J Biochem 130, 581–587 (2001)

Coexpression of the CUG-Binding Protein Reduces DM Protein Kinase Expression in COS Cells[1]

Nobuhiro Takahashi,*[2] Noboru Sasagawa,* Fusako Usuki,† Yoshihiro Kino,* Hiroyuki Kawahara,‡[3] Hiroyuki Sorimachi,‡ Tatsuya Maeda,‡ Koichi Suzuki,‡[4] and Shoichi Ishiura*[5]

Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, †Department of Clinical Medicine, National Institute of Minamata Disease, 4058-18 Hama, Minamata, Kumamoto 867, ‡Laboratory of Molecular Structure and Functions, Department of Molecular Biology, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-0032

Received May 25, 2001, accepted August 14, 2001

Myotonic dystrophy (DM) is the most common form of adult onset muscular dystrophy. Patients have a large CTG repeat expansion in the 3′ untranslated region of the *DMPK* gene, which encodes DM protein kinase. RNA *trans*-dominant models, which hypothesize that the expanded CUG trinucleotide repeat on *DMPK* mRNA sequesters a factor or disrupts the RNA metabolism of the *DMPK* mRNA itself and other mRNAs in a trans dominant manner, have been proposed. A candidate for the sequestered factor, termed CUG-binding protein (CUG-BP), exists in several alternatively spliced isoforms. We found a human isoform with a twelve base insertion (deduced amino acids Leu-Tyr-Leu-Gln) and an isoform with a three base insertion (deduced amino acid Ala) insertion. In order to elucidate the effects of CUG-BP on *DMPK* expression, we introduced *CUG-BP* and *DMPK* cDNA transiently into COS-7 cells. Cotransfection of CUG-BP did not significantly affect the expression of either wild type or mutant *DMPK* at the mRNA level. On the other hand, cotransfection of CUG-BP significantly affected the expression of both the wild type and mutant *DMPK*s at the protein level. This reduction was remarkable when the mutant *DMPK* construct was used.

Key words: CUG-BP, DMPK, myotonic dystrophy, triplex repeat.

Myotonic dystrophy (DM) is the most common form of adult onset muscular dystrophy with an incidence of 1 in 8,000 births. Patients have a large CTG repeat expansion in the 3′ untranslated region (3′UTR) of the *DMPK* gene, which encodes DM protein kinase (*1–3*). The clinical symptoms in adult patients vary widely. Mildly affected patients suffer from cataracts and myotonia while the more severely affected suffer from muscle weakness, progressive muscle wasting, cardiac abnormalities, diabetes, and testicular atrophy. The most severely affected congenital myotonic dystrophy (CDM) patients display mental retardation and muscle hypotonia. The severity of the symptoms correlates with the length of the repeat In normal populations, the repeat number ranges from 5 to 37. In DM patients, however, the repeat number increases to 50–100 repeats in patients with mild symptoms to thousands of repeats in those with the most severe symptoms. There is a tendency for progeny to have greater repeat expansions than the affected parent, resulting in earlier onset and more severe symptoms, a phenomenon referred to as anticipation.

The molecular mechanism underlying myotonic dystrophy remains unknown, although three distinct but probably complementary hypotheses have been proposed (*4, 5*). One hypothesis is based on the fact that the expression of the *DMPK* protein is reduced in DM patients (*6, 7*). Haploinsufficiency has been proposed as a mechanism to explain the dominant nature of DM. *DMPK* knockout mice develop late-onset, progressive skeletal myopathy (*8, 9*), while heterozygous mice show atrioventricular conduction abnormalities (*10*). These mice, however, display neither the myotonia nor cataracts that are the hallmarks of DM. Although reduced levels of *DMPK* expression may contribute to some extent to the pathogenesis of DM, some other mechanisms must exist in order to fully explain the complex array of symptoms associated with the disease.

A second model considers the effects of the CTG repeat expansion on the expression of neighboring genes. The *SIX5* gene (formerly *DMAHP*) is located just downstream of *DMPK* and the CTG repeat exists simultaneously in the 5′ control region of *SIX5* (*11*). The *DMWD* gene (formerly 59) is located just upstream of the *DMPK* gene (*12*). Some studies have indicated that the expansion of the *DMPK* (CTG) repeat reduces the expression of the *SIX5* gene (*13,*

[1] This work was supported in part by a grant from the Ministry of Health and Welfare, Japan, and Grant-in-Aid for Scientific Research on Priority Area (C) —Advanced Brain Project— from the Ministry of Education, Culture, Sports, and Technology of Japan.
Present addresses: [2] Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institute of Health, Building 6B/Room 413, 9000 Rockville Pike, Bethesda, Maryland 20892-2790, USA; [3] Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Kita-ku, Sapporo 060-0812, [4] Tokyo Metropolitan Institute of Gerontology, 35-2 Sakaecho, Itabashi-ku, Tokyo 173-0015
[5] To whom correspondence should be addressed. Tel: +81-3-5454-6739, Fax +81-3-5454-6739, E-mail cishiura@mail ecc.u-tokyo ac.jp

14) while others have shown that the expression level of the *SIX5* gene is not altered in DM patients (*15*). Recently, the tissue-specific and allele-specific reduction of *SIX5* expression upon CTG repeat expansions was described (*16*). Similarly, an allele-specific reduction in the *DMWD* RNA level in DM patient samples has been reported (*15, 17*). Mice that overexpress or are deficient in these genes have not yet been described, and the extent of their contribution to DM pathogenesis remains unknown

RNA *trans*-dominant models have also been proposed (*18–20*). These models hypothesize that the expanded CUG trinucleotide repeat on *DMPK* mRNA sequesters a factor or disrupts the RNA metabolism of the *DMPK* mRNA itself and other mRNAs in a *trans*-dominant manner. Decreases in both the mutant and normal *DMPK* messages in poly(A) RNA from DM patients have been observed (*20*). Others have reported the nuclear retention of the *DMPK* transcript and the formation of nuclear foci in cultured cell lines from DM patients (*18*). A candidate for a sequestered factor has been identified and termed CUG-binding protein (CUG-BP) (*21*). The phosphorylation state and intracellular distribution of CUG-BP is altered in DM patients and *DMPK* knockout mice (*19*). CUG-BP regulates the alternative splicing of the human cardiac troponin T (cTNT) premessenger RNA, and this regulation is lost in DM patients (*22*).

For this study, we developed a cell culture system in which to investigate the molecular mechanisms underlying the phenomena observed in DM patient samples.

We introduced wild type ($(CTG)_5$) and mutant ($(CTG)_{130}$) *DMPK* cDNAs transiently and examined the expression of the *DMPK* mRNA and protein. We show that a $(CTG)_{130}$ repeat has significant negative effects on the expression of both the *DMPK* mRNA and protein We cotransfected CUG-BP in these circumstances and further examined the expression of the *DMPK* mRNA and protein CUG-BP has significant negative effects on the expression of *DMPK* proteins

MATERIALS AND METHODS

Construction of Plasmids—Human CUG-BP cDNA was amplified from a human cDNA library by PCR using the oligonucleotides 5′-GCTCAAAGAAAATGAACGGC-3′ and 5′-AAGAGACAGGGATTTGGACG-3′ as sense (CUG-BP forward) and antisense (CUG-BP reverse) primers, respectively. The PCR mixture in 50 μl [5 μl of 10 × PCR buffer, 10 nmol of dNTP, 25 pmol of each primer, 0.1 μg of cDNA library (CLONTECH), and 1.25 units of *Pfu* polymerase (STRATAGENE)] was subjected to 30 thermal cycles of 94°C for 45 s, 59°C for 1 min, and 72°C for 4 min. Mouse CUG-BP cDNA was amplified from mouse total RNA by RT-PCR. First strand cDNA was reverse transcribed with CUG-BP reverse primer and the ThermoScript™ RT-PCR System (Gibco BRL). The RT-PCR reaction was performed essentially according to the manufacturer's protocol. One microgram of total RNA and CUG-BP reverse primer (10 pmol) were incubated at 65°C and placed on ice. An aliquot (10 μl) of the cDNA synthesis mixture (4 μl of 5 × cDNA Synthesis Buffer, 0.1 μmol DTT, 20 nmol each of dNTP, 40 units of RNaseOUT™, 1 μl of DEPC-treated water, and 15 units of ThermoScript™ RT) was added to the tube and the mixture was incubated at 55°C for 45 min. The reaction was terminated by incubating at 85°C for 5 min. The PCR mixture in 50 μl [5 μl of 10 × PCR buffer, 0 1 μmol $MgCl_2$, 10 nmol of dNTP, 25 pmol of each primer, 5 μl of the RT-PCR product, and 2.5 units of LA *Taq* polymerase (TaKaRa)] was subjected to thermal cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 2 min.

pSRD (a mammalian expression vector having SV40 promotor, kindly provided by Prof. Shigeo Ohno, Yokohama City University School of Medicine)/CUG-BP was constructed by amplifying a fragment of about 1.6 kbp from a human skeletal muscle cDNA library by PCR using the CUG-BP forward and reverse primers. This amplified fragment was ligated into the *Pvu*II site of pSRD-MCS vector, generating pSRD/CUG-BP

The full-length *DMPK* cDNA [pTV118N/*DMPK*$(CTG)_5$] was obtained as described before (*23*) This cDNA contained a total of five (CTG) repeats. *DMPK* cDNA with an *Eco*T22I site in the 3′UTR instead of a CTG repeat [pTV118N/*DMPK*$(CTG)_0$] was prepared as described before (*24*). A $(CTG/CAG)_{130}$ repeat DNA was artificially synthesized as described before (*25*) and ligated into the *Eco*T22I site of the pTV118N/*DMPK*$(CTG)_0$ vector, creating the mutant pTV118N/*DMPK*$(CTG)_{130}$ cDNA. Wild type and mutant *DMPK* cDNAs were ligated into the *Eco*RI site of pSRD vector, generating pSRD/*DMPK*$(CTG)_5$ and pSRD/*DMPK*$(CTG)_{130}$.

Cell Culture and Transfection Experiment—COS-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, SIGMA) supplemented with 10% fetal bovine serum (FBS, SIGMA) The cells were washed once with phosphate-buffered saline without magnesium and calcium [PBS(–)], lifted with trypsin, washed once again with PBS(–), and resuspended in K-PBS (30.8 mM NaCl, 120.7 mM KCl, 8 1 mM Na_2HPO_4, 1 46 mM KH_2PO_4, and 25 mM $MgCl_2$) at 1 × 10^6 cells/ml. This suspension (500 μl) was placed in an electroporation cuvette (Gene Pulser cuvette 0.4 cm, BIO-RAD) with 5 μg of each plasmid. Electroporation was performed under the following conditions: resistance at infinity, voltage at 0.22 kV, and capacitance at 0.975 μF (Gene Pulser II Electroporation System, BIO-RAD).

RNA Extraction and Northern Blotting—Mouse tissues were washed with PBS(–), frozen immediately in liquid nitrogen, and stored at –80°C until use. Tissues were homogenized with a Polytron homogenizer and total RNA was extracted by the standard acid guanidium-phenol-chloroform (AGPC) method. The integrity of the RNA was confirmed by agarose gel electrophoresis and ethidium bromide staining.

Sixty hours after transfection, cells were collected and total RNA was extracted by the AGPC method. Total RNA was separated by electrophoresis in 1% agarose-18% formaldehyde denaturing gels in MOPS buffer [20 mM 3-(*N*-morpholino) propanesulfonic acid, pH 7.0, 5 mM sodium acetate, 1 mM EDTA] and transferred to Biodyne Transfer Membranes (Pall). The blots were hybridized in ULTRAhyb™ buffer (Ambion) containing the ^{32}P-labeled probes overnight at 42°C. The final wash conditions were 2× SSC in 0.1% SDS at 42°C two times for 5 min and 0.1× SSC in 0 1% SDS at 42°C two times for 15 min. The filters were exposed to an Imaging Plate (BAS-IP MS 2040, FUJI PHOTO FILM) for 3–30 h and analyzed with a BAS1000 system (FUJI PHOTO FILM).

For the *DMPK*-specific probe, a 478 bp *Eco*T14I fragment of *DMPK* cDNA was used For the human CUG-BP–specific probe, a 744 bp *Kpn*I fragment of human CUG-BP cDNA was used. For the mouse CUG-BP–specific probe, a 534 bp *Eco*T14I fragment of mouse CUG-BP cDNA was used. PCR was performed to prepare the β-actin– and GAPDH–specific probes. A 321 bp fragment of the β-actin cDNA was PCR amplified from a mouse cDNA library using the forward primer 5′-GTGACATTAAGGAGAAGCTGTGC-3′ and the reverse primer 5′-TCTCCTTCTGCATCCTGTCGGC-3′. A 348 bp fragment of the GAPDH cDNA was PCR amplified from a mouse cDNA library using the forward primer 5′-GCCAAAAGGGTCATCATCTCTG-3′ and the reverse primer 5′-CATGCCAGTGAGCTTCCCGT-3′. These fragments were cloned into the pGEM-T Easy vector. The fragments, 0.1–0.2 μg of each, were used as templates for the DNA probes. The DNA probes were labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) using a Megaprime DNA labelling system (Amersham Pharmacia Biotech)

H-Mapping—H-mapping was performed essentially as described before (*23*). Briefly, total RNA was dissolved in 20 μl of DEPC-treated water supplemented with 1 mM EDTA (pH 7 4) RNA was denatured by heating for 10 min at 70°C. Antisense oligo DNA (5′-GCCGAAAGAAAGAAATGGTC-3′), 0.5 μg, was added and the solution was incubated for 15 min at 20°C. One microliter of 4 M KCl was added and the solution was incubated for an additional 15 min at 20°C. Twenty microliters of TM buffer (40 mM Tris, pH 7.4, and 60 mM $MgCl_2$) and 0.8 unit of Ribonuclease H (Gibco BRL) were added and the reaction was incubated for 30 min at 37°C. RNA was extracted one time with phenol-chloroform and chloroform and ethanol precipitated. This RNA was analyzed by the standard Northern blotting procedure. For the *DMPK* 3′UTR-specific probe, a 305 bp *Pst*I fragment of *DMPK* cDNA was used.

Antibodies and Immunoblotting—The antibodies used for analyses were raised in rabbits using peptides synthesized from the predicted peptide sequence as antigens. The *DMPK* antibody recognizes *DMPK* amino acid residues 593–625 and the CUG-BP antibody recognizes CUG-BP amino acid residues 469–482.

Tissues were homogenized with a Polytron homogenizer in RIPA buffer (0.15 M NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl pH 8.0, and 1 mM EDTA) and the total protein was extracted. Sixty hours after transfection, the cells were collected and the total protein was extracted by sonication in buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100). Total protein concentration was measured with a DC Protein Assay Kit II (BIO-RAD). Protein (20–50 μg) was loaded onto a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane (NT-32, NIHON EIDO). The membrane was blocked with 5% skim milk prepared with TPBS buffer (2% NaCl, 0.725% $Na_2HPO_4 \cdot 12H_2O$, 0.05% KCl, 0 05% KH_2PO_4, and 0.125% Tween 20) for 1 h at room temperature. The membrane was washed and incubated with primary antibody in TPBS buffer for 1 h at 37°C, then washed and incubated with secondary antibody (Anti-Rabbit HRP-Linked Antibody, New England Biolabs) for 30 min. Immunoreactive proteins were detected using a POD Immunostain Set (Wako Pure Chemical Industries). Protein expression was analyzed with an Image Master™ System (Amersham Pharmacia Biotech).

RESULTS

The CUG-Binding Protein Exists in Multiple Isoforms—First, we amplified a PCR fragment of CUG-BP from human skeletal muscle and human brain cDNA libraries. The polymerase chain reaction yielded a single band of the predicted size (1.6 kbp) and the product was cloned. Sequence analyses revealed that CUG-BP exists in several alternatively spliced isoforms (Fig. 1a). In addition to the originally reported isoform, we found an isoform with a twelve base (deduced amino acids Leu-Tyr-Leu-Gln) insertion at the 688th nucleotide from the initiation codon when we sequenced clones from human skeletal muscle. However, an isoform with a three base (deduced amino acid Ala) insertion at the 880th nucleotide was found exclusively when we sequenced clones from human brain. These insertions lie in a linker domain between the second and third RNA binding domains (RBDs) (*26*). We termed these isoforms CUG-BP+LYLQ (GenBank accession no. AF267533) and CUG-BP+A (GenBank accession no. AF267534), respectively.

We also cloned CUG-BP from a mouse liver cDNA library. Sequence analyses revealed that this clone contains the same twelve base insertion at the same position as human CUG-BP+LYLQ. We termed this isoform mCUG-BP+LYLQ (GenBank accession no. AF267535). Human and mouse CUG-BP share 93.7 and 98.8% homology at the nucleotide and amino acid levels, respectively (Fig. 1, a and b).

Tissue-Specific Distribution of CUG-BP—When considering the multisystemic nature of DM, it is very important to examine the tissue distribution of the CUG-BP mRNA and protein. Although previous studies have shown the existence of CUG repeat–binding activities in various tissues (*5*), quantitative analysis has not yet been described. In order to investigate the tissue distribution of the CUG-BP mRNA and protein, total RNA and total proteins were extracted from various mouse tissues CUG-BP mRNA was detected with a mouse CUG-BP–specific probe. We observed moderate signals in brain and testis and relatively weak signals in heart, skeletal muscle, lung, and spleen (Fig. 2a)

Next, we developed a rabbit polyclonal antibody raised against the C-terminal 14 amino acids of CUG-BP. CUG-BP was detected with this antibody, and a tissue-specific distribution of the protein was observed. CUG-BP is moderately expressed in lung and spleen and weakly expressed in brain, eye, and testis (Fig. 2b). No signals were detectable in heart, skeletal muscle, liver, or kidney. In previous studies (*5, 7*), CUG-BP was found as a doublet of 49 and 51 kDa, but we observed a major band at about 50 kDa in brain, lung, and spleen. This is exactly the same size as that of CUG-BP expressed ectopically in COS-7 cells (Fig. 2b, COS). However, two bands were detected in eye and testis, the molecular mass of the latter the same as reported. The bands disappear when the CUG-BP antibody is preincubated with antigen polypeptide, confirming that these bands represent CUG-BP (data not shown).

Cotransfection of CUG-BP Does Not Affect the Expression of DMPK mRNA—In order to elucidate the effects of CUG-BP on *DMPK* expression, we introduced CUG-BP (pSRD/

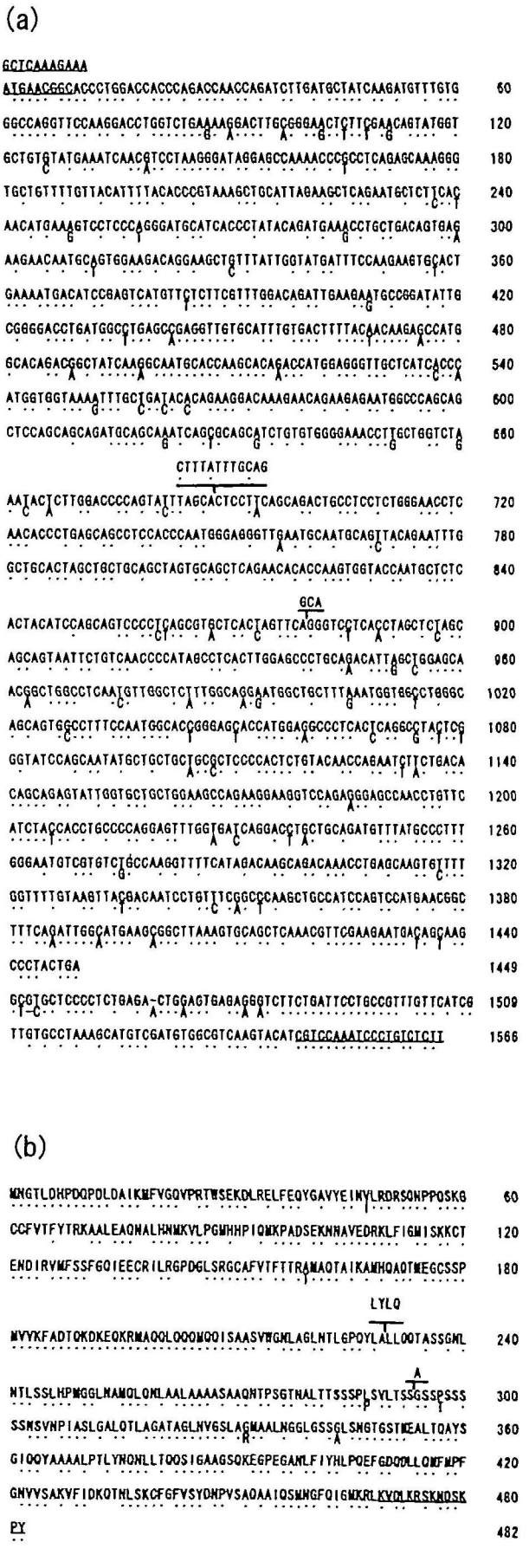

Fig. 1. **Nucleotide and deduced amino acid sequences of human and mouse CUG-BP cDNA.** (a) Alignment of the human and mouse CUG-BP messages. The human sequence is shown on top and the mouse sequence below, identical nucleotides are indicated by dots and gaps by hyphens. Sequence positions of CUG-BP forward and reverse primers are underlined. Twelve or three nucleotide insertions found in CUG-BP+LYLQ and mCUG-BP+LYLQ or CUG-BP+A, respectively, are indicated above the sequences. ATG initiation codons and TGA termination codons are shown in bold face. (b) Alignment of the deduced amino acid sequence of human and mouse CUG-BP. The human sequence is shown on top and the mouse sequence below, identical amino acids are indicated by dots. Insertions of four or one amino acids found in CUG-BP+LYLQ and mCUG-BP+LYLQ or CUG-BP+A, respectively, are indicated above the sequences. The 14 amino acid sequence recognized by the CUG-BP antibody is underlined

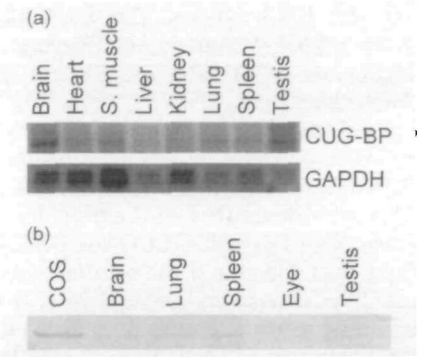

Fig 2 **Tissue distribution of mouse CUG-BP mRNA and protein.** (a) Northern blot analysis of CUG-BP mRNA expression in various mouse tissues. Each lane contains 15 μg of total RNA. The membrane was rehybridized with radioactive GAPDH cDNA as an internal reference for the amount of RNA loaded. (b) Western blot analysis of CUG-BP protein expression. Each lane contains 50 μg of total protein

CUG-BP) and *DMPK* [pSRD/*DMPK*$(CTG)_5$ or pSRD/*DMPK*$(CTG)_{130}$] cDNAs transiently into COS-7 cells. We constructed a mutant *DMPK* cDNA artificially (see "MATERIALS AND METHODS"). Total RNA and protein were extracted from the cells 60 h after transfection. *DMPK* transcripts were analyzed by Northern blotting and detected with a ^{32}P-labeled *DMPK*-specific probe.

Expression of both *DMPK*$(CTG)_5$ and *DMPK*$(CTG)_{130}$ mRNA was observed (Fig. 3a: lanes 2 and 3), with the *DMPK*$(CTG)_{130}$ mRNA migrating more slowly than the *DMPK*$(CTG)_5$ mRNA, as expected. Autoradiography of the bands revealed that the level of *DMPK*$(CTG)_{130}$ mRNA expression was slightly lower than that of *DMPK*$(CTG)_5$ (Fig. 3b· bars 2 and 3), but the difference was not significant. When we cotransfected CUG-BP with *DMPK*, the expression of both wild type and mutant *DMPK* mRNA was reduced (Fig. 3a: lanes 5 and 6 and Fig. 3b bars 5 and 6), but, again, not significantly.

Cotransfection of CUG-BP Reduces the Expression of DMPK at the Protein Level—The expression of the *DMPK* protein was analyzed by Western blotting and immunostaining with an antibody that recognizes the carboxyl terminus of *DMPK*. *DMPK* expression was observed when wild type or mutant *DMPK* cDNA was introduced into cells (Fig. 4a: lanes 2 and 3). In each case, the relative molecular mass of *DMPK* was about 70 kDa, as expected, because the

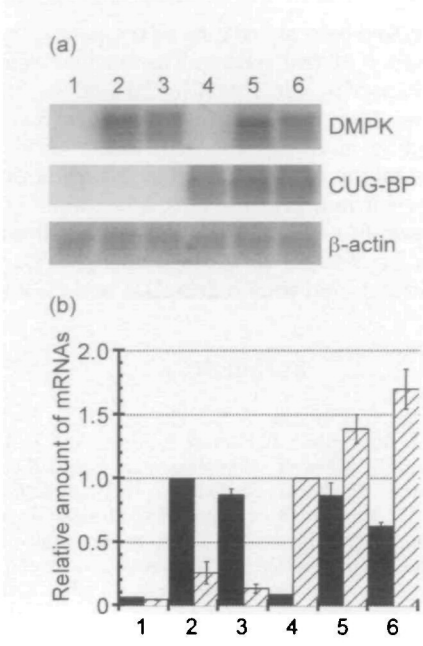

Fig 3 **CUG-BP weakly suppresses the expression of DMPK mRNA.** (a) Northern blot analysis of DMPK and CUG-BP mRNAs in cells cotransfected with the corresponding cDNAs. Each lane contains 10 μg of total RNA extracted from cells transfected with vector (lane 1), pSRD/DMPK(CTG)$_5$ (2), pSRD/DMPK(CTG)$_{130}$ (3), vector and pSRD/CUG-BP (4), pSRD/DMPK(CTG)$_5$ and pSRD/CUG-BP (5), or pSRD/DMPK(CTG)$_{130}$ and pSRD/CUG-BP (6) A representative result of three independent experiments is shown (b) Quantitative analysis of DMPK and CUG-BP mRNAs. Solid bars represent relative radioactivities of bands probed with a DMPK-specific probe (lane 2 = 1 0). Hatched bars represent those probed with a CUG-BP-specific probe (lane 4 = 1 0) Error bars denote the standard deviations for experiments performed in triplicate

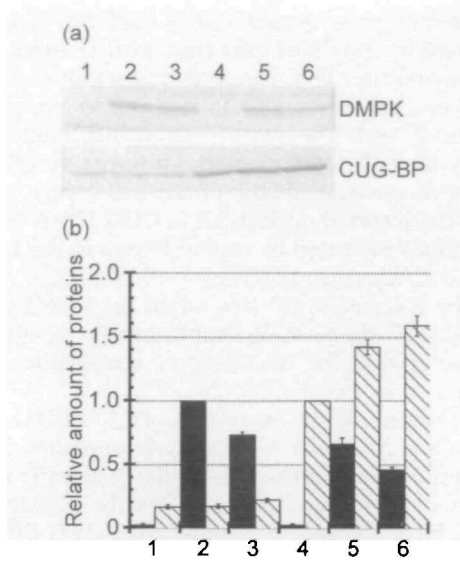

Fig 4 **CUG-BP significantly suppresses the expression of the DMPK protein.** (a) Western blot analysis of DMPK and CUG-BP proteins in cells cotransfected with the corresponding cDNAs. Each lane contains 20 μg of total protein extracted from cells transfected with vector (lane 1), pSRD/DMPK(CTG)$_5$ (2), pSRD/DMPK(CTG)$_{130}$ (3), vector and pSRD/CUG-BP (4), pSRD/DMPK(CTG)$_5$ and pSRD/CUG-BP (5), or pSRD/DMPK(CTG)$_{130}$ and pSRD/CUG-BP (6). A representative result of three independent experiments is shown (b) Quantitative analysis of the DMPK and CUG-BP proteins. Solid bars represent relative densities of bands probed with a DMPK-specific antibody (lane 2 = 1 0) Hatched bars represent those probed with a CUG-BP–specific antibody (lane 4 = 1.0) Error bars denote the standard deviations for experiments performed in triplicate

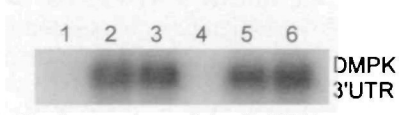

Fig 5 **Poly(A) shortening of the DMPK transcript is not observed, regardless of the CTG repeat length and coexpression of CUG-BP.** Poly(A) shortening was assayed as follows: A deoxyoligonucleotide complementary to a region immediately downstream of the CTG repeat was annealed to the mRNA. The hybrids were treated with RNase H to cleave the mRNA at the RNA-DNA duplex, and the RNA fragments were separated by electrophoresis, blotted, and hybridized to a ^{32}P-labeled probe specific for the 3′ end fragment Each lane contains 10 μg of total RNA extracted from cells transfected with vector (lane 1), pSRD/DMPK(CTG)$_5$ (2), pSRD/DMPK(CTG)$_{130}$ (3), vector and pSRD/CUG-BP (4), pSRD/DMPK-(CTG)$_5$ and pSRD/CUG-BP (5), or pSRD/DMPK(CTG)$_{130}$ and pSRD/CUG-BP (6)

CTG repeat lay within the 3′UTR of *DMPK* Quantitation of the bands with a densitometer revealed that the expression of the *DMPK* protein from the *DMPK*(CTG)$_{130}$ construct was reduced significantly by 20–30% as compared with the *DMPK*(CTG)$_5$ construct ($p < 0.05$) (Fig. 4b: bars 2 and 3). When we immunostained the same samples with the CUG-BP antibody, a single band of endogenous CUG-BP was observed. When we cotransfected CUG-BP with *DMPK*, the expression of the *DMPK* protein from both wild type and mutant constructs was significantly reduced by 30–40% ($p < 0.05$) (Fig. 4a: lanes 5 and 6 and Fig. 4b: bars 5 and 6). Unexpectedly, the expression of the CUG-BP mRNA and protein was significantly increased by *DMPK* ($p < 0.05$) (Fig. 3b: bars 4–6 and Fig 4b: bars 4–6). Ectopically expressed CUG-BP migrated at exactly the same position as endogenous CUG-BP and was still observed as a single band (Fig. 4a).

Poly(A) Lengths of Wild Type and Mutant DMPK mRNA Are Not Altered upon CUG-BP Expression—A previous study showed that the levels of both the mutant and normal *DMPK* messages in the poly(A) RNA of DM patients are decreased (*20*). Poly(A) lengths of the wild type and mutant *DMPK* mRNAs can not be compared directly because the two massages differ originally in length and are relatively large. Therefore, we performed so-called "H-mapping," originally described by Brewer and Ross (*27*) (see legend of Fig. 5). The sizes of the *DMPK* 3′UTRs generated by RNase H digestion were almost the same (Fig. 5: lanes 2 and 3), and the poly(A) lengths were estimated to be 200–300 bases long. Coexpression of CUG-BP did not altered the poly(A) lengths of either the wild type or mutant *DMPK* mRNA (Fig. 5: lanes 5 and 6).

DISCUSSION

We cloned alternatively spliced forms of CUG-BP from human skeletal muscle (CUG-BP+LYLQ) and human brain (CUG-BP+A) cDNA libraries. The mouse poly(C)-binding protein exists in multiple alternatively spliced isoforms with small peptide insertions (*28*). Alternative splicing

within two contiguous acceptor sites (ag/CAG) in an exon/intron boundary produces isoforms with or without one peptide insertions. The inserted nucleotides of CUG-BP+LYLQ also contain a potential splice acceptor sequence (AG) at the 3′ end. The twelve nucleotides might not be encoded by an individual exon, but alternative splicing at the 5′ end of an exon might produce isoforms with the insertion. The inserted nucleotides of CUG-BP+A can occur at the positions indicated by capital letters in the following sequences: tCAGcaggg, tcAGCaggg, tcaGCAggg, or tcagCAGgg. The inserted nucleotide might be a CAG trinucleotide and an AG dinucleotide might function as alternative splice acceptor site. The RNA-binding specificities of alternatively spliced isoforms differ (*26*).

Next, we examined the expression of CUG-BP in various mouse tissues. Northern blot analysis revealed that the CUG-BP mRNA is expressed in a tissue-specific manner, moderately in brain and testis and weakly in heart, skeletal muscle, lung, and spleen. Similarly, the CUG-BP protein is moderately expressed in lung and spleen and weakly expressed in brain, eye, and testis. Unexpectedly, CUG-BP was not observed in skeletal muscle, a tissue primarily affected in DM patients. The binding of CUG-BP to expanded CUG repeats may have effects on both the metabolism of *DMPK* mRNA and CUG-BP itself. The molar ratio of the two molecules may be a determinant, and further studies are needed in order to understand fully the contribution of CUG-BP in the respective tissues.

In order to elucidate the effects of CUG-BP on *DMPK* expression, we introduced CUG-BP and *DMPK* cDNA transiently into COS-7 cells. Cotransfection of CUG-BP had no significant effect on the expression of either wild type or mutant *DMPK* at the mRNA level. On the other hand, cotransfection of CUG-BP significantly affected the expression of both wild type and mutant *DMPK*s at the protein level. This reduction was remarkable when the mutant *DMPK* construct was used. Multiple CUG-BPs might bind the expanded CUG trinucleotide repeat of the mutant *DMPK* mRNA and exert greater effects Previous reports have shown that the expanded CTG trinucleotide repeat reduces the expression of *DMPK* at the protein level (*24*) and the expression of the upstream reporter gene product (*29*). The amount of *DMPK* protein is reduced in DM patients (*6, 7*). This reduction in *DMPK* at the protein level might be caused by the binding of CUG-BP to the expanded CUG trinucleotide repeat within the *DMPK* mRNA.

It has been reported that shortening of the poly(A) tail of an mRNA precedes the degradation of the mRNA itself (*30–32*), and that the poly(A) length correlates positively with the efficiency of translation of the mRNA (*33*). EDEN-binding protein (EDEN-BP), an *Xenopus* homologue of CUG-BP, has been reported to be a factor that mediates the shortening of the poly(A) tail of the bound mRNA (*34*). It has been argued that *Xenopus* EDEN-BP and human CUG-BP might have analogous functions because of their very high sequence conservation (88.4% in the amino acid sequence), and this might explain the decreases in the levels of *DMPK* messages in poly(A) RNA from DM patients (*34*). Our results, however, showed that the length of the poly(A) tail is not altered upon expansion of the CTG trinucleotide repeat and coexpression of CUG-BP. This suggests that the reduction in the amount of *DMPK* at the protein level is not caused by a shortening of the poly(A) tail lowering the efficiency of translation. The nuclear retention of the *DMPK* transcript and formation of nuclear foci in cultured cell lines from DM patients have been reported (*18, 35*) The binding of CUG-BP to expanded CUG trinucleotide repeats might result in the nuclear retention of the *DMPK* transcript and prevent its translation. Our results raise the possibility that CUG-BP may contribute to DM pathogenesis by binding to the expanded CUG repeat of the *DMPK* mRNA, and thus reduce DM protein kinase.

REFERENCES

1 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., Sohn, R , Zemelman, B., Snell, R.G , Rundle, S.A., Crow, S., Davies, J , Shelbourne, P , Buxton, J , Jones, C , Juvonen, V , Johnson, K., Harper, P S , Shaw, D J , and Housman, D E. (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

2 Fu, Y-H., Pizzuti, A., Fenwick, R.G , King, J., Rajnarayan, S , Dunne, P W , Dubel, J , Nasser, G.A., Ashizawa, T., de Jong, P , Wieringa, B., Korneluk, R , Perryman, M B., Epstein, H F., and Caskey, C T (1992) An unstable triplet repeat in a gene related to myotonic muscular dystrophy *Science* **255**, 1256–1258

3 Mahadevan, M., Tsilfidis, C , Sabourin, L., Shutler, G , Amemiya, C., Jansen, G , Neville, C , Narang, M , Barcelo, J., O'Hoy, K., Leblond, S , Earle-Macdonald, J , de Jong, P J , Wieringa, B , and Korneluk, R.G. (1992) Myotonic dystrophy mutation an unstable CTG repeat in the 3′ untranslated region of the gene. *Science* **255**, 1253–1255

4 Harris, S , Moncrieff, C , and Johnson, K. (1996) Myotonic dystrophy. will the real gene please step forward! *Hum Mol Genet* **5**, 1417–1423

5 Korade-Mirnics, Z , Babitzke, P , and Hoffman, E (1998) Myotonic dystrophy. molecular windows on a complex etiology *Nucleic Acids Res.* **26**, 1363–1368

6 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 , and Caskey, C T (1993) Decreased expression of myotonin-protein kinase messenger RNA and protein in adult form of myotonic dystrophy *Science* **260**, 235–238

7 Koga, R , Nakao, Y , Kurano, Y , Tsukahara, T , Nakamura, A., Ishiura, S., Nonaka, I , and Arahata, K. (1994) Decreased myotonin-protein kinase in the skeletal and cardiac muscles in myotonic dystrophy *Biochem. Biophys. Res. Commun* **202**, 577–585

8 Jansen, G , Groenen, P J T.A., Bachner, D., Jap, P H.K., Coerwinkel, M , Oerlemans, F., van den Broek, W , Gohlsch, B , Pette, D , Plomp, J J , Molenaar, P.C., Nederhoff, M G.J., van Echteld, C J.A., Dekker, M , Berns, A , Hameister, H , and Wieringa, B. (1996) Abnormal myotonic dystrophy protein kinase levels produce only mild myopathy in mice. *Nat Genet* **13**, 316–324

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

10 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 results in atrioventricular conduction abnormalities in a mouse myotonic dystrophy model. *J. Clin. Invest.* **103**, R1–R7

11. Boucher, C.A., King, S.K., Carey, N., Krahe, R., Winchester, C.L , Rahman, S , Creavin, T , Meghji, P., Bailey, M E.S , Chartier, F L , Brown, S D , Sicilliano, M J , and Johnson, K.J (1995) A novel homeodomain-encoding gene is associated with a large

CpG island interrupted by the myotonic dystrophy unstable $(CTG)_n$ repeat. *Hum Mol Genet* **4**, 1919–1925
12 Shaw, D J, McCurrach, M, Rundle, S.A., Harley, H G, Crow, S R, Sohn, R, Thirion, J-P., Hamshare, M.G, Buchler, A.J, Harper, P S, Housman, D E, and Brook, J D (1993) Genomic organization and transcriptional units at the myotonic dystrophy locus *Genomics* **18**, 673–679
13 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
14 Thornton, C.A., Wymer, J P, Simmons, Z, McClain, C, and Moxley III, R T (1997) Expansion of the myotonic dystrophy CTG repeat reduces expression of the flanking DMAHP gene. *Nat Genet.* **16**, 407–409
15 Eriksson, M, Ansved, T, Edstrom, L, Anvret, M, and Carey, N (1999) Simultaneous analysis of expression of the three myotonic dystrophy locus genes in adult skeletal muscle samples the CTG expansion correlates inversely with DMPK and 59 expression levels, but not DMAHP levels. *Hum Mol Genet* **8**, 1053–1060
16 Korade-Mirnics, Z., Tarleton, J, Servidei, S, Casey, R R, Gennarelli, M, Pegoraro, E, Angelini, C, and Hoffman, E P (1999) Myotonic dystrophy tissue-specific effect of somatic CTG expansions on allele-specific DMAHP/SIX5 expression *Hum Mol. Genet* **8**, 1017–1023
17 Alwazzan, M, Newman, E, Hamshere, M G, and Brook, J D (1999) Myotonic dystrophy is associated with a reduced level of RNA from the DMWD allele adjacent to the expanded repeat *Hum Mol Genet* **8**, 1491–1497
18 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**, 7388–7397
19 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
20. Wang, J, Pegoraro, E, Menegazzo, E, Gennarelli, M, Hoop, R C, Angelini, C, and Hoffman, E P (1995) Myotonic dystrophy evidence for a possible dominant-negative RNA mutation *Hum Mol Genet* **4**, 599–606
21 Timchenko, L T, Miller, J W, Timchenko, N A., DeVore, D R., Datar, K.V, Lin, L, Roberts, R, Caskey, C T, and Swanson, M S (1996) Identification of a $(CUG)_n$ triplet RNA-binding protein and its expression in myotonic dystrophy *Nucleic Acids Res.* **24**, 4407–4414
22. 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
23 Sasagawa, N, Sorimachi, H, Maruyama, K., Arahata, K., Ishiura, S., and Suzuki, K. (1994) Expression of a novel human myotonin protein kinase (MtPK) cDNA clone which encodes a protein with a thymopoietin-like domain in COS cells. *FEBS Lett* **351**, 22–26
24 Sasagawa, N, Saitoh, N, Shimokowa, M, Sorimachi, H., Maruyama, K., Arahata, K., Ishiura, S., and Suzuki, K. (1996) Effect of artificial CTG repeat expansion of the expression of myotonin protein kinase (MtPK) in COS-1 cells. *Biochim. Biophys. Acta* **1315**, 112–116
25. Takahashi, N, Sasagawa, N, Suzuki, K., and Ishiura, S. (1999) Synthesis of long trinucleotide repeats in vitro *Neurosci Lett.* **262**, 45–48
26 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
27 Brewer, G and Ross, J (1990) Messenger RNA turnover in cell-free extracts. *Methods Enzymol* **181**, 202–209
28 Funke, B, Zuleger, B, Benavente, R, Schuster, T, Goller, M, Stevenin, J, and Horak, I (1996) The mouse poly C-binding protein exists in multiple isoforms and interacts with several RNA-binding proteins. *Nucleic Acids Res.* **24**, 3821–3828
29. Amack, J D, Paguio, A.P., and Mahadevan, M S (1999) Cis and trans effects of the myotonic dystrophy (DM) mutation in a cell culture model *Hum Mol Genet* **8**, 1975–1984
30 Beelman, C.A. and Parker, R (1995) Degradation of mRNA in eukaryotes *Cell* **81**, 179–183
31 Colgan, D F and Manley, J L (1997) Mechanism and regulation of mRNA polyadenylation *Genes Dev* **11**, 2755–2766
32 Decker, C J and Parker, R (1994) Mechanisms of mRNA degradation in eukaryotes. *Trends Biochem. Sci* **19**, 336–340
33 Jackson, R J (1993) Cytoplasmic regulation of mRNA function the importance of the 3′ untranslated region *Cell* **74**, 9–14
34 Paillard, L, Omilli, F, Legagneux, V, Bassez, T, Maniey, D, and Osborne, H B (1998) EDEN and EDEN-BP, a cis element and an associated factor that mediate sequence-specific mRNA deadenylation in *Xenopus* embryos. *EMBO J* **17**, 278–287
35 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