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 multi-systemic 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 (CIC-1) mRNA and protein in DM1 skeletal muscle tissue due to aberrant splicing of the CIC-1 pre-mRNA. The splicing regulator, CUG binding protein (CUG-BP), which is elevated in DM1 striated muscle, binds to the CIC-1 pre-mRNA, and overexpression of CUG-BP in normal cells reproduces the aberrant pattern of CIC-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\(^{CUG}\)) 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 (Kiesert et al., 1997; Thornton et al., 1997). However, several lines of evidence indicate that a gain of function for RNA\(^{CUG}\), rather than haploinsufficiency of DMPK and/or SIX5, plays the prominent role in DM1 pathogenesis. First, only CTV 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; Kiesert 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\(^{CUG}\) and RNA\(^{CUG}\) 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\(^{CUG}\), 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\(^{CUG}\) 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...
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 HSA10 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-1-GFP/H9004) in which the N-terminal 151 amino acids of ClC-1 were 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-1-GFP, lanes 3 and 6). Anti-GFP antibody (Clontech) was used to demonstrate expression of the ClC-1-GFP fusion protein (46 kDa) (lane 3). Detection of the ClC-1-GFP 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.

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). 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.

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-1-GFP 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-1-GFP, lanes 3 and 6). Anti-GFP antibody (Clontech) was used to demonstrate expression of the ClC-1-GFP fusion protein (46 kDa) (lane 3). Detection of the ClC-1-GFP 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.
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 CIC-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 CIC-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 CIC-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 CIC-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 CIC-1 mRNA was 15.3% (±1.5%) that of cyclophilin in normal skeletal muscle and ≤0.7% that of cyclophilin in DM1 skeletal muscle. The CIC-1 probe was designed to detect correctly spliced CIC-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 CIC-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., 1998; Cannon, 1996; Garnett et al., 1995; Rhodes et al., 1999; Steinmeyer et al., 1991). We propose that the CIC-1 deficiency demonstrated here is sufficient to explain the myotonia observed in DM1 patients.

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

We hypothesized that the CIC-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 CIC-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 CIC-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 CIC-1 mRNAs contain an insert of 134 nt immediately preceding exon 7 (CIC-1 6b7a and CIC-1 6b7a/H9004; Figure 3A); one of these RNAs (CIC-1 6b7a/H9004) lacks exon 6. The third aberrant mRNA resulted from splicing of exon 5 to exon 8 (CIC-1 5-8a; Figure 3A). Sequence comparisons of the PCR products to the human CLCN1 gene (GenBank AC073342) revealed that the 134 nt insert corresponds to a previously unreported exon located between exons 6 and 7. The exons are designated as exons 6b and 7a. The ratio of RT-PCR products suggested that very little of the normal CIC-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 CIC-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 CIC-1 protein and mRNA deficiencies demonstrated above. Indeed, evidence that CIC-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 (Namagishi et al., 2000).

Results described in the companion paper (Mankodi et al., 2002) indicated that a fraction of CIC-1 mRNA from HSA skeletal muscle retained intron 2. Based on this information, we performed RT-PCR analysis of the 5′ region of the CIC-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_{6b7a/9004}, and ClC-1_{5-8} 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 CIC-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_CUGBP induces aberrant splicing of the CIC-1 pre-mRNA and subsequent loss of CIC-1 mRNA. We next addressed the mechanism of the aberrantly regulated CIC-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 CIC-1 pre-mRNA, we coexpressed a CIC-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 CIC-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 UV crosslinking assay using bacterially expressed GST-CUG-BP and 11...
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The fact that inclusion of the mutated intron 2 is weakly induced is consistent with the residual binding of CUG-BP to RNA Jmut (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, 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, (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. 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 Jmut; Figures 4B and 4C).

The Jmut 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 Jmut (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.

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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 (Nee-lands et al., 2001). As the membrane resting potential of skeletal muscle is primarily maintained by chloride and not potassium conductance, functional CIC-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 (Kloczke et al., 1994; Rich et al., 1999). We propose that the CIC-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 CIC-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 CIC-1 deficiency in the myotonia of DM1.

The Mechanism of an RNA Gain of Function for RNA CUG<sub>1200</sub>

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 CIC-1 and IR pre-mRNAs, respectively. Our working model (Figure 5) is that nuclear accumulation of RNA CUG<sub>1200</sub> leads to increased CUG-BP activity, which induces inappropriate alternative splicing of its pre-mRNA targets. CIC-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 CIC-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 (CIC-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<sub>1200</sub> results in increased CUG-BP steady-state levels. Transient expression of RNA CUG<sub>1200</sub> 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-
phosphoisoforms 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 CUGn repeats longer than ~20 repeats form double-stranded RNA containing U-U mismatches (Michalowski et al., 1999; Napiarewa 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 CUGn and does not colocalize with RNA CUGn (Fardaei et al., 2001; Michalowski et al., 1999). However, expression of RNA CUGn 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 CUGn, sequestration of MBNL on RNA CUGn, 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 CIC-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 CUGn 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 CIC-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 and mice 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. CIC-1 mRNA levels increase rapidly during postnatal development (Steinemeyer et al., 1991). If the aberrant splicing patterns observed in DM1 patients reflect reiteration of an embryonic program, the developmentally regulated induction of CIC-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 MgCl2, 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 10,000 × 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-NaK ATPase were from Biogenesis (Kingston, NH) and Sigma, respectively. Anti-peptide antibodies against CIC-1 were generated in rabbits against the N-terminal 14 amino acid peptide MEQSSRQQRGGQGS(C) that was conjugated to keyhole limpet hemocyanin by an additional cysteine (Anaspec, San Jose, CA). For Westerns, the CIC-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 CIC-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 poly linker sequence. The cytoplasmic 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 (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.

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CAGGCTGT-T3 and downstream (5'-GCATCCTTGTTCCACT-3') primers, and 1.5 ng of 5'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 were 67a = 568 bp, 66b7a = 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 CIG-1, as 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'-TATCCCTGCAAGCAACCCCCCGC-3'). PCR was performed using 200 ng of upstream (5'-AGGGATGCGC GAAGAACAGGCTG-3') and downstream (5'-GCATCCTGAAGCCCACTCAC-3') and 1.5 ng of 5'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 in 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 muscle sarcolemmal chloride channel C. A PCR product of 435 bp, 5'-AGGGATGCGC GAAGAACAGGCTG-3' was digested with HindIII and the 435 bp fragment ligated into the HindIII site of the expression vector pBluescript II KS (Stratagene). The resulting construct was sequenced to verify the authenticity of the construct.

In Vitro Transcription and UV Crosslinking
The templates for in vitro transcription were PCR products derived from regions of the intron 2 mini-gene with the T7 promoter included in the upstream primer. Uniformly 32P-labeled transcripts ranging in size from 90 to 172 nt in length were used for UV crosslinking assays 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) x 100.

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