REFERENCES AND NOTES

1. S. H. Leppla, in *Handbook of Natural Toxins*, J. Moss, B. Iglewski, M. Vaughan, A. T. Tu, Eds. (Dekker, New York, 1995), vol. 8, pp. 543–572.
2. V. M. Gordon, S. H. Leppla, E. L. Hewlett, *Infect. Immun.* **56**, 1066 (1988); J. C. Milne, D. Furlong, P. C. Hanna, J. S. Wall, R. J. Collier, *J. Biol. Chem.* **269**, 20607 (1994).
3. S. H. Leppla, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3162 (1982).
4. C. Pezard, P. Berche, M. Mock, *Infect. Immun.* **59**, 3472 (1991).
5. J. W. Ezzell, B. E. Ivins, S. H. Leppla, *ibid.* **45**, 761 (1984).
6. A. M. Friedlander, *J. Biol. Chem.* **261**, 7123 (1986).
7. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
8. K. R. Klimpel, N. Arora, S. H. Leppla, *Mol. Microbiol.* **13**, 1093 (1994).
9. S. K. Kochi, G. Schiavo, M. Mock, C. Montecucco, *FEMS Microbiol. Lett.* **124**, 343 (1994); A. Menard, E. Papini, M. Mock, C. Montecucco, *Biochem. J.* **320**, 687 (1996).
10. J. N. Weinstein *et al.*, *Science* **275**, 343 (1997); H.-M. Koo *et al.*, *Cancer Res.* **56**, 5211 (1996).
11. D. T. Dudley, L. Pang, S. J. Decker, A. J. Bridges, A. R. Saltiel, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7686 (1995).
12. For reviews, see T. Moriguchi, Y. Gotoh, E. Nishida, *Adv. Pharmacol.* **36**, 121 (1996); M. S. Murakami and G. F. Vande Woude, *Methods Enzymol.* **283**, 584 (1997); W. T. Matten and G. F. Vande Woude, *Sem. Dev. Biol.* **5**, 173 (1994).
13. N. S. Duesbery and G. F. Vande Woude, unpublished data.
14. M. Glotzer, A. W. Murray, M. W. Kirschner, *Nature* **349**, 132 (1991).
15. M. A. White *et al.*, *Cell* **80**, 533 (1995).
16. Y. Singh, K. R. Klimpel, N. Arora, M. Sharma, S. H. Leppla, *J. Biol. Chem.* **269**, 29039 (1994).
17. S. Mansour, J. M. Candia, K. K. Gloor, N. G. Ahn, *Cell Growth Differ.* **7**, 243 (1996); S. J. Mansour *et al.*, *Science* **265**, 966 (1994).
18. Wild-type MAPKK1 (0.5 μg) was incubated with LF (0.2 μg) as described in Fig. 2A for 30 min at 30°C. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride membrane in CAPS transfer buffer {3-[cyclohexylamino]-1 propanesulfonic acid (10 mM, pH 11) and 10% methanol} at 300 mA constant current for 30 min. After transfer, membranes were quickly stained with Ponceau S solution (0.1% Ponceau S and 5% acetic acid) and rinsed with distilled deionized water. The appropriate band was cut from the membrane and subjected to automated Edman degradation in an Applied Biosystems 477A gas-phase sequencer, and phenylthiohydantoin derivatives were identified online with a 120 phenylthiohydantoin analyzer.
19. S. J. Mansour, J. M. Candia, J. E. Matsuura, M. C. Manning, N. G. Ahn, *Biochemistry* **35**, 15529 (1996).
20. M. Fukuda, Y. Gotoh, E. Nishida, *EMBO J.* **16**, 1901 (1997).
21. S. E. Hammond and P. C. Hanna, abstract presented at the 97th General Meeting of the American Society of Microbiology, Miami Beach, FL (1997).
22. N. S. Duesbery *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9165 (1997).
23. S. H. Leppla, *Methods Enzymol.* **165**, 103 (1988).
24. E. K. Shibuya, T. G. Boulton, M. H. Cobb, J. V. Ruderman, *EMBO J.* **11**, 3963 (1992).
25. N. Yew, M. L. Mellini, G. F. Vande Woude, *Nature* **355**, 649 (1992).
26. MAPKK and LF were diluted in 16 μl of assay buffer {assay dilution buffer [ADB, Upstate Biotechnology; consisting of 20 mM Mops (pH 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol] containing 10 mM protein kinase C inhibitor peptide, 1 mM protein kinase A inhibitor peptide, and 10 mM compound R24571}. After incubation at 30°C for 0.5 hours, 11 μl of kinase buffer {[γ-^{32}P]ATP (Amersham; 10 mCi/ml, 3000 mCi/mmol) diluted 1:9 in ADB containing 0.5 mM ATP, 75 mM $MgCl_2$, 0.35 μg of MAPK [ERK2; D. J. Robbins *et al.*, *J. Biol. Chem.* **268**, 5097 (1993)], and 10 μg of MBP (Upstate Biotechnology)} was added, and samples were incubated for a further 15 min at 30°C. Proteins were separated by SDS-PAGE on 14% gels and processed for autoradiography.
27. A MAPKK1 construct containing a seven–amino acid NH_2-terminal deletion was generated by polymerase chain reaction using primer pairs A and B and pKH-1, encoding His^6-tagged human wild-type MAPKK1 [S. J. Mansour *et al.*, *J. Biochem.* **116**, 304 (1994)], as a template. The amplified fragment was digested with Xho I and Hind III and introduced into pRSETA (Invitrogen). The primers used were 5′-AAGAAGCGG**CTCGAG**ATCCAGCTGAACCCG-3′ (A) and 5′-CTTTGTTAGCAGCCGGATCAAGCTTCGAAT-3′ (B). Constructs containing MAPKK1 mutations $Pro^{5\ or\ 7}$ to Ala were generated by introducing the mutations into pKH-1 with the use of the Quickchange Site-Directed Mutagenesis Kit (Stratagene). The primers used were 5′-ATGCCCAAGAAGAAG**G**CGACGCCCATCCAGCTGAACCCG-3′ for P5A, 5′-CCCAAGAAGAAGCCGACG**G**CCATCCAGCTGAACCCGGCC-3′ for P7A, and their respective complementary sequences. The sequences of these constructs were confirmed by direct DNA sequencing. To produce recombinant protein, *Escherichia coli* strain TOP10F′ was transformed with these constructs. Cultures were grown at 37°C to an optical density at 600 nm of 0.3 in 750 ml of SOB and ampicillin (100 μg/ml) and then incubated for 1 hour in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside, at which point M13/T7 helper phage was added to a concentration of three plaque-forming units per cell. Cells were harvested by centrifugation 3 hours later, resuspended in 36 ml of extraction buffer (EB) [50 mM potassium phosphate (pH 8.0); 10% (v/v) glycerol; 0.25% (v/v) Tween-20; and 1 mM dithiothreitol (DTT), containing protease inhibitors (EDTA-free Complete tablets; Boehringer Mannheim)], and lysed by four sonication/freeze/thaw cycles. Soluble protein was separated by centrifugation (at 14,000 rpm for 30 min with an SS-34 rotor), and the supernatant was sterile-filtered and adjusted to 1% Tween-20, 1 M KCl, and 20% glycerol. The supernatant was then loaded on a Ni^{2+}-charged HiTrap Chelating column (Pharmacia), washed with EB containing 10 mM imidazole, and eluted with EB containing 200 mM imidazole. Fractions containing recombinant protein were then pooled and desalted and concentrated in buffer containing 50 mM tris (pH 8.0), 0.1 M NaCl, 10% glycerol, 0.01% Triton X-100, and 1 mM DTT by centrifugation with a Centriprep-30 centrifugal concentrator (Amicon, Beverely, MA, XX).

28. This paper is dedicated to the memory of Ken Paull, whose untimely death is a significant loss to NCI and the cancer research community. The authors thank A. Cline and R. Frederickson for preparing the manuscript, L. Miller for technical assistance, H.-M. Koo for helpful discussions, M. Jeffers and S. Koochekpour for critical comments on the manuscript, and A. Murray for providing Δ90 cyclin. This research was sponsored in part by NCI, Department of Health and Human Services, under contract with Advanced BioScience Laboratories. By acceptance of this article, the publisher or recipient acknowledges the right of the U.S. government and its contractors and agents to retain a nonexclusive royalty-free license in and to any copyright covering the article.

8 December 1997; accepted 10 March 1998

Disruption of Splicing Regulated by a CUG-Binding Protein in Myotonic Dystrophy

Anne V. Philips, Lubov T. Timchenko, Thomas A. Cooper*

Myotonic dystrophy (DM) is caused by a CTG expansion in the 3′ untranslated region of the *DM* gene. One model of DM pathogenesis suggests that RNAs from the expanded allele create a gain-of-function mutation by the inappropriate binding of proteins to the CUG repeats. Data presented here indicate that the conserved heterogeneous nuclear ribonucleoprotein, CUG-binding protein (CUG-BP), may mediate the trans-dominant effect of the RNA. CUG-BP was found to bind to the human cardiac troponin T (cTNT) pre–messenger RNA and regulate its alternative splicing. Splicing of cTNT was disrupted in DM striated muscle and in normal cells expressing transcripts that contain CUG repeats. Altered expression of genes regulated posttranscriptionally by CUG-BP therefore may contribute to DM pathogenesis.

Myotonic dystrophy (DM) is a dominantly inherited neuromuscular disorder caused by a trinucleotide (CTG) expansion in the 3′ untranslated region (UTR) of *DM*, the gene that encodes DM protein kinase (*1*). The severity of symptoms correlates with the length of the expansion (*2*). It has been proposed that the CUG repeats in the *DM* RNA create a gain-of-function mutation that disrupts processing or transport of heterologous RNAs in a trans-dominant manner (*3–5*). This hypothesis is supported by the observation that transcripts of the expanded *DM* allele accumulate in the nucleus (*6*). One possible mediator of a trans-dominant effect is CUG-BP (or hNab50), a conserved heterogeneous nuclear ribonucleoprotein (hnRNP) that binds specifically to RNA that contains CUG repeats (*7, 8*). CUG-BP is ubiquitously expressed, distributes to both the nucleus and cytoplasm, and exists in two isoforms that differ by the extent of phosphorylation (*7, 9*). The hypophosphorylated isoform accumulates in the nucleus in tissues and cultured cells

A. Philips and T. Cooper, Department of Pathology, Baylor College of Medicine, Houston, TX 77030, USA.
L. Timchenko, Department of Medicine, Section of Cardiology, Baylor College of Medicine, Houston, TX 77030, USA.

*To whom correspondence should be addressed. E-mail: tcooper@bcm.tmc.edu

from DM patients (*7*, *9*). Altered posttranscriptional processing of transcripts that require CUG-BP has been hypothesized to contribute to DM pathogenesis; however, target genes for CUG-BP activity have not been identified.

We noticed that CUG repeats occur within muscle-specific splicing enhancers (MSEs) of the chicken cTNT gene (*10*) (Fig. 1, A and B). Chicken cTNT is expressed in embryonic and adult cardiac muscle and embryonic skeletal muscle, and alternative splicing of exon 5 is regulated so that the exon is included in mRNAs produced during early development of heart and skeletal muscle but not in adult heart. The gene is transcriptionally silent in adult skeletal muscle (*11*). The MSEs (*10*) and the CUG repeats within the MSEs (*12*) are required for the enhanced inclusion of ccTNT exon 5 in embryonic striated muscle. Regulation of cTNT alternative splicing is conserved in chickens and humans (*13*), and a similar CUG motif occurs in human cTNT, 31 nucleotides (nt) downstream of exon 5 (Fig. 1B).

To investigate whether cTNT splicing patterns are altered in DM, we analyzed cTNT transcripts in cardiac tissue from two unrelated DM patients by reverse transcriptase–polymerase chain reaction (RT-PCR) (Fig. 2, A and B). In both patients, the fraction of cTNT mRNAs that included the alternative exon was greatly increased compared with that in normal adult cardiac tissue or several cardiomyopathies. Primary cell lines derived from DM skeletal muscle also showed an abnormal shift to the embryonic splicing pattern (Fig. 2B) (*14*). Altered splicing of cTNT in DM striated muscle was not due to nonspecific changes in splicing efficiency, as splicing of the hnRNP A2/B1 alternative exon, which is not regulated in striated muscle and is not flanked by CUG-containing elements, was unaffected (Fig. 2, A and C).

To explore the basis for the aberrant splicing, we inserted a genomic fragment from the human cTNT gene containing exon 5 into the intron of a constitutively spliced minigene (Fig. 3A) (*10*). The hcTNT minigene was transiently transfected into normal and DM primary skeletal muscle cell lines (*15*), and regulation of exon 5 splicing was assayed by RT-PCR. Substantially more minigene transcripts containing exon 5 were present in DM than in normal skeletal muscle cells (Fig. 3B). Thus, the alteration in cTNT splicing in DM cells is mediated by factors acting in trans.

To determine whether the proximal CUG repeats in the downstream intron

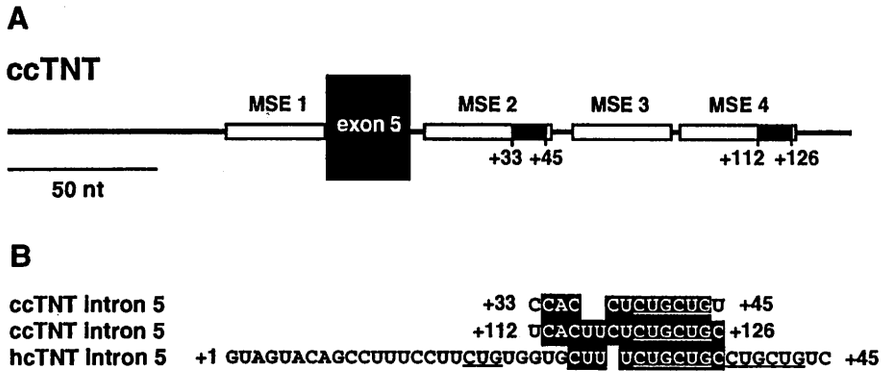

Fig. 1. A conserved muscle-specific splicing element downstream of exon 5 in human cTNT. (**A**) Diagram of the ccTNT genomic fragment necessary and sufficient for regulated splicing of exon 5 (black box) in striated muscle. Four MSEs (white boxes) are required for enhanced exon inclusion in striated muscle (*10*). (**B**) A common sequence in chicken cTNT MSEs and human (hcTNT) intron 5 [highlighted in black in (A) and (B)] contains CUG repeats (underlined).

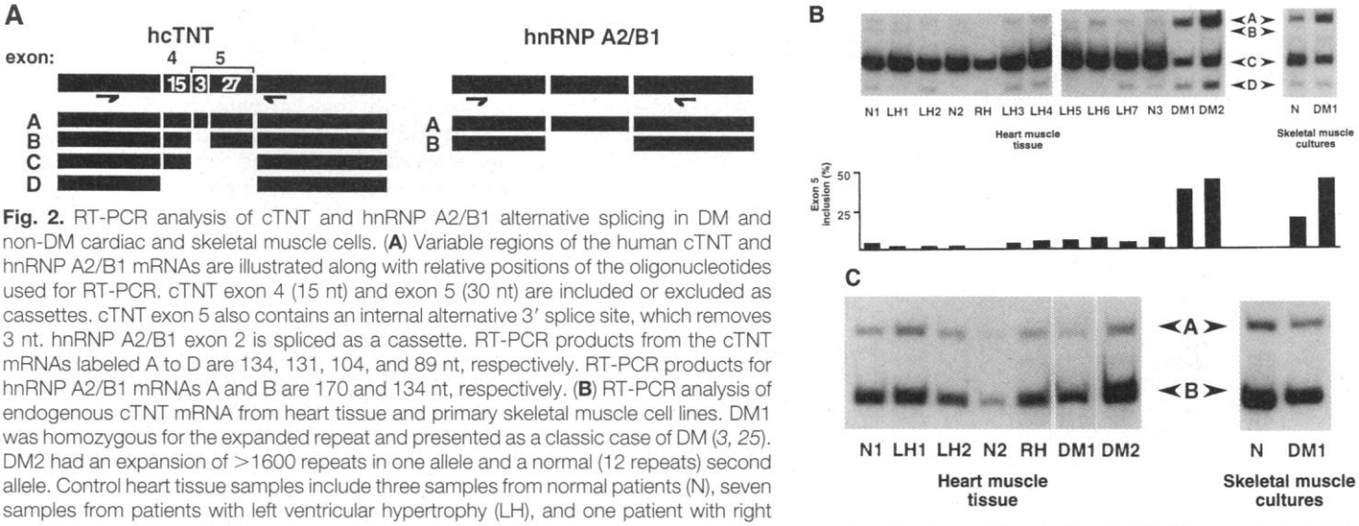

Fig. 2. RT-PCR analysis of cTNT and hnRNP A2/B1 alternative splicing in DM and non-DM cardiac and skeletal muscle cells. (**A**) Variable regions of the human cTNT and hnRNP A2/B1 mRNAs are illustrated along with relative positions of the oligonucleotides used for RT-PCR. cTNT exon 4 (15 nt) and exon 5 (30 nt) are included or excluded as cassettes. cTNT exon 5 also contains an internal alternative 3′ splice site, which removes 3 nt. hnRNP A2/B1 exon 2 is spliced as a cassette. RT-PCR products from the cTNT mRNAs labeled A to D are 134, 131, 104, and 89 nt, respectively. RT-PCR products for hnRNP A2/B1 mRNAs A and B are 170 and 134 nt, respectively. (**B**) RT-PCR analysis of endogenous cTNT mRNA from heart tissue and primary skeletal muscle cell lines. DM1 was homozygous for the expanded repeat and presented as a classic case of DM (*3*, *25*). DM2 had an expansion of >1600 repeats in one allele and a normal (12 repeats) second allele. Control heart tissue samples include three samples from normal patients (N), seven samples from patients with left ventricular hypertrophy (LH), and one patient with right ventricular hypertrophy (RH) secondary to pulmonary hypertension. The patients' ages (years) were as follows: N1 (58), N2 (63), N3 (20–78, pooled), LH1 (65), LH2 (54), LH3 (69), LH4 (69), LH5 (83), LH6 (66), LH7 (81), RH (53), DM1 (21), DM2 (16 days). DM skeletal muscle cultures were from DM1. Reverse transcription was done on 4 μg of total RNA with 10 ng of the reverse primer (*12*). One-quarter of the cDNA was added to 40-μl PCR mixtures containing Taq polymerase buffers (Promega), 2 ng of the forward primer ^{32}P-labeled by polynucleotide kinase, 120 ng each of unlabeled forward and reverse primers, and 2.5 units of DNA Taq polymerase. Twenty cycles were carried out at annealing and extension temperatures of 70° and 72°C, respectively. Forward and reverse oligonucleotides for cTNT were ATAGAAGAGGTGGTGGAAGAGTAC and GTCTCAGCCTCTGCTTCAGCATCC, respectively. PCR products were resolved on 5% nondenaturing polyacrylamide gels. Bands were quantitated with a PhosphorImager (Molecular Dynamics). The percent of exon 5 inclusion is calculated as {[counts per minute (cpm) of bands A + B]/(cpm of bands A + B + C + D) × 100}. Small amounts of band B are detectable in some lanes. Because the results are expressed as a ratio, lane-to-lane variation in the amount of mRNA is internally controlled. (**C**) RT-PCR analysis of hnRNP A2/B1 mRNA. Forward and reverse oligonucleotides were CTGAAGCGACTGAGTCCGCG and ACAGTCTGTAAGCTTTCCCC, respectively.

were targets for these factors, we introduced 5-nt substitutions into the hcTNT minigene to create hcTNT CUG → CAG (Fig. 3A). The CUG → CAG mutation had no effect in normal cells (*16*) but nearly eliminated the enhanced exon 5 inclusion in DM cells (Fig. 3B). This indicates that the effect on cTNT alternative splicing in DM cells is mediated, at least in part, through the proximal downstream CUG repeats.

To determine whether CUG-BP plays a role in the regulation of cTNT alternative splicing, we cotransfected the hcTNT minigene with a cDNA expression vector encoding human CUG-BP into normal human skeletal muscle cells. The amount of exon 5 inclusion directly correlated with the expression of CUG-BP and the minigene containing the five CUG → CAG substitutions did not respond to CUG-BP overexpression (Fig. 3C). Immunoblot analysis confirmed that the amounts of CUG-BP protein were increased in these experiments (*17*). We conclude that CUG-BP activates inclusion of the cTNT alternative exon 5 and that this activation requires conserved CUG repeats in the downstream intron.

We next investigated whether the CUG repeats are required for muscle-specific splicing of cTNT exon 5 by testing the hcTNT and hcTNT CUG → CAG minigenes in a transient transfection system (*10*). Inclusion of cTNT exon 5 was enhanced in differentiated embryonic skeletal muscle cells compared with fibroblasts and the five CUG → CAG mutations nearly eliminated enhanced exon 5 inclusion in muscle cultures (Fig. 3D) (*18*). These results indicate that the CUGs are required for enhanced splicing of cTNT exon 5 in embryonic striated muscle.

To determine whether CUG-BP binds directly to the CUG repeats, we did ultraviolet (UV) cross-linking experiments with recombinant CUG-BP expressed as a fusion protein with maltose-binding protein (MBP). The cTNT genomic fragment that responded to CUG-BP overexpression (Fig. 3A) was subcloned downstream of the T7 promoter and transcribed in vitro as several uniformly labeled RNAs (Fig. 4, RNAs A

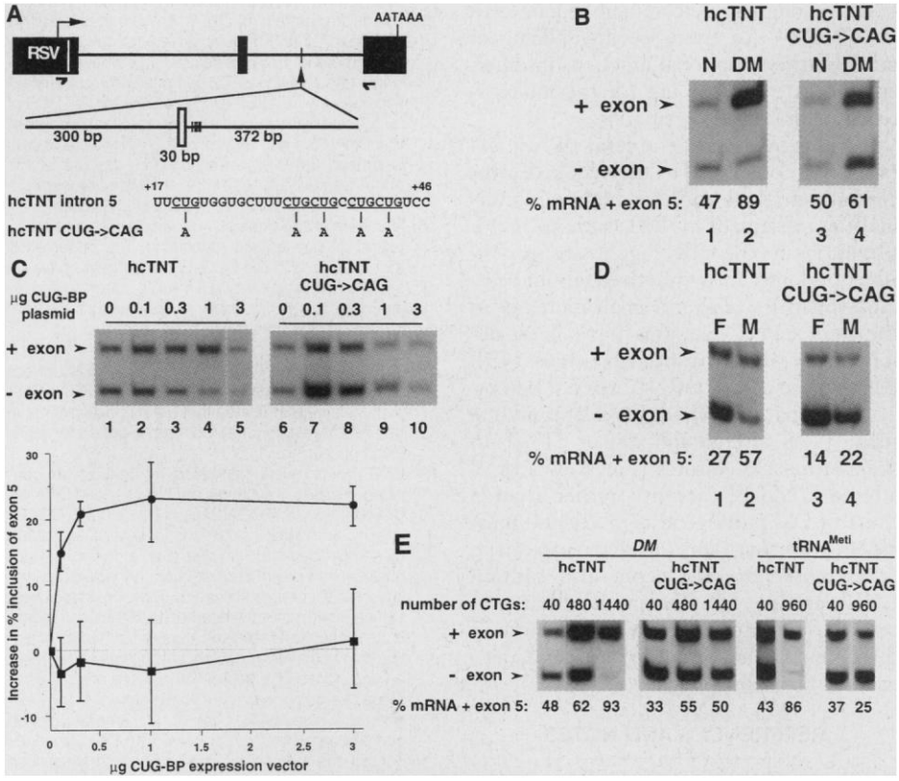

Fig. 3. Transient transfection analysis of human cTNT alternative splicing. (**A**) Structure of hcTNT and hcTNT CUG → CAG minigenes. Five vertical lines represent the proximal CUGs that are mutated in the hcTNT CUG → CAG minigene. Arrows indicate positions of the oligonucleotides used for RT-PCR analysis. The enhancer and transcription initiation site are from Rous sarcoma virus (RSV). (**B**) Transient transfection of hcTNT and hcTNT CUG → CAG minigenes into primary skeletal muscle cell lines from normal individuals (N) and DM patients. For the experiment shown, the percent of spliced mRNAs that include exon 5 is indicated below each lane. The averaged results from five or more independent experiments (lanes 1 to 4) are 41 ± 6.7%, 89 ± 4.5%, 41 ± 9.3%, and 50 ± 19%. (**C**) Response of hcTNT (circles) or hcTNT CUG → CAG (boxes) to increasing amounts of cotransfected CUG-BP expression vector in normal human primary skeletal muscle cell lines (n = 5 independent transfections). Full-length CUG-BP cDNA replaced the chloramphenicol acetyltransferase (CAT) gene in the pOPRS-VICat expression vector (Stratagene). The increase in percent inclusion of exon 5 was calculated as the difference in the percent of spliced mRNAs that include exon 5 with and without the cotransfected CUG-BP expression vector. (**D**) hcTNT and hcTNT CUG → CAG minigenes were transiently transfected into the QT35 quail fibroblast cell line (lanes F) and primary chicken embryo skeletal muscle cultures (lanes M) as described (*10*). Enhanced exon inclusion in chicken muscle cultures requires positive-acting factors that are induced as part of the myogenic program (*10*). The averaged results from five independent experiments (lanes 1 to 4) are 26 ± 4.6%, 57 ± 3.9%, 11 ± 3.0%, and 23 ± 2.0%. (**E**) Expression of an expanded repeat reproduces the trans-dominant effect. hcTNT and hcTNT CUG → CAG minigenes were transiently transfected (*15*) with the *DM* (3 µg) and $tRNA^{Met_i}$ (1 µg) expression vectors containing the number of CTG repeats indicated above each lane. Averaged results from four (*DM*) and three ($tRNA^{Met_i}$) independent experiments (left to right) are 49 ± 14%, 63 ± 14%, 79 ± 9%, 33 ± 6%, 44 ± 12%, 46 ± 12%, 50 ± 10%, 79 ± 7%, 29 ± 4%, and 23 ± 5%. RT-PCR analysis detected large amounts of *DM* transcripts in all samples shown.

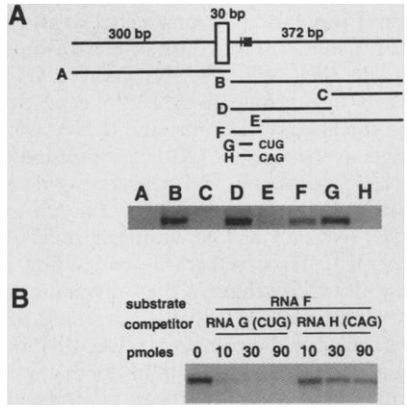

Fig. 4. Binding of CUG-BP to cTNT pre-mRNA. (**A**) The indicated PCR-generated fragments from the human cTNT gene were transcribed in vitro (*27*) from the T7 promoter by using guanosine [^{32}P]triphosphate. For UV cross-linking, 800 ng of bacterial rMBP–CUG-BP was preincubated with 3 µl (20 µg of protein) of HeLa cell nuclear extract (*28*) at 30°C for 10 min (*29*). The labeled RNA was added with a reaction mixture [containing 3.3 mM $MgCl_2$, 2.5 mM adenosine triphosphate, 25 mM creatine phosphate, and buffer D (*28*)] to 10 µl and incubated at 30°C for 10 min. Reaction mixtures were placed on an aluminum block prechilled in ice water and UV irradiated 4 cm from a germicidal lamp (Philips G15T8) for 8 min. Samples were digested with ribonuclease (RNase) A (0.5 µg) and RNase T1 (0.5 µg) at 37°C for 20 min, mixed with an equal volume of protein loading buffer, denatured at 90°C, and subjected to electrophoresis on an 11% SDS-polyacrylamide gel. (**B**) Binding of rMBP–CUG-BP to uniformly labeled RNA F in the presence of increasing amounts of unlabeled G or H RNAs.

to H). Recombinant MBP–CUG-BP did not bind to RNA containing the upstream intron and the alternative exon (Fig. 4A, RNA A), but it did bind to the downstream intron (RNA B). Recombinant MBP–CUG-BP binding localized to a 30-nt RNA (Fig. 3A, positions +17 to +46 of intron 5) containing the five CUG repeats (RNA G). Specific rMBP–CUG-BP binding to this segment was eliminated by the CUG → CAG mutations (RNA H). CUG-BP binding to RNA F was inhibited by RNA G but not by the identical RNA fragment H that contained the CUG → CAG substitutions (Fig. 4B). Thus, CUG-BP binds exclusively to the CUG motifs located 19 to 43 nt downstream of the alternative exon.

To determine whether the trans-dominant effect could be reproduced in normal muscle cells, we cotransfected the hcTNT and hcTNT CUG → CAG minigenes with *DM* minigenes that contained 40, 480, or 1440 CTG repeats in the 3′ UTR (*19*). Coexpression of *DM* minigenes containing 480 and 1440 repeats with the hcTNT minigene resulted in increased exon 5 inclusion (Fig. 3E). Furthermore, the amount of exon 5 inclusion appeared to correlate with the length of the expanded repeat. Splicing of exon 5 was essentially unaffected when the hcTNT minigene lacked the CUG-BP binding site (Fig. 3E). We also inserted CTG repeats into a human $tRNA^{Met_i}$ gene that was modified to accumulate tRNA transcripts in the nucleus (*20*). Coexpression of tRNAs containing 960 repeats promoted exon inclusion for the wild-type but not the CUG → CAG hcTNT minigene mRNAs (Fig. 3E). Thus, transcripts containing at least 480 CUGs have a trans-dominant effect on splicing of cTNT exon 5 and this effect requires the conserved CUG-BP binding site. In addition, the trans-dominant effect is independent of gene context and whether the RNA is transcribed by polymerase (Pol) II or Pol III.

Our results support the hypothesis that nuclear accumulation of CUG-BP in DM cells alters expression of genes that require CUG-BP for posttranscriptional processing. CUG-BP activity is increased in DM cells as assayed by the enhanced inclusion of cTNT exon 5, suggesting that the trans-dominant effect is not simply due to sequestration of CUG-BP on the expanded *DM* transcripts as originally proposed (*3*, *7*). Increased CUG-BP activity could be due to the increased nuclear concentration of CUG-BP. It remains to be determined whether CUG-BP colocalizes with transcripts from the expanded allele. It is also likely that the phosphorylation state of CUG-BP plays a role in its nuclear-cytoplasmic distribution as well as its intrinsic splicing activity, as demonstrated for other splicing factors (*21*). Altered ratios of CUG-BP phosphorylated isoforms with different splicing activities could have a strong effect on overall splicing activity.

The hnRNPs such as CUG-BP perform multiple posttranscriptional regulatory functions in the nucleus and cytoplasm (*22*). Thus, a wide array of aberrant RNA processing phenotypes could result from an overabundance of CUG-BP in the nucleus and a deficiency in the cytoplasm. Because CUG-BP is ubiquitously expressed, its role in muscle-specific splicing is most likely as a constitutive component of a muscle-specific splicing complex. A common requirement for CUG-BP for tissue-specific splicing or other posttranscriptional functions in different tissues could account for the multisystemic nature of DM symptoms.

Overexpression of the fetal cTNT isoform may contribute to the reduced myocardial function and conduction abnormalities observed in DM patients (*23*). Mutations in the cTNT gene are responsible for dominantly inherited familial cardiomyopathies (*24*) and small increases in the fetal cTNT isoforms have been described in some cardiomyopathies (*13*). Because the cTNT mRNA was not detected in normal or DM adult skeletal muscle tissue (*17*), altered splicing of cTNT in skeletal muscle cultures is an indicator of altered CUG-BP activity rather than a cause of DM pathogenesis in skeletal muscle. Introns that flank alternative exons in other muscle-specific genes also contain CUG repeats (*12*). We predict that processing of transcripts from one or more of these genes is disrupted in DM and contributes to pathogenesis.

REFERENCES AND NOTES

1. P. Harper, *Myotonic Dystrophy* (Saunders, Philadelphia, 1989).
2. A. Hunter *et al.*, *J. Med. Genet.* **29**, 774 (1992); C. Tsilfidis, A. E. MacKenzie, G. Mettler, J. Barcelo, R. G. Korneluk, *Nature Genet.* **1**, 192 (1992); J. B. Redman, R. G. Fenwick Jr., Y. H. Fu, A. Pizzuti, C. T. Caskey, *J. Am. Med. Assoc.* **269**, 1960 (1993).
3. L. T. Timchenko, C. T. Caskey, M. S. Swanson, *Cold Spring Harbor Symp. Quant. Biol.* **61**, 607 (1996).
4. Z. Korade-Mirnics, P. Babitzke, E. Hoffman, *Nucleic Acids Res.* **26**, 1363 (1998).
5. J. Wang *et al.*, *Hum. Mol. Genet.* **4**, 599 (1995).
6. B. M. Davis, M. E. McCurrach, K. L. Taneja, R. H. Singer, D. E. Housman, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7388 (1997); K. L. Taneja, M. McCurrach, M. Schalling, D. Housman, R. H. Singer, *J. Cell Biol.* **128**, 995 (1995); M. G. Hamshere, E. E. Newman, M. Alwazzan, B. S. Athwal, J. D. Brook, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7394 (1997).
7. L. T. Timchenko *et al.*, *Nucleic Acids. Res.* **24**, 4407 (1996).
8. L. T. Timchenko, N. A. Timchenko, C. T. Caskey, R. Roberts, *Hum. Mol. Genet.* **5**, 115 (1996).
9. R. Roberts *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13221 (1997).
10. K. R. Ryan and T. A. Cooper, *Mol. Cell. Biol.* **16**, 4014 (1996).
11. T. A. Cooper and C. P. Ordahl, *Science* **226**, 979 (1984); *J. Biol. Chem.* **260**, 11140 (1985).
12. T. A. Cooper, unpublished data.
13. P. J. Townsend, P. J. R. Barton, M. H. Yacoub, H. Farza, *J. Mol. Cell. Cardiol.* **27**, 2223 (1995); P. A. Anderson, N. N. Malouf, A. E. Oakeley, E. D. Pagani, P. D. Allen, *Circ. Res.* **69**, 1226 (1991).
14. Cardiac TNT is not expressed in adult skeletal muscle tissue but is reexpressed in cultures prepared from adult skeletal muscle as the satellite cells recapitulate the embryonic program [N. Toyota and Y. Shimada, in *Muscular Dystrophy: Biomedical Aspects*, S. Ebashi and E. Ozawa, Eds. (Japan Science Society Press, Tokyo, 1983), pp. 89–97; F. Edom, V. Mouly, J. P. Barbet, M. Y. Fiszman, G. S. Butler-Browne, *Dev. Biol.* **164**, 219 (1994)].
15. Normal human skeletal muscle cells were purchased from Clonetics (San Diego). DM skeletal muscle cells were from patient DM1 (*3*, *25*). CUG-BP localizes in nuclei in this DM cell line (*3*, *7*) and these cells have been shown to express reduced amounts of *DM* transcripts and protein (*3*, *26*). Cells were maintained as described (*9*) and transfected by the calcium phosphate method. RNA was extracted 48 hours later for single transfections or 72 hours later for cotransfections with the CUG-BP expression vector. RT-PCR of minigene mRNA was carried out as in Fig. 2B except that RNA from one-quarter of a 60-mm plate of cells was used for reverse transcription, one-half of the cDNA was used for PCR, the annealing temperature was 68°C, and PCR was done with 25 cycles.
16. Normal and DM skeletal muscle cultures were maintained as proliferating myoblasts. The observation that mutations in the CUG-BP binding site have no effect on splicing in normal muscle cultures is probably due to the absence of coregulators that are present in differentiated striated muscle.
17. Data not shown.
18. The fact that the CUG → CAG mutations did not completely eliminate exon 5 inclusion in cTNT mRNA in muscle cells indicates that additional elements in the 702-nt genomic fragment are required for regulation of splicing.
19. CTG repeats were generated by head-to-tail concatamerization of synthetic double-stranded DNA oligonucleotides 5′-TCGA$(CTG)_{20}$C-3′ and 5′-TCGAG-$(CAG)_{20}$-3′ containing 20 CTG trinucleotides flanked by compatible restriction sites (Sal I and Xho I). Oligonucleotides were phosphorylated, annealed, gel-isolated, and concatamerized by ligation in solution. Concatamers not in the head-to-tail orientation regenerate Sal I or Xho I sites and were eliminated by digestion with both enzymes. Concatamers were gel isolated and cloned into the Sal I–Xho I sites of sp72 (Promega). Large concatamers were "grown" in the plasmid by sequential cloning of smaller cloned concatamers into the Sal I site. The *DM* minigenes contain the cytomegalovirus enhancer/transcription initiation site fused to a *DM* genomic fragment containing exons 11 (exon position 38) to 15. Exon 15 is truncated at the position of the natural CTG repeats (exon position 376) by the synthetic CTG repeats of lengths indicated in Fig. 3E. Presumably the transcript utilizes a cryptic polyadenylation site within the downstream plasmid sequence. We found that expression from constructs that lacked the *DM* polyadenylation site was more consistent than that from constructs that contained the *DM* polyadenylation site (*17*).
20. The modified human $tRNA^{Met_i}$ gene contains an internal Pol III promoter and is truncated at position 62 by a Sal I site into which the CTG concatamers were cloned. In the original construct, truncation of the mature tRNA prevents 3′ end processing and causes nuclear accumulation of transcripts [E. Bertrand *et al.*, *RNA* **3**, 75 (1997)].
21. S. H. Xiao and J. L. Manley, *Genes Dev.* **11**, 334 (1997); J. F. Caceres, G. R. Screaton, A. R. Krainer, *ibid.* **12**, 55 (1998).
22. F. Weighardt, G. Biamonti, S. Riva, *Bioessays* **18**, 747 (1996).
23. M. F. Phillips and P. S. Harper, *Cardiovasc. Res.* **33**, 13 (1997); L. S. Tokgozoglu *et al.*, *J. Am. Med. Assoc.* **274**, 813 (1995).
24. H. Watkins *et al.*, *N. Engl. J. Med.* **332**, 1058 (1995); L. Thierfelder *et al.*, *Cell* **77**, 701 (1994).
25. E. Roeder *et al.*, *Am. J. Hum. Genet.* **55**, A7 (1994).
26. R. Krahe *et al.*, *Genomics* **28**, 1 (1995).

27. T. A. Cooper, *J. Biol. Chem.* **267**, 5330 (1992).
28. J. D. Dignam, R. M. Lebovitz, R. G. Roeder, *Nucleic Acids. Res.* **5**, 1475 (1983).
29. The sequence specificity of rMBP–CUG-BP binding was increased by a 10-min preincubation in HeLa cell nuclear extracts, which suggests that modification of the protein, most likely phosphorylation, is required for optimal activity.
30. We thank J. Seidman and L. Thierfelder for genomic clones containing hcTNT exon 5; C. Lo for excellent technical assistance; and B. Casey, D. Nelson, and S. Berget for critical comments on the manuscript. Supported by grants from the Muscular Dystrophy Association and the National Institutes of Health (HL45565 to T.A.C. and AR 44387 to L.T.T.).

23 December 1997; accepted 25 March 1998

Yeast Ku as a Regulator of Chromosomal DNA End Structure

Serge Gravel, Michel Larrivée, Pascale Labrecque, Raymund J. Wellinger*

During telomere replication in yeast, chromosome ends acquire an S-phase–specific overhang of the guanosine-rich strand. Here it is shown that in cells lacking Ku, a heterodimeric protein involved in nonhomologous DNA end joining, these overhangs are present throughout the cell cycle. In vivo cross-linking experiments demonstrated that Ku is bound to telomeric DNA. These results show that Ku plays a direct role in establishing a normal DNA end structure on yeast chromosomes, conceivably by functioning as a terminus-binding factor. Because Ku-mediated DNA end joining involving telomeres would result in chromosome instability, our data also suggest that Ku has a distinct function when bound to telomeres.

The Ku protein, comprising two subunits of about 70 and 85 kD, appears to be present in all eukaryotic cells, which suggests that it is involved in a conserved and important function. Originally identified as an autoimmune antigen localized to the nucleus, this protein together with a third component, the catalytic subunit of a DNA-dependent protein kinase (DNA-PK_{cs}), is now known to be critical for nonhomologous DNA double-strand break repair and the site-specific recombination of the V(D)J gene segments (*1*). In vitro, mammalian Ku binds directly to double-stranded (ds) DNA ends in a sequence-independent fashion (*2*). Thus, it has been postulated that Ku associates with dsDNA ends produced by DNA damage or during recombination and may recruit additional factors necessary for successful end joining (*1*).

The yeast *Saccharomyces cerevisiae* contains two genes, *HDF1* and *YKU80/HDF2* (hereafter referred to as *YKU80*), which are homologs of the two mammalian Ku subunits (*3*–*5*). Like its mammalian counterpart, yeast Ku binds DNA ends only in the heterodimeric form (*5*). Consistent with the proposed roles for Ku in mammalian cells, yeast cells lacking Ku activity are severely impaired in Rad52-independent nonhomologous DNA end joining (NHEJ) (*4*–*6*). Furthermore, yeast strains devoid of Ku do not grow at elevated temperatures (*3*, *4*) and

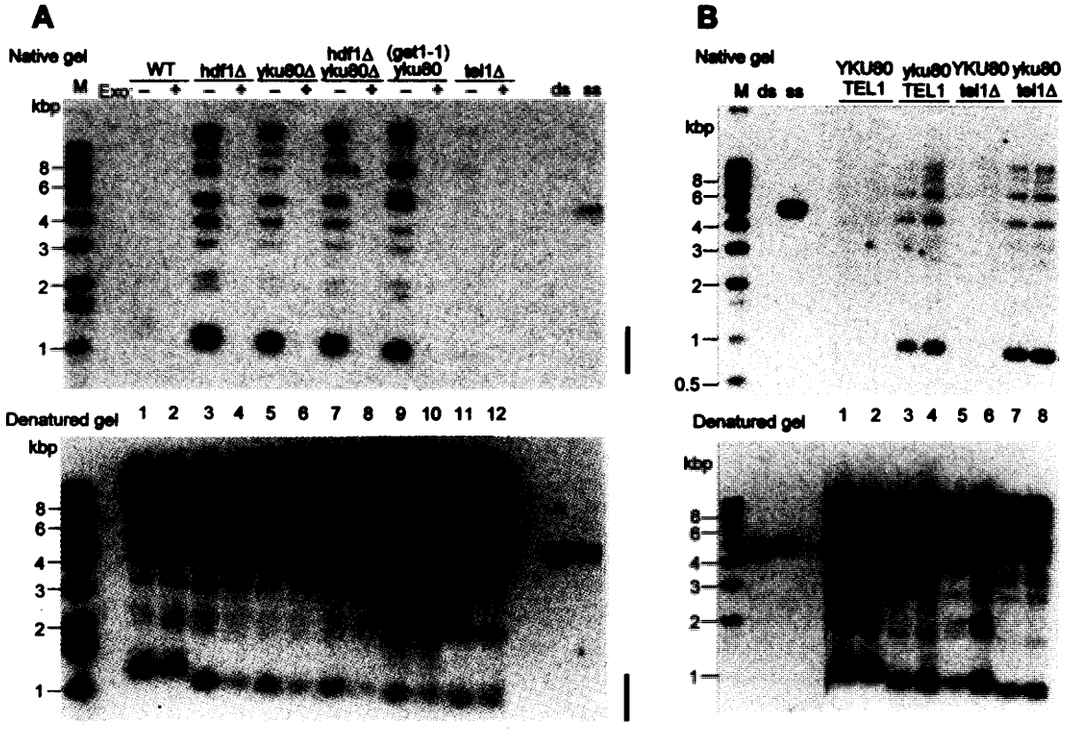

Fig. 1. Altered telomeric end structure caused by mutations in yeast Ku genes. (**A**) (Top) DNA from strains with the indicated genotypes and grown at 23°C was mock treated (lanes 1, 3, 5, 7, 9, and 11) or digested with *E. coli* exonuclease I (lanes 2, 4, 6, 8, 10, and 12). All samples were then digested with Xho I and analyzed by nondenaturing in-gel hybridization with a 22-mer oligonucleotide probe composed of telomeric $C_{1-3}A$ repeats (*12*). About two-thirds of the terminal restriction fragments are of a size indicated by the bar. Additional bands derive from individual telomeres lacking a subtelomeric repeated element Y′. Lanes ss and ds contain DNA with telomeric TG_{1-3} repeats in ss and ds form, respectively. Lane M, end-labeled 1-kb ladder DNA (Gibco-BRL) serving as size standard. (Bottom) Same gel after denaturation of the DNA and rehybridization with the same telomeric probe. The following strains were used: DWY291 (lanes 1 and 2), DWY290 (lanes 3 and 4), DWY292 (lanes 5 and 6), DWY293 (lanes 7 and 8), RWY737d (lanes 9 and 10), KWRY100 (lanes 11 and 12) (*29*). (**B**) A diploid yeast strain formed with haploids KWRY100 (*tel1Δ::HIS3, YKU80*) and RWY739b (*TEL1, yku80*) was sporulated and individual tetrads were dissected. All four spores from a tetratype tetrad were grown at 23°C (lanes 1, 3, 5, and 7) or incubated at 37°C (lanes 2, 4, 6, and 8) before DNA isolation and analysis by nondenaturing hybridization (top), followed by denaturation of the DNA and rehybridization (bottom) as in (A).