expression of small conductance calcium-activated potassium (SK) channels is elevated in skeletal muscle of DM1 patients (Kimura et al., 2000; Renaud et al., 1986). Inhibition of these channels with apamin, injected into the thenar muscle of DM1 patients, resulted in decreased myotonic discharges, supporting a proposal that increased SK channel expression contributes to myotonia (Behrens et al., 1994). Denervated muscle also displays hyperexcitability, and it has been proposed that increased expression of SK channels in the T tubular system of these cells causes a local increase in potassium concentration leading to hyperexcitability (Neelands et al., 2001). As the membrane resting potential of skeletal muscle is primarily maintained by chloride and not potassium conductance, functional ClC-1 channels are likely to inhibit the depolarization that would result from increased potassium conductance. Given that there is no demonstration of myotonia resulting directly from abnormal potassium conductance, it is probable that myotonia triggered by potassium accumulation requires that chloride conductance is reduced or absent. Consistent with this proposal, chloride channel mRNA expression is reduced in denervated muscle (Klocke et al., 1994; Rich et al., 1999). We propose that the ClC-1 deficiency demonstrated here is the primary cause of the myotonia observed in DM1 patients. This does not rule out additional contributions from sodium and potassium conduction abnormalities.

A recent report using RT-PCR analysis described finding similar levels of ClC-1 mRNA in normal and DM1 skeletal muscle samples (Kimura et al., 2000). Based on a direct comparison of RT-PCR and RNase protection analysis using known amounts of in vitro transcribed RNA (data not shown), we believe that direct analysis of mRNA levels by RNase protection is more accurate for quantitation of low-abundance mRNAs.

The striking correlations between our results and those described in the companion paper (Mankodi et al., 2002) provide particularly strong support for the primary role of ClC-1 deficiency in the myotonia of DM1.

The Mechanism of an RNA Gain of Function for $RNA^{CUG)n}$

The results presented here and in a recent report (Savkur et al., 2001) indicate that two predominant clinical features of DM1, myotonia and insulin resistance, result from misregulated alternative splicing of the ClC-1 and IR pre-mRNAs, respectively. Our working model (Figure 5) is that nuclear accumulation of $RNA^{CUG)n}$ leads to increased CUG-BP activity, which induces inappropriate alternative splicing of its pre-mRNA targets. ClC-1, IR, and a third pre-mRNA that is aberrantly regulated in DM1 heart muscle, cTNT, are likely to be natural targets of CUG-BP. CUG-BP binds to specific intronic sites in each of these three pre-mRNAs and induces different splicing patterns (cTNT exon 5 inclusion, IR exon 11 skipping, and ClC-1 intron 2 retention). For all three pre-mRNAs, the splicing patterns induced by CUG-BP match the aberrant patterns observed for the endogenous pre-mRNAs in DM1 striated muscle tissue. Furthermore, aberrant splicing of cTNT in DM1 cells requires the CUG-BP binding site, indicating that aberrant splicing is due to increased activity of CUG-BP (Philips et al., 1998). This aberrant regulation ultimately produces symptoms by the loss of mRNA due to nonsense-mediated decay (ClC-1) or expression of protein isoforms that are inappropriate for mature striated muscle (cTNT and IR).

The most likely explanation for increased CUG-BP activity is the increase in CUG-BP steady-state levels recently demonstrated in DM1 skeletal (Savkur et al., 2001) and heart muscle tissue (Timchenko et al., 2001). It is unclear how nuclear accumulation of $RNA^{CUG)n}$ results in increased CUG-BP steady-state levels. Transient expression of $RNA^{CUG)n}$ in cultured cells increases the half-life of endogenous CUG-BP protein (Timchenko et al., 2001), but the half-life of CUG-BP in DM1 cells remains to be examined. Alterations of phosphorylation and nuclear:cytoplasmic distribution of CUG-BP phos-

phoisoforms in DM1 cells have also been described (Roberts et al., 1997); however, specific roles for these changes in aberrant splicing regulation remain to be established. We predict that other CELF family members are also involved in DM1 pathogenesis since some CELF family members are nearly identical to CUG-BP with regard to binding specificity and splicing activity (Ladd et al., 2001; and data not shown).

$RNA^{CUG)n}$ repeats longer than ~20 repeats form double-stranded RNA containing U-U mismatches (Michalowski et al., 1999; Napieraa and Krzyosiak, 1997) and bind dsRNA binding proteins (Miller et al., 2000; Tian et al., 2000). One of these, muscleblind (MBNL), exhibits striking sequence-specific binding to double-stranded CUG repeats. Endogenous and exogenously expressed MBNL colocalizes with expanded repeats in nuclear foci in DM1 cultured cells and skeletal muscle tissue (Fardaei et al., 2001; Mankodi et al., 2001; Miller et al., 2000). In contrast to MBNL, CUG-BP does not bind double-stranded $RNA^{CUG)n}$ and does not colocalize with $RNA^{CUG)n}$ (Fardaei et al., 2001; Michalowski et al., 1999). However, expression of $RNA^{CUG)n}$ in COS cells causes a shift in CUG-BP to a high molecular weight form that cofractionates with the repeat RNA by gel filtration chromatography (Timchenko et al., 2001). Therefore, it is currently unclear whether increased CUG-BP is a consequence of direct interactions with $RNA^{CUG)n}$, sequestration of MBNL on $RNA^{CUG)n}$, or an alternative pathway yet to be discovered.

We mapped the intron 2 binding site for CUG-BP to U/G-rich motifs just upstream of the ClC-1 exon 3. Binding of CUG-BP to this site is required for induction of intron 2 retention, demonstrating the functional relevance of CUG-BP binding. Similar U/G-rich motifs flanking cTNT exon 5 and IR exon 11 are required for induction of the aberrant splicing patterns by CUG-BP (Philips et al., 1998; Savkur et al., 2001). U/G repeats were the preferred binding sequence of CUG-BP in a yeast three-hybrid assay (Takahashi et al., 2000), consistent with the natural binding sites identified in these three pre-mRNAs. Tracts of U/G repeats have been described as positive or negative *cis* regulators of alternative splicing (Buratti et al., 2001; Gabellini, 2001). We predict that exons associated with U/G motifs are potential targets of the CUG-BP or other CELF protein family members that could be misregulated in DM1, contributing to other aspects of the DM phenotype.

Accumulating evidence indicates that CELF proteins regulate alternative splicing during development (Ladd et al., 2001; Zhang et al., 1999). Our results suggest that $RNA^{CUG)n}$ induces reiteration of embryonic splicing patterns mediated by CELF proteins. cTNT and IR revert to an embryonic or nonmuscle splicing pattern in adult DM1 muscle by a mechanism that is distinct from the regenerative changes associated with satellite cell proliferation (Savkur et al., 2001). Inclusion of ClC-1 exon 7a is also an embryonic splicing pattern in wild-type mice (the mouse gene does not contain an exon homologous to 6b) (Mankodi et al., 2002), suggesting that inclusion of exon 7a in HSA^{LR} mice and exons 6b and 7a in DM1 patients also reflects reiteration of an embryonic splicing pattern. It is unclear whether intron 2 retention represents aberrant regulation of a natural alternative splice or an aberrant splice induced by fortuitous binding of CUG-BP to the 3′ splice site of intron 2. ClC-1 mRNA levels increase rapidly during postnatal development (Steinmeyer et al., 1991). If the aberrant splicing patterns observed in DM1 patients reflect reiteration of an embryonic program, the developmentally regulated induction of ClC-1 could be due to an alternative splicing switch to in-frame RNAs.

Experimental Procedures

Tissue Samples

All DM skeletal muscle biopsy and autopsy samples were from adult-onset DM1 patients. The diagnosis of DM1 was confirmed by repeat lengths and histological and clinical examinations.

Membrane Fractions and Western Blot Analysis

Autopsy skeletal muscle tissues were pulverized in liquid nitrogen and then homogenized in a Dounce homogenizer in cold lysis buffer (20 mM HEPES [pH 7.5], 10 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$, 0.5 mM DTT). The soluble fraction was passed through a 26-gauge needle eight times using a 1 ml syringe. Nuclear:cytoplasmic separations were performed by centrifugation twice at 600 × g for 5 min. The membrane fraction was prepared by centrifuging the cytoplasmic fraction 15 min at 20,000 × g. The pellet was washed in lysis buffer containing 600 mM KCl, 0.1% NP40, then recentrifuged 15 min at 20,000 × g, and solubilized in 40 μl of protein loading buffer. Fractions were separated by SDS-PAGE on an 8% gel and Western blot analysis was performed as described (Ladd et al., 2001). Sodium channel Westerns were performed using a monoclonal anti-sodium channel pan antibody (1:1000) (Sigma, St. Louis, MO). Anti-hnRNP C was provided by G. Dreyfuss (University of Pennsylvania). Anti-GAPDH and anti-Na^+K^+ATPase were from Biogenesis (Kingston, NH) and Sigma, respectively. Anti-peptide antibodies against ClC-1 were generated in rabbits against the N-terminal 14 amino acid peptide MEQSRSQQRGGEQS(C) that was conjugated to keyhole limpet hemocyanin by an additional cysteine (Anaspec, San Jose, CA). For Westerns, the ClC-1 primary antibody was used at 1:1,000 and the secondary anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) at 1:20,000. Both were incubated in PBS containing 0.1% Tween 20, 5% nonfat milk, 0.08% SDS.

RNase Protection Assay

The probe used to quantify endogenous ClC-1 mRNA spanned 241 nt, consisting of 39 nt of exon 7a, 79 nt of exon 7, 98 nt of exon 8, and 25 nt of polylinker sequence. The cyclophilin probe was 151 nt long and is protected by the first 127 nt of the open reading frame. The 243 nt probe used to study the intron 2 minigene was obtained by PCR and consisted of 159 nt of exon 2 and backbone vector sequence, 59 nt of the 5′ end of intron 2, and 25 nt of noncomplementary sequence. Hybridization reactions were performed using 7–8 μg of total cellular RNA and 0.3–1 fmol of uniformly ^{32}P-labeled RNA probe in 20 μl of hybridization buffer (80% deionized formamide, 400 mM NaCl, 40 mM PIPES [pH 6.4], 1 mM EDTA). Reaction mixtures were denatured at 90°C for 6 min and hybridized at 58°C for 16 hr. RNase digestions were performed by the addition of 150 μl of digestion buffer (10 mM Tris-HCl [pH 7.5], 200 mM NaCl, 5 mM EDTA) containing 125 units/ml RNase T1, 25 μg/ml RNase A at 37°C for 30 min, and terminated with 225 μl of inactivation solution (Ambion, Austin, TX). RNase-resistant hybrids recovered by ethanol precipitation were resuspended in 10 μl formamide-dye mix (Ambion), heated to 90°C for 6 min, and analyzed on 5% polyacrylamide-8 M urea gels. Gels were dried and exposed to Kodak BiomaxMR film. The radioactivity associated with each band was quantified using a PhosphorImager (Molecular Dynamics, Piscataway, NJ).

RNA Extraction and RT-PCR

Total cellular RNA was extracted from skeletal muscle tissues as described (Savkur et al., 2001). For exon 6b/7a inclusion, first strand cDNA was synthesized using 10 ng of RT primer (5′-CTCCTACCAG CCTTCCAAAT-3′). PCR amplification was carried out using half of the synthesized cDNA, 200 ng upstream (5′-CATCTCTCCC

CAGGCTGT-3′) and downstream (5′-GCATCCTTGTTCCACACT-3′) primers, and 1.5 ng of 5′ ^{32}P-end-labeled upstream primer as described (Ladd et al., 2001). Amplification was performed using 25 cycles of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, followed by a final 10 min extension at 72°C. PCR product sizes are 6b7a = 568 bp, 6Δ6b7a = 490 bp, N = 435 bp, 5-8 = 278 bp. RT-PCR conditions were optimized on mixtures of the four in vitro transcribed RNAs shown in Figure 3A. This analysis demonstrated that ClC-$1_{5\text{-}8}$ was amplified disproportionally to the longer products. In addition, the RT-PCR conditions required to detect amplified product precluded comparisons of mRNA abundance between samples. For intron 2 inclusion, the RT primer was (5′-TATTCCTTCAGGACAACCCCACG-3′). PCR was performed using 200 ng of upstream (5′-AGGGATGCC CAAGAAGACAGGCT-3′) and downstream (5′-GCCATCAGCAGTC CCAGAAGCAC-3′) and 1.5 ng of 5′ ^{32}P-end-labeled downstream primer. These primers generate a 161 bp product corresponding to the spliced product or a 948 bp product corresponding to the unspliced pre-mRNA. Amplification was performed using 25 cycles of 30 s at 95°C, 30 s at 65°C, 1 min at 72°C, followed by a final 10 min extension at 72°C. The products of PCR amplification were resolved on 4% nondenaturing polyacrylamide gels and quantified using a PhosphorImager (Molecular Dynamics).

Intron 2 Minigene

A PCR-amplified segment of the *CLCN1* gene containing from the last 74 nt of exon 2 to the first 85 nt of exon 3 was cloned between the RSV enhancer/transcription start site and the terminal exon and 3′ flanking genomic sequence of chicken skeletal α-actin. Transfection in QT35 fibroblasts and RNA extraction was performed as described previously (Ladd et al., 2001). Percent intron retention was calculated as (cpm intron 2 band)/(cpm intron 2 band + cpm spliced mRNA band) × 100.

In Vitro Transcription and UV Crosslinking

The templates for in vitro transcription were PCR products derived from regions of the intron 2 minigene with the T7 promoter included in the upstream primer. Uniformly ^{32}P-labeled transcripts ranging in size from 90 to 172 nt in length were used for UV crosslinking assays as described (Ladd et al., 2001). UV crosslinking assays included 300 ng of purified GST-CUG-BP in the presence of 15 μg HeLa nuclear extract to reduce nonspecific binding.

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References

Beck, C.L., Fahlke, C., and George, A.L., Jr. (1996). Molecular basis for decreased muscle chloride conductance in the myotonic goat. Proc. Natl. Acad. Sci. USA *93*, 11248–11252.

Behrens, M.I., Jalil, P., Serani, A., Vergara, F., and Alvarez, O. (1994). Possible role of apamin-sensitive K^+ channels in myotonic dystrophy. Muscle Nerve *17*, 1264–1270.

Berul, C.I., Maguire, C.T., Aronovitz, M.J., Greenwood, J., Miller, C., Gehrmann, J., Housman, D., Mendelsohn, M.E., and Reddy, S. (1999). DMPK dosage alterations result in atrioventricular conduction abnormalities in a mouse myotonic dystrophy model. J. Clin. Invest. *103*, R1–R7.

Brook, J.D., McCurrach, M.E., Harley, H.G., Buckler, A.J., Church, D., Aburatani, H., Hunter, K., Stanton, V.P., Thirion, J.P., Hudson, T., et al. (1992). Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3′ end of a transcript encoding a protein kinase family member. Cell *68*, 799–808.

Bryant, S.H., and DeCoursey, T.E. (1980). Sodium currents from cut skeletal muscle fibers from normal and myotonic goats. J. Physiol. *307*, 31P.

Buratti, E., Dork, T., Zuccato, E., Pagani, F., Romano, M., and Baralle, F.E. (2001). Nuclear factor TDP-43 and SR proteins promote in vitro and in vivo CFTR exon 9 skipping. EMBO J. *20*, 1774–1784.

Cannon, S.C. (1996). Ion-channel defects and aberrant excitability in myotonia and periodic paralysis. Trends Neurosci. *19*, 3–10.

Charlet-B., N., Logan, P., Singh, G., and Cooper, T.A. (2002). Dynamic antagonism between ETR-3 and PTB regulates cell type-specific alternative splicing. Mol. Cell *9*, 649–658.

Davis, B.M., McCurrach, M.E., Taneja, K.L., Singer, R.H., and Housman, D.E. (1997). Expansion of a CUG trinucleotide repeat in the 3′ untranslated region of myotonic dystrophy protein kinase transcripts results in nuclear retention of transcripts. Proc. Natl. Acad. Sci. USA *94*, 7388–7393.

Fardaei, M., Larkin, K., Brook, J.D., and Hamshere, M.G. (2001). In vivo co-localisation of MBNL protein with DMPK expanded-repeat transcripts. Nucleic Acids Res. *29*, 2766–2771.

Franke, C., Hatt, H., Iaizzo, P.A., and Lehmann-Horn, F. (1990). Characteristics of Na^+ channels and Cl^- conductance in resealed muscle fibre segments from patients with myotonic dystrophy. J. Physiol. *425*, 391–405.

Franke, C., Iaizzo, P.A., Hatt, H., Spittelmeister, W., Ricker, K., and Lehmann-Horn, F. (1991). Altered Na^+ channel activity and reduced Cl^- conductance cause hyperexcitability in recessive generalized myotonia (Becker). Muscle Nerve *14*, 762–770.

Fu, Y.H., Pizzuti, A., Fenwick, R.G., King, J., Rajnarayan, S., Dunne, P.W., Dubel, J., Nasser, G.A., Ashizawa, T., Dejong, P., et al. (1992). An unstable triplet repeat in a gene related to myotonic muscular dystrophy. Science *255*, 1256–1258.

Fu, Y.H., Friedman, D.L., Richards, S., Pearlman, J.A., Gibbs, R.A., Pizzuti, A., Ashizawa, T., Perryman, M.B., Scarlato, G., Fenwick, R.G., et al. (1993). Decreased expression of myotonin-protein kinase messenger RNA and protein in adult form of myotonic dystrophy. Science *260*, 235–238.

Gabellini, N. (2001). A polymorphic GT repeat from the human cardiac $Na^+Ca_2^+$ exchanger intron 2 activates splicing. Eur. J. Biochem. *268*, 1076–1083.

Goldin, A.L. (1999). Diversity of mammalian voltage-gated sodium channels. Ann. N Y Acad. Sci. *868*, 38–50.

Gurnett, C.A., Kahl, S.D., Anderson, R.D., and Campbell, K.P. (1995). Absence of the skeletal muscle sarcolemma chloride channel ClC-1 in myotonic mice. J. Biol. Chem. *270*, 9035–9038.

Harper, P.S. (2001). Myotonic Dystrophy, Volume 37, Third Edition (London: W.B. Saunders).

Hentze, M.W., and Kulozik, A.E. (1999). A perfect message: RNA surveillance and nonsense-mediated decay. Cell *96*, 307–310.

Iaizzo, P.A., Franke, C., Hatt, H., Spittelmeister, W., Ricker, K., Rudel, R., and Lehmann-Horn, F. (1991). Altered sodium channel behaviour causes myotonia in dominantly inherited myotonia congenita. Neuromuscul. Disord. *1*, 47–53.

Jansen, G., Groenen, P.J., Bachner, D., Jap, P.H., Coerwinkel, M., Oerlemans, F., van den Broek, W., Gohlsch, B., Pette, D., Plomp, J.J., et al. (1996). Abnormal myotonic dystrophy protein kinase levels produce only mild myopathy in mice. Nat. Genet. *13*, 316–324.

Jentsch, T.J., Friedrich, T., Schriever, A., and Yamada, H. (1999). The CLC chloride channel family. Pflugers Arch. *437*, 783–795.

Kimura, T., Takahashi, M.P., Okuda, Y., Kaido, M., Fujimura, H., Yanagihara, T., and Sakoda, S. (2000). The expression of ion channel mRNAs in skeletal muscles from patients with myotonic muscular dystrophy. Neurosci. Lett. *295*, 93–96.

Klesert, T.R., Otten, A.D., Bird, T.D., and Tapscott, S.J. (1997). Trinucleotide repeat expansion at the myotonic dystrophy locus reduces expression of DMAHP. Nat. Genet. *16*, 402–406.

Klesert, T.R., Cho, D.H., Clark, J.I., Maylie, J., Adelman, J., Snider, L., Yuen, E.C., Soriano, P., and Tapscott, S.J. (2000). Mice deficient

in Six5 develop cataracts: implications for myotonic dystrophy. Nat. Genet. *25*, 105–109.

Klocke, R., Steinmeyer, K., Jentsch, T.J., and Jockusch, H. (1994). Role of innervation, excitability, and myogenic factors in the expression of the muscular chloride channel ClC-1. A study on normal and myotonic muscle. J. Biol. Chem. *269*, 27635–27639.

Ladd, A.N., Charlet-B., N., and Cooper, T.A. (2001). The CELF family of RNA binding proteins is implicated in cell-specific and developmentally regulated alternative splicing. Mol. Cell. Biol. *21*, 1285–1296.

Lehmann-Horn, F., and Jurkat-Rott, K. (1999). Voltage-gated ion channels and hereditary disease. Physiol. Rev. *79*, 1317–1372.

Lipicky, R.J. (1977). Studies in myotonic dystrophy. In Pathogenesis of Muscular Dystrophy, L.P. Rowlan, ed. (Amsterdam: Excerpta), pp. 728–738.

Liquori, C.L., Ricker, K., Moseley, M.L., Jacobsen, J.F., Kress, W., Naylor, S.L., Day, J.W., and Ranum, L.P. (2001). Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of ZNF9. Science *293*, 864–867.

Lu, X., Timchenko, N.A., and Timchenko, L.T. (1999). Cardiac elav-type RNA-binding protein (ETR-3) binds to RNA CUG repeats expanded in myotonic dystrophy. Hum. Mol. Genet. *8*, 53–60.

Mahadevan, M., Tsilfidis, C., Sabourin, L., Shutler, G., Amemiya, C., Jansen, G., Neville, C., Narang, M., Barcelo, J., Ohoy, K., et al. (1992). Myotonic dystrophy mutation—an unstable CTG repeat in the 3′ untranslated region of the gene. Science *255*, 1253–1255.

Mankodi, A., Logigian, E., Callahan, L., McClain, C., White, R., Henderson, D., Krym, M., and Thornton, C.A. (2000). Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat. Science *289*, 1769–1773.

Mankodi, A., Urbinati, C.R., Yuan, Q.P., Moxley, R.T., Sansone, V., Krym, M., Henderson, D., Schalling, M., Swanson, M.S., and Thornton, C.A. (2001). Muscleblind localizes to nuclear foci of aberrant RNA in myotonic dystrophy types 1 and 2. Hum. Mol. Genet. *10*, 2165–2170.

Mankodi, A., Takahashi, M.P., Jiang, H., Beck, C., Bowers, W.J., Moxley, R.T., Cannon, S.C., and Thornton, C.A. (2002). Expanded CUG repeats in myotonic dystrophy trigger aberrant splicing of ClC-1 chloride channel pre-mRNA and hyperexcitability in skeletal muscle. Mol. Cell *10*, this issue, 35–44.

Michalowski, S., Miller, J.W., Urbinati, C.R., Paliouras, M., Swanson, M.S., and Griffith, J. (1999). Visualization of double-stranded RNAs from the myotonic dystrophy protein kinase gene and interactions with CUG-binding protein. Nucleic Acids Res. *27*, 3534–3542.

Miller, J.W., Urbinati, C.R., Teng-Umnuay, P., Stenberg, M.G., Byrne, B.J., Thornton, C.A., and Swanson, M.S. (2000). Recruitment of human muscleblind proteins to (CUG)n expansions associated with myotonic dystrophy. EMBO J. *19*, 4439–4448.

Nagamitsu, S., Matsuura, T., Khajavi, M., Armstrong, R., Gooch, C., Harati, Y., and Ashizawa, T. (2000). A "dystrophic" variant of autosomal recessive myotonia congenita caused by novel mutations in the CLCN1 gene. Neurology *55*, 1697–1703.

Napieraa, M., and Krzyosiak, W.J. (1997). CUG repeats present in myotonin kinase RNA form metastable "slippery" hairpins. J. Biol. Chem. *272*, 31079–31085.

Neelands, T.R., Herson, P.S., Jacobson, D., Adelman, J.P., and Maylie, J. (2001). Small-conductance calcium-activated potassium currents in mouse hyperexcitable denervated skeletal muscle. J. Physiol. *536*, 397–407.

Philips, A.V., Timchenko, L.T., and Cooper, T.A. (1998). Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy. Science *280*, 737–741.

Reddy, S., Smith, D.B., Rich, M.M., Leferovich, J.M., Reilly, P., Davis, B.M., Tran, K., Rayburn, H., Bronson, R., Cros, D., et al. (1996). Mice lacking the myotonic dystrophy protein kinase develop a late onset progressive myopathy. Nat. Genet. *13*, 325–335.

Renaud, J.F., Desnuelle, C., Schmid-Antomarchi, H., Hugues, M., Serratrice, G., and Lazdunski, M. (1986). Expression of apamin receptor in muscles of patients with myotonic muscular dystrophy. Nature *319*, 678–680.

Rhodes, T.H., Vite, C.H., Giger, U., Patterson, D.F., Fahlke, C., and George, A.L. (1999). A missense mutation in canine ClC-1 causes recessive myotonia congenita in the dog. FEBS Lett. *456*, 54–58.

Rich, M.M., Kraner, S.D., and Barchi, R.L. (1999). Altered gene expression in steroid-treated denervated muscle. Neurobiol. Dis. *6*, 515–522.

Roberts, R., Timchenko, N.A., Miller, J.W., Reddy, S., Caskey, C.T., Swanson, M.S., and Timchenko, L.T. (1997). Altered phosphorylation and intracellular distribution of a (CUG)n triplet repeat RNA-binding protein in patients with myotonic dystrophy and in myotonin protein kinase knockout mice. Proc. Natl. Acad. Sci. USA *94*, 13221–13226.

Rudel, R., Ruppersberg, J.P., and Spittelmeister, W. (1989). Abnormalities of the fast sodium current in myotonic dystrophy, recessive generalized myotonia, and adynamia episodica. Muscle Nerve *12*, 281–287.

Sarkar, P.S., Appukuttan, B., Han, J., Ito, Y., Ai, C., Tsai, W., Chai, Y., Stout, J.T., and Reddy, S. (2000). Heterozygous loss of Six5 in mice is sufficient to cause ocular cataracts. Nat. Genet. *25*, 110–114.

Savkur, R.S., Philips, A.V., and Cooper, T.A. (2001). Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy. Nat. Genet. *29*, 40–47.

Steinmeyer, K., Klocke, R., Ortland, C., Gronemeier, M., Jockusch, H., Grunder, S., and Jentsch, T.J. (1991). Inactivation of muscle chloride channel by transposon insertion in myotonic mice. Nature *354*, 304–308.

Takahashi, N., Sasagawa, N., Suzuki, K., and Ishiura, S. (2000). The CUG-binding protein binds specifically to UG dinucleotide repeats in a yeast three-hybrid system. Biochem. Biophys. Res. Commun. *277*, 518–523.

Taneja, K.L., McCurrach, M., Schalling, M., Housman, D., and Singer, R.H. (1995). Foci of trinucleotide repeat transcripts in nuclei of myotonic dystrophy cells and tissues. J. Cell Biol. *128*, 995–1002.

Tapscott, S.J., and Thornton, C.A. (2001). Reconstructing myotonic dystrophy. Science *293*, 816–817.

Thornton, C.A., Wymer, J.P., Simmons, Z., McClain, C., and Moxley, R.T. (1997). Expansion of the myotonic dystrophy CTG repeat reduces expression of the flanking DMAHP gene. Nat. Genet. *16*, 407–409.

Tian, B., White, R.J., Xia, T., Welle, S., Turner, D.H., Mathews, M.B., and Thornton, C.A. (2000). Expanded CUG repeat RNAs form hairpins that activate the double-stranded RNA-dependent protein kinase PKR. RNA *6*, 79–87.

Timchenko, L.T., Miller, J.W., Timchenko, N.A., Devore, D.R., Datar, K.V., Lin, L.J., Roberts, R., Caskey, C.T., and Swanson, M.S. (1996). Identification of a (CUG)n triplet repeat RNA-binding protein and its expression in myotonic dystrophy. Nucleic Acids Res. *24*, 4407–4414.

Timchenko, N.A., Cai, Z.J., Welm, A.L., Reddy, S., Ashizawa, T., and Timchenko, L.T. (2001). RNA CUG repeats sequester CUGBP1 and alter protein levels and activity of CUGBP1. J. Biol. Chem. *276*, 7820–7826.

Zhang, L., Liu, W., and Grabowski, P.J. (1999). Coordinate repression of a trio of neuron-specific splicing events by the splicing regulator PTB. RNA *5*, 117–130.