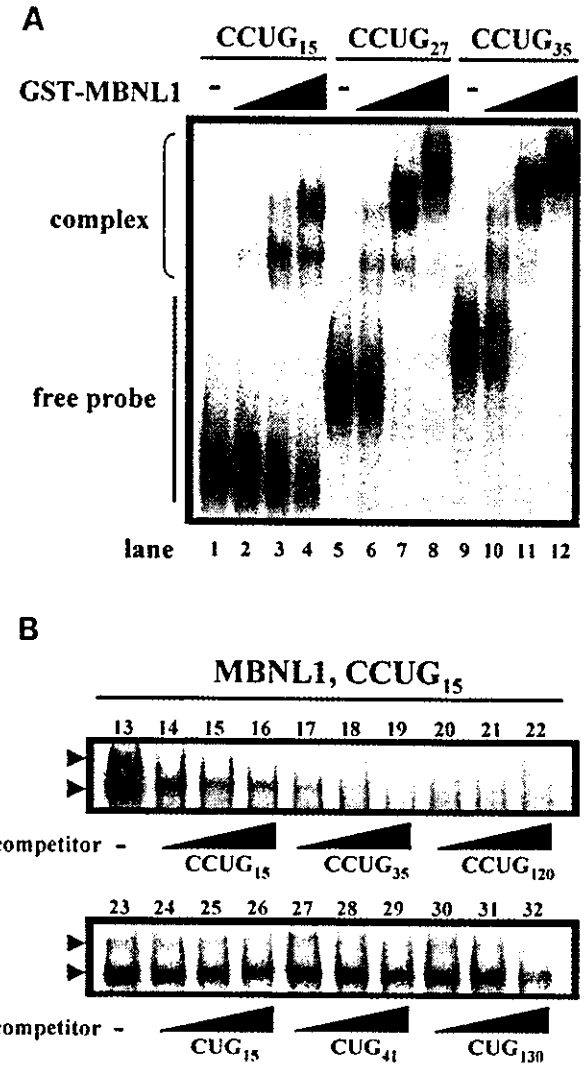

Figure 6. Length-dependent binding of GST–MBNL1 to CCUG repeats. (**A**) $CCUG_{15}$, $CCUG_{27}$ and $CCUG_{35}$ probes were examined with increasing doses of GST–MBNL1 in the gel retardation assay. GST–MBNL1 was added to the reaction at amounts of 0 ng (lanes 1, 5 and 9), 10 ng (lanes 2, 6 and 10), 50 ng (lanes 3, 7 and 11), or 250 ng (lanes 4, 8 and 12). Free $CCUG_{15}$ probe remains even at the highest dose of GST–MBNL1 (lane 4), while those of $CCUG_{27}$ and $CCUG_{35}$ are almost undetectable (lanes 8 and 12). (**B**) All lanes contain GST–MBNL1 and ^{32}P-labeled $CCUG_{15}$ probe. Increasing doses of $CCUG_{15}$ (lanes 14–16), $CCUG_{35}$ (lanes 17–19), $CCUG_{120}$ (lanes 20–22), CUG_{15} (lanes 24–26), CUG_{41} (lanes 27–29) and CUG_{130} (lanes 30–32) were added as cold competitors in advance of the probe addition. The amount of RNA was 100 ng (lanes 14, 17, 20, 24, 27 and 30), 300 ng (lanes 15, 18, 21, 25, 28 and 31) or 600 ng (lanes 16, 19, 22, 26, 29 and 32). The competition by $CCUG_{120}$ was more efficient than that by $CCUG_{15}$ or $CCUG_{35}$ (lanes 14, 17 and 20). CUG_{130} also competed best among CUG repeats (lane 32).

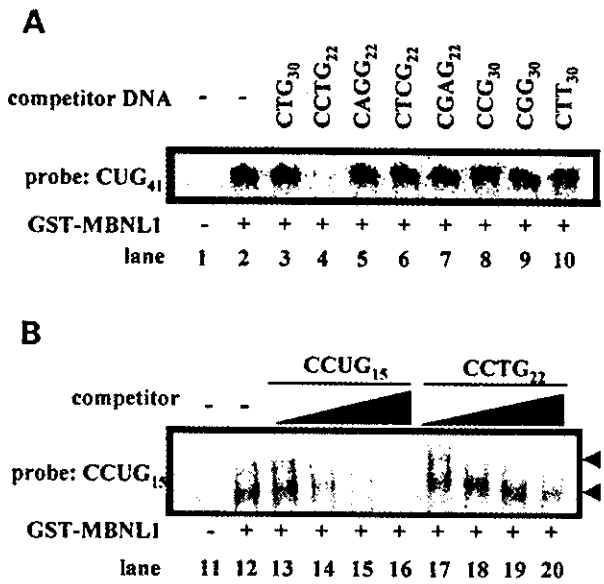

Figure 7. *In vitro* binding of GST–MBNL1 to CCTG DNA repeat. (**A**) Unlabeled oligo DNAs (300 ng each) were added as competitors into the reaction of GST–MBNL1 and CUG_{41} probe. The complex of CUG_{41} and GST–MBNL1 is indicated as a band in the panels. Competition by $CCTG_{22}$ was observed (lane 4). (**B**) Comparison of unlabeled CCUG RNA repeats and CCTG DNA repeats in the inhibition of the binding of GST–MBNL1 to the $CCUG_{15}$ probe. Increasing doses of $CCUG_{15}$ or $CCTG_{22}$ were added at amounts of 50 ng (lanes 13 and 17), 300 ng (lanes 14 and 18), 1000 ng (lanes 15 and 19), 3000 ng (lanes 16 and 20).

the abnormal splicing, because if CUG–BP interacts with these expanded repeats, the binding of CUG–BP to its target RNAs should be reduced, not leading to the aberrant splicing. Rather, since the affinity of CUG–BP to double-stranded CUG and CCUG repeats is low, the presence of expanded repeats may not interrupt the binding of CUG–BP to its target RNAs of splicing. Even if CUG–BP is sequestered by these repeats, the up-regulation of CUG–BP does not appear to be explained directly by the sequestration. Therefore, it seems reasonable to postulate an indirect mechanism as leading to the up-regulation of CUG–BP.

The other type of cellular effect by the expanded repeat is a defect in myogenesis accompanied by the altered expression of myogenic markers. Mahadevan and colleagues have suggested the abnormalities in differentiation of their cell culture models that expresses expanded CUG repeats in the 3′-UTR of *DMPK* (45). In the same cells, MyoD has been suggested to be down-regulated in the post-transcriptional level (46). In another report, an impaired cell cycle withdrawal due to a reduced expression of p21 in DM1 cells was also suggested (24). Interestingly, the reduction of MyoD protein was also suggested in the report (24). These properties may be involved in the muscle development abnormalities in congenital DM1 and/or defects in muscle regeneration, possibly leading to muscle wasting in adult patients (1). Thus, myogenic regulation is also a noteworthy feature of DM. However, it is probable that the abnormalities in splicing and myogenic differentiation may share relevance to each other rather than being two separable events.

Since the expression of MBNL1 is induced during myoblast differentiation (31), this protein might be involved in myogenic differentiation as in the case of muscleblind in fly. Interestingly, the expression of MBNL3 has recently been shown to be reduced during differentiation (47), in contrast to MBNL1. Furthermore, MBNL3 has an inhibitory role in myogenesis, in which the overexpression of MBNL3 suppresses the progress of differentiation (47). One speculation is that MBNL1 is a positive regulator of muscle differentiation and competes with MBNL3 for RNA substrates. FISH analysis has shown that not only MBNL1 but also MBNL2 and MBNL3 co-localize with

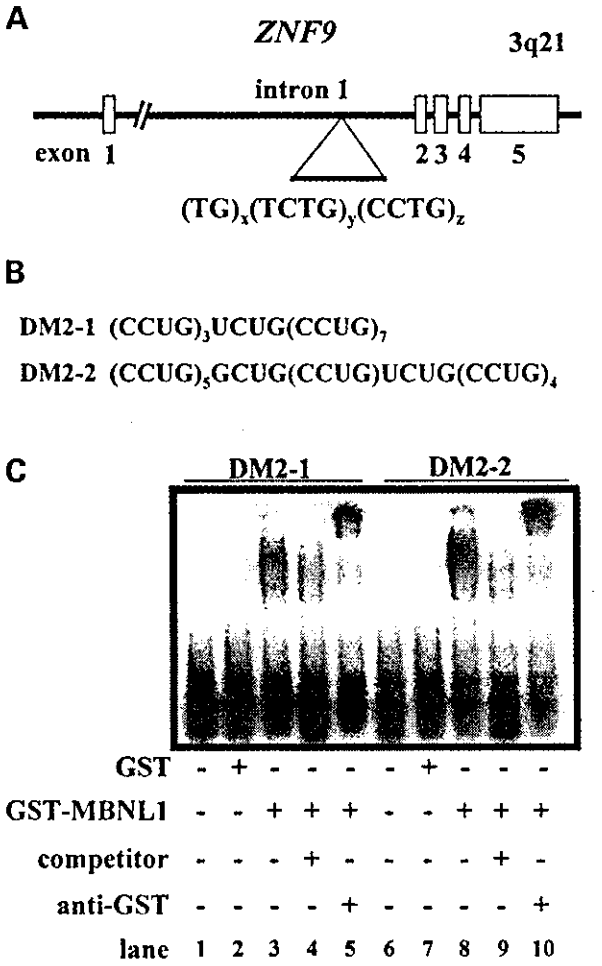

Figure 8. GST–MBNL1 bound to minimal CCUG repeat motifs in *ZNF9*. (**A**) Gene structure of *ZNF9*. Exons are indicated as open boxes. A repetitive sequence containing variable lengths of TG, TCTG and CCTG repeat elements is located in intron 1. (**B**) Two of the shortest CCUG elements of *ZNF9* ever reported were chosen as probes for the experiment below. Both contains non-CCUG tetranucleotide(s). (**C**) *In vitro* binding of GST–MBNL1 to both DM2-1 and DM2-2 RNAs. GST or GST–MBNL1 was incubated with ^{32}P-labeled probes. RNA–protein complexes are observed in the gel. The addition of unlabeled DM2-1 or DM2-2 reduced the shifted band (lanes 4 and 9), while the addition of anti-GST antibody induced supershifts of the complex (lanes 5 and 10).

CUG and CCUG repeats (26), suggesting that these three proteins have similar RNA-binding properties. Indeed, our preliminary results in the yeast system have suggested that MBNL2 also interacts with CCUG and CCCG repeats (unpublished data). However, it is also conceivable that, even though the binding specificities of MBNL proteins overlap partially, they may have different target RNAs. Since the myogenic defect is an important issue in DM, the myogenic roles of MBNL proteins must be clarified by further experiments.

RNA-binding properties of MBNL1

Although the length of CUG and CCUG repeats used in this study are smaller than those found in DM1 or DM2, competition analysis demonstrates the length-dependent interaction of MBNL1 with up to CUG_{130} or $CCUG_{120}$ *in vitro*, whose lengths are longer than the minimum lengths of pathogenesis. Although we cannot exclude the possibility that longer expanded CUG/CCUG repeats form other RNA structures and have other binding properties, the tendency of length-dependent binding may allow the prediction that MBNL1 binds to even longer expansion than the lengths used in this experiment, which is supported by the FISH results.

Previously, long CUG repeats have been shown to form a hairpin structure comprising a long stem and a single loop rather than with multiple stem-loops (34). In addition, detailed biochemical analyses in a recent report have shown that all CNG repeats and CCUG repeats form stable hairpin structures *in vitro*, and these RNAs contain heterogeneous conformers (folding variants) in the population due to alternative alignment of the hairpin structure (48). Notably, $CNG_{16\text{-}17}$ and $CCUG_{14}$ are sufficient lengths to form stable hairpins (48), suggesting that the $CCUG_{15}$ probe used in this study may be double-stranded. In the gel retardation assays, some portion of $CCUG_{15}$ probe remained unbound even in the overdose of MBNL1, and such population decreased when longer CCUG repeats were analyzed. This might indicate that some of $CCUG_{15}$ conformers do not bind to MBNL1, and as the length gets longer, the relative amount of such conformers decreases due to the stabilization of hairpin structure. It is not known what kind of conformers MBNL1 binds to or not. It is also unclear whether unbound conformers contain single-stranded RNAs. Next, the tendency that G-rich sequences such as CGG and CGGG are not preferred by MBNL1 seems to reflect the sequence selectivity of MBNL1 rather than its structure selectivity, because at least CGG repeats form a hairpin structure similar to those by CHG repeats (48). However, the authors also pointed out the peculiarity of CCG repeats in the formation of conformers and loop structure of the hairpins, may be due to the stability of CCG repeats highest among CNG repeats. These issues will be clarified by further biochemical and structural analyses of MBNL1.

According to the deletion analysis, some isoforms of MBNL1 did not bind to CCUG repeats. Nevertheless, they might bind to some RNAs other than CCUG, since all these isoforms have at least one zinc finger motif. Indeed, a peptide containing only the first CCCH motif of TTP (tristetraprolin), an RNA-binding protein with two CCCH motifs involved in the deadenylation of tumor necrosis factor-α mRNA (49), has been shown to bind to a UUAUU RNA, suggesting that each CCCH motif has the ability to bind to RNA by itself (50). The existence of multiple MBNL1 isoforms implies that there is some functional antagonism or feedback regulation among MBNL1 isoforms through the interaction with common RNAs or proteins.

Target RNA motifs of MBNL1

Molecular targets of MBNL1 are important for understanding the effect of its sequestration by the expanded repeats and could be involved in the pathogenesis of DM. From our results, targets of MBNL1 may be CHHG or CHG repeat-containing RNAs. Strikingly, CCUG repeats showed much higher affinity than CUG and other CHG/CHHG repeats assayed in the present study. A simple interpretation of the results is that the major target RNAs of MBNL1 may have a CCUG repeat

sequence. However, the interactions with CUG repeats or other minor sequences are also important because these interactions might be interfered easily by the presence of expanded pathogenic repeats. At least, the fact that expanded CUG repeats can sequester MBNL1 *in vivo* suggests the physiological interaction between MBNL1 and CUG repeats, although the length of CUG repeats in physiological target genes might be much shorter than the mutant RNA in DM1.

We have also shown that MBNL1 is able to interact with CCUG repeats containing insertions (DM2-1 and DM2-2). These insertions are seen in normal alleles of *ZNF9* and might be related to the stability of the CCTG tract in *ZNF9* (51). The insertions may alter the predicted secondary structure of CCUG repeats, but both DM2-1 and DM2-2 probes still have affinity to MBNL1. Currently, we do not know whether these insertions increase or decrease the affinity of MBNL1 to RNA repeats. Some of the target RNAs of MBNL1 might have such insertions in repetitive motifs. Importantly, length-dependent binding of MBNL1 to CUG/CCUG repeats suggests MBNL1's preference for stem and bulge structure rather than stem–loop structure, if a long and uninterrupted CUG or CCUG repeat actually folds as a long and stable hairpin structure. Mixture of CHHG and CHG motifs is also possible interactors of MBNL1. Indeed, two mixed sequences, $(CUUG)(CCUG)_4(CCCG)(CCUG)(CCAG)$ and $(CCUG)_7(CCCG)(CCUG)_5$ interacted with GST–MBNL1 in gel retardation assays (unpublished data), suggesting that MBNL1 binds to CHHG-mixed sequences and that an RNA containing eight tetranucleotides has sufficient length for binding to MBNL1. Thus, complex variations in repetitive sequences can be predicted as the binding motifs of MBNL1.

We searched the human and mouse genome databases for CHHG-containing genes and found many such genes. Since MBNL1 is a conserved protein, we postulated that CHHG motifs in these genes are also conserved between human and mouse. However, we have not still found conserved motifs between humans and mice in these genes, suggesting that physiological target motifs of MBNL1 may be complex configurations and/or that MBNL1 might have broader RNA-binding specificities. The CCUG tract in *ZNF9*, a potential target of MBNL1 as shown above, is conserved among human, chimpanzee and gorilla, but it is not found in mouse or rat, suggesting that CCUG motifs were acquired in the process of evolution (51). If *ZNF9* is an actual target of MBNL1, it is possible that MBNL1 might be involved in the metabolism of *ZNF9* pre-mRNA in the former three species. Interestingly, more than 500 bp up- or downstream of the CCUG tract is highly conserved among these three species. In addition, the UG repeat franking with the CCUG repeat (Fig. 8A) is conserved among all five above species (51). However, we do not know whether these regions have any physiological role or not. As we have examined CCUG tract in *ZNF9* only *in vitro*, the physiological significance of the interaction between MBNL1 and the CCUG tract should be examined *in vivo*.

Notably, a knockout mouse model of MBNL1 showed myotonia, cataracts and abnormal muscle histology in a recent report (52), strongly supporting the hypothesis that a loss of function of this protein by the expanded repeats leads to pathogenesis of DM. These mice also exhibit abnormalities in splicing of mouse CLC-1 (clc-1), and cardiac and fast skeletal muscle troponin T (tnnt2 and tnnt3). Although the molecular pathway causing these abnormalities is still unclear, MBNL1 may have some roles in the regulation of alternative splicing. From our results, it is possible that MBNL1 acts with CHHG/CHG repeat-like sequences in the process of splicing. To date, abnormal splicing of CLC-1 has been suggested to be caused by the altered expression of CUG–BP. It is mysterious whether both or either of these two proteins are involved in the splicing of CLC-1. There might be some direct or indirect crosstalk between CUG–BP and MBNL proteins. Our results presented here will be a help for further understanding of MBNL1 as well as the pathogenesis of DM.

MATERIALS AND METHODS

Vectors

For protein expression in yeast, $MBNL1_{40}$ and its deletion mutants, the structures of which are indicated in Figure 3, were amplified by polymerase chain reaction (PCR) so as to have *Bam*HI and *Sal*I sites in the 5′ and 3′ regions, respectively, of the open reading frame, and subcloned into pGAD424 (Clontech). pGAD424, containing CUG–BP, was the same as previously described (28). The p20 fragment of PKR was amplified using primers 5′-CATGGCTGGTGATCTTTCAGC AGGTTTCGG-3′ and 5′-ACGCGTCGACAGTAGCAAAAG AACCAGAGG-3′, and inserted into the *EcoR*I and *Sal*I sites of pGAD424. For bacterial expression, pET32b (Novagen) was adopted by replacing thioredoxin-tag and S-tag with GST. The resulting plasmid was cut by *Sac*I in the polylinker region, blunted with T_4 DNA polymerase, and self-ligated to adjust the reading frame of the His-tag. The newborn vector, designated pET-GX, is able to express a fusion protein with GST in the N-terminus and a His-tag in the C-terminus. Fragments of MBNL1 and CUG–BP were inserted into the polylinker sites of pET-GX. Oligo DNAs of repetitive sequences were purchased from Proligo. To make longer repetitive sequences, oligo DNAs were amplified and extended by the non-template PCR method (53), and inserted into the *Hinc*II site of either pBluescript SK+ (Stratagen) or pUC118 (Takara). After sequencing, repetitive fragments were cut off with appropriate restriction enzymes, blunted with T_4 DNA polymerase and inserted into the *Sma*I site of pIIIA/MS2-2 vector (38). For *in vitro* transcription, we used pBluescriptII SK+ vector, which was cut by *Sac*I and *Kpn*I, and ligated with linker nucleotides consisting of 5′-CGTCGACGAGCT-3′ and 5′-CGTCGACGG TAC-3′, both of which have a *Hinc*II site. The resulting vector was named pBSDM. Some of the repetitive DNA fragments above were inserted into the *Hinc*II site of pBSDM. DM2-1 and DM2-2 fragments (their sequences are shown in Fig. 8B) were also purchased and inserted into the *Hinc*II site of pBSDM. All constructs were confirmed by sequencing.

Yeast three-hybrid system

The transformation of yeast cells and reporter gene assays were performed as previously described (28,38). In the *HIS3* assay, yeast strain *L40-coat* was transformed with pGAD and pIIIA/MS2-2 vectors coding RNA-binding proteins and RNA, respectively, and

selected on plates lacking leucine and uracil. Yeast transformants were picked up and spotted onto selection plates lacking in leucine, uracil, and histidine, with or without 0.1, 0.5 or 1 mM 3-AT. The plates were incubated at 30°C for about 1 week and the viability of the yeast transformants was analyzed. We classified the binding activity as (+++++), (++++), (+++) and (++), when yeast growth was observed on the plates containing 1, 0.5, 0.1 and 0 mM 3-AT, respectively; (+) yeast grew in the absence of 3-AT after more than 1 week, (−) no growth of yeast transformants was observed even after prolonged incubation. Positive and negative controls were included to confirm the validity of this system using combinations of iron response element (IRE) and iron regulatory protein (IRP), or pIIIA/MS2-2 and pGAD424 empty vectors, respectively. According to the classification above, yeast transformants of IRE and IRP were (++++), while those of pIIIA/MS2-2 and pGAD424 were (−). The liquid β-gal assay was performed as described previously (28,38).

Bacterial expression and purification of recombinant proteins

pET-GX vector containing $MBNL1_{40}$ or CUG-BP was transformed into BL21(DE3) strain. An overnight culture of transformant in LB medium was diluted and shaken at 37°C for 1.5 h (or until the OD reached 0.3–0.4), then 0.1 mM IPTG was added. During induction by IPTG, the culture was shaken at 27°C for 1.5 h. Bacterial cells were collected by centrifugation, washed with PBS and suspended in 0.05 culture volumes of sonication buffer containing 50 mM sodium phosphate (pH 7.5), 150 mM NaCl, 1 mM PMSF, and 1/1000 volume of protease inhibitor cocktail (Sigma). Lysozyme was added at a final concentration of 1 mg/ml, then incubated for 30 min on ice. The reaction was sonicated on ice nine times for 10 s each. Triton-X was added to a final concentration of 1% and the lysate was centrifuged. The supernatant of the lysate was subjected to affinity purification using Glutathione Sepharose 4B (Amersham Biosciences) and Talon Metal Affinity Resin (Clontech), according to the manufacturer's protocol. The quantity and purity were checked on SDS-polyacrylamide electrophoresis gels by staining with Coomassie brilliant blue (CBB). The identity of GST–MBNL1 was verified by peptide mass finger printing with a mass spectrometry (AXIMA-CFR, SHIMADZU) following trypsinization of GST–MBNL1.

In vitro binding assay

To synthesize RNAs, pBSDM with repetitive sequences were cut by *Pvu*II and either *Kpn*I or *Sac*I, depending on the direction of the transcription, and blunted with T_4 DNA polymerase. Restriction fragments containing repetitive sequences were purified by agarose gel electrophoresis. *In vitro* transcription was performed using mMESSAGE mMACHINE (Ambion) according to the manufacturer's directions. When labeling RNAs, 1 μl of [α-^{32}P]-CTP (~800 Ci/mmol) was added to the reaction. Unincorporated nucleotides were discarded using NucAway Spin Columns (Ambion). The gel mobility shift assay was performed as described (34). In brief, a purified protein and RNA were mixed in reaction buffer containing 50 mM Tris–HCl (pH 7.6), 100 mM NaCl, 5% glycerol, 1 mg/ml yeast tRNA, 1 mg/ml bovine serum albumin and 1 mM DTT, and incubated for 10 min at room temperature. Typically, ~5×10^5 cpm RNA was added to the reaction. When necessary, non-labeled RNA competitors, anti-GST antibody (Santa Cruz) or RNaseV1 (Ambion), were added as indicated in each figure legend. The reaction was resolved by native PAGE using 4–5% polyacrylamide gels and 0.5× Tris borate buffer. In the case of RNase treatment as shown in Figure 4D, denaturing 13% polyacrylamide gels containing 8 M urea were used. The gels were dried after fixation with 10% methanol and 10% acetic acid, autoradiographed, and visualized by a BAS-2500 imaging analyzer (Fujifilm).

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Altered expression of CUG binding protein 1 mRNA in myotonic dystrophy 1: possible RNA–RNA interaction

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Abstract

The triplet repeats mutation, which causes myotonic dystrophy 1 (DM1), is thought to have a dominant negative effect on RNA levels. In light of previous results using differential display analysis, the present study focused on the expression of CUG binding protein 1 (CUGBP1) mRNA. Northern blot analysis demonstrated that the quantity of CUGBP1 mRNA in three DM1 patients was approximately 70% of that observed in three normal controls ($P < 0.05$). In addition, a semi-quantitative RT-PCR assay showed that the relative amount of CUGBP1 mRNA was reduced in muscle biopsy samples from 10 DM1 patients compared to that from five normal individuals ($P < 0.01$) and 10 myopathic disease controls ($P < 0.01$). The amount of CUGBP1 mRNA was negatively correlated with the size of the CTG expansion ($r = -0.85$, $P < 0.05$). In vitro RNA–RNA binding experiments demonstrated that the incubation of expanded CUG repeats with CUGBP1 RNA generated a higher molecular weight band, which was digested by RNase III. The CUGBP1 mRNA was found to contain several CAG repeat sequences. These results suggest that the CUG expansion may bind to complementary sequences within the CUGBP1 mRNA and that this molecular interaction may affect CUGBP1 mRNA expression in DM1.

Keywords: Myotonic dystrophy (DM); CTG repeat; CUG binding protein (CUGBP); DM protein kinase (DMPK); mRNA; RNA–RNA interaction

1. Introduction

Myotonic dystrophy 1 (DM1) is a multi-system disorder that is characterized by myotonia and progressive muscle weakness. Patients with DM1 have a large number of CTG triplet repeats in the 3′-untranslated region of the DM protein kinase (DMPK) gene, which has been mapped to chromosome 19q 13.3. The length of the CTG triplet repeats correlates well with the age of onset and severity of the disease (Harley et al., 1992).

The mechanisms that a non-translated CTG repeat in a single allele results in the severe dominant phenotype of DM1 remains unclear. One possible explanation is that this series of repeats may disrupt the expression of other genes at either the DNA or RNA level (Harris et al., 1996). The repeat expansion has been identified as a *cis*-acting determinant, which affects neighboring genes (Thornton et al., 1997; Klesert et al., 1997). Another hypothesis is that a triplet expansion in the 3'UTR of the DMPK gene may affect the expression of other mRNAs. Several studies have suggested that the DM1 mutation has a *trans*-effect on cellular RNA expression (Sabourin et al., 1997; Sasagawa et al., 1999; Amack et al., 1999).

Here, we screened mRNAs expressed in DM1 muscle using fluorescent differential display analysis, which has been used to detect differentially expressed genes (Liang and Pardee, 1992). CUGBP1 mRNA has been shown to be differentially expressed in patients with DM1. The product of CUGBP mRNA is one of a family of RNA binding proteins. While the biological function of CUGBP has not yet been established, it is speculated that (CUG)n repeat region in DMPK mRNA is a binding site for the CUGBP protein, and triplet repeat expansion leads to sequestration of this protein on mutant DMPK transcript (Timchenko et al., 1996). Recent report proposed that CUGBP might cause disruption of alternative splicing of pre-mRNA of muscle specific

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chloride channel in DM1 (Charlet et al., 2002). In the present study, we hypothesized alteration of CUGBP mRNA might be involved in a *trans*-RNA interaction between CUG repeats and CUGBP1 mRNA. Therefore, we performed an in vitro RNA–RNA binding assay to examine this hypothesis.

2. Materials and methods

2.1. Muscle biopsy, total RNA preparation and cDNA synthesis

Twenty-five open biopsied samples of biceps muscle from 10 DM1 patients (mean age ± S.D., 32.6 ± 5.6 years, seven men and three women), 10 myopathic disease controls (5 limb-girdle muscular dystrophy, three distal myopathy and two facioscapulohumeral muscular dystrophy patients, 29.1 ± 7.9 years, six men and four women), and five normal individuals (33.3 ± 2.6 years, three men and two women) who exhibited no pathological findings were analyzed. Written, informed consent was obtained from all subjects or their parents before biopsy. All samples were immediately frozen in liquid nitrogen after biopsy and were stored at −70 °C until analysis. Total RNA was prepared from the biopsies by the guanidinium–HCl/thiocyanate/phenol/chloroform method. To remove any contaminating DNA, the RNA was treated with DNase I (TaKaRa, Tokyo, Japan) for 30 min at 37 °C. After acid phenol extraction and isopropanol precipitation, 1 μg of DNA-free total RNA was placed in 8 μl of DEPC-treated water. The solution was heated to 65 °C for 10 min, and then cooled to 37 °C. To this solution was added 15 mM DTT, 1.8 mM of each dNTP, 1 μM of the appropriate primers, and 5 U of reverse transcriptase (Amersham Pharmacia Biotech, UK).

2.2. Southern blot analysis

Peripheral blood leukocytes were prepared for extraction of DNA for Southern blot analysis from all of the DM1 patients and one normal individual. For detection of mutant alleles, 10 μg per lane of *Bam*HI restriction fragments were resolved by electrophoresis on a 1.0% agarose gel and were transferred to Hybond-N+ nylon membranes (Amersham Pharmacia Biotech). The primer set for amplification of the 3′ end of the human DMPK gene, not including the repeat region, was designed as follows: Forward: 5′-CAC GGA TCC ACC TTC CCA TG-3′ (corresponding to nucleotides 9568-9587); Reverse: 5′-CCA TCT AGC TGG AGA GAG AA-3′ (9864–9883). The 316 bp PCR product, which was subcloned into the PUC118 cloning vector (Takara) and labeled with [^{32}P]dCTP using a Megaprime DNA Labeling Kit (Amersham Pharmacia Biotech), was used as the DNA probe. The bands were visualized using a BAS 2000 Image Analyzer (Fuji Medical Systems, Stanford, USA).

2.3. Fluorescent differential display analysis

Fluorescent differential display analysis was performed using RNA prepared from biopsied skeletal muscles taken from three DM patients and three normal control individuals. Conditions for the experiment have been described previously (Watanabe et al., 1999). Briefly, three fluorescein isothiocyanate (FITC)-labeled 3′-anchored oligo (d)T primers (FITC-5′-CGTACGCGT$_{15}$N-3′, where N = one of A, C or G) and 60 arbitrary primers were used for PCR. Isomigrating bands, which were amplified to a different degree depending upon whether the sample came from a patient or control individual, were excised and re-amplified using the same set of primers. The re-amplified bands were subcloned into the pT7Blue-cloning vector (Novagen) and fully sequenced in both directions. Comparison studies were performed using the GenBank database of the National Center for Biotechnology Information (Bethesda, MD, USA). Subcloned bands of interest were used as probes for Northern blot analysis, and labeled with [^{32}P] dCTP using a Megaprime DNA Labeling Kit (Amersham Pharmacia Biotech). For standardization of lane loading differences, membranes were subsequently hybridized with a probe specific for human β-actin. The bands were visualized using a BAS 2000 Image Analyzer (Fuji Medical Systems).

2.4. Semi-quantitative multiplex PCR assay

For quantitative analysis of CUGBP1 mRNA, a semi-quantitative multiplex RT-PCR assay was developed. Primer sequences were as follows: CUGBP1, Forward: 5′-AAC AAT GCA GTG GAA GAC AGG-3′ (corresponding to nucleotides 441–460), Reverse: 5′-CTC CAG CTA ATG TCT GCA GG-3′ (1076–1095); β-actin (internal control), Forward: 5′-CTA CAA TGA GCT GCG TGT GG-3′ (343–362), Reverse: 5′-CAT ACT CCT GCT TGC TGA TCC-3′ (1040–1060). PCR using these amplimers yielded PCR products of 655 bp and 818 bp, respectively. The reaction volume was 20 μl and contained 1 μl of cDNA solution, 10x PCR buffer, 0.25 mM of each dNTP, 0.5 μM each of the forward and backward primers, and 0.5 U of Ex Taq DNA polymerase. Initially, we defined the kinetics of co-amplification of the CUGBP1- and β-actin-specific PCR products using normal control samples. PCR was performed as follows: denaturation at 94 °C for 1 min for 1 cycle; then 18 to 23 cycles each of denaturation at 94 °C for 15 s, annealing at 57 °C for 1 min, and elongation at 74 °C for 3 min. The reactions were amplified through 21 cycles. The reactions were sampled after every cycle, resolved by electrophoresis on a 5% polyacrylamide sequencing gel, and the bands were stained with SYBR Green I (TaKaRa). The intensity of SYBR Green I luminescence was measured using a FluoroImager scanner (Molecular Dynamics) and was analyzed with ImageQuant software (Molecular Dynamics). It was demonstrated that reaction cycle-intensity curves fit the linear portion of

semi-logarithmic graphs from 18 to 22 cycles of reaction, and both CUGBP1- and β-actin-specific PCR products were presumed to be amplified with comparable efficiency under these conditions (Fig. 2A and B). Thus, we examined the level of expression of the mRNAs in all biopsied samples using a quantitative multiplex RT-PCR assay, as described above.

2.5. Binding of CUG repeat to CUGBP1 RNA in vitro

Fluorescein 12-UTP-labeled single strand riboprobes were generated from cloned human DMPK, CUGBP1 and β-actin cDNAs. Primer sequences to amplify DMPK cDNA were as follows: Forward: 5′-CCT AGA ACT GTC TTC GAC TCC G-3′ (corresponding to nucleotides 2669–2690), Reverse: 5′-TTG CGA ACC AAC GAT AGG TGG G-3′ (3040–3061). Primers for CUGBP1 and β-actin were identical to those used for the quantitative multiplex PCR described above. The amplified 655 and 818 bp products were subcloned into the pT7 Blue T-vector (Novagen, Madison, USA), while those for DMPK amplified 374 bp (one of normal individuals) and 518 bp (one of DM1 cases) products containing 12 and 60 CTG repeats respectively were subcloned into the pSPT18 vector (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany). The CUGBP1 cDNA was cleaved with Ear I (position 591 and 778) and AlwNI (position 836) into three fragments, named R1 (187 bp), R2 (58 bp) and R3 (259 bp). The fragments were re-ligated (Fig. 3A). All subcloned cDNAs were linearized, and sense and anti-sense riboprobes were transcribed in vitro by a RNA labeling kit (Boehringer Mannheim) using T7 or SP6 RNA polymerases and were concentrated to 20 pM. Each combination of riboprobe was incubated in 20 μl of standard binding buffer (Tomizawa, 1985) containing 10 mM $MgCl_2$ and 100 mM NaCl in 20 mM Tris-HCl (pH 7.6) at 70 °C for 3 min in the same tubes, followed by slow cooling at 4 °C, as previously described (Skripkin et al., 1996; Besancon and Wagner, 1999). Some of reactions were incubated with 0.0001, 0.001 or 0.01 Unit RNase III (Epicentre Technologies, Wisconsin, USA) at 37 °C for 15 min. Samples were analyzed by 1% agarose gel electrophoresis, and scanned with the FluoroImager machine (Molecular Dynamics) at the high sensitive mode.

2.6. Statistics

Since the sample groups were small, comparisons between the relative levels of the mRNAs and proteins in DM1 patient tissues versus controls were performed using the non-parametric Mann–Whitney test. Correlations between the CTG-repeat size and the relative amount of the mRNAs and proteins in DM1 patients were expressed as Pearson's correlation coefficients. Significance was set at $P < 0.05$ in all tests.

3. Results

3.1. CTG expansion in DM1 patients

The normal BamH1 fragment of 1376 bp was observed in the control WBCs, while the expanded allele, varying between approximately 1760 and 5540 bp, was identified exclusively in DM1 patients. Thus, the number of triplet nucleotide repeats was calculated to range from 128 to 1388. Fig. 1A shows representative triplet repeats of 1388, 1229, and 128 in lanes 1, 2, and 3, respectively.

3.2. Decreased level of CUGBP1 mRNAs expression in DM1 biopsies

One PCR product was differentially amplified between the two samples (Fig. 1B). This product, which was expressed to a lesser degree in the DM1 patients than in normal controls, was identical to Homo sapiens CUGBP1 mRNA (Accession no. U63289). Northern blot analysis showed that the quantity of CUGBP1 mRNA in the controls was approximately 113% greater than that of the affected individuals ($P < 0.05$) (Fig. 1C and D).

The RT-PCR based assay was performed on biopsied muscle samples from 10 DM1 patients, 10 myopathy controls and five normal individuals. Fig. 2C shows a representative analysis of the tissue expression levels of CUGBP1 and β-actin mRNAs. In DM1 muscle biopsies, the ratio of CUGBP1 to β-actin band density was significantly greater than in the myopathy controls ($P < 0.01$) and normal controls ($P < 0.01$) (Fig. 2D). There was no significant difference between the myopathy controls and normal individuals ($p = 0.378$) (Fig. 2D). In DM1 patients, the number of CTG repeats negatively correlated with the ratio of CUGBP1 to β-actin band density ($r = -0.85$, $P < 0.05$) (Fig. 2E).

3.3. The CUG expansion binds to CAG repeats within CUGBP1 mRNA in vitro

The primers for CUGBP1 cDNA were designed to amplify a partial ORF of the 655 bp product, which has 32 CAG trinucleotides containing five of (CAG)2 and 1 of (CAG)3. A quarter of the CAG trinucleotides are located within nucleotide 778–836 (Fig. 3A). Thus, we assessed the anti-sense binding of the CUG repeats to the complementary sequence in CUGBP1 mRNA. Initially we generated fluorescein 12-UTP-labeled riboprobes comprising (CUG)12, (CAG)12, (CUG)60 or (CAG)60. Fig. 3B demonstrates that the incubation of (CUG)60 with (CAG)12, as well as with (CAG)60 produced a band shift, which was slightly observed in the incubation of (CUG)12 with (CAG)12. On the other hand, (CAG)12 showed almost identical migration to that of (CAG)60, even though both has significantly different length. This was also seen in (CUG)12 and (CUG)60. It was suggested that (CAG)60 or (CUG)60 formed secondary structure to increase migration to migrate in the

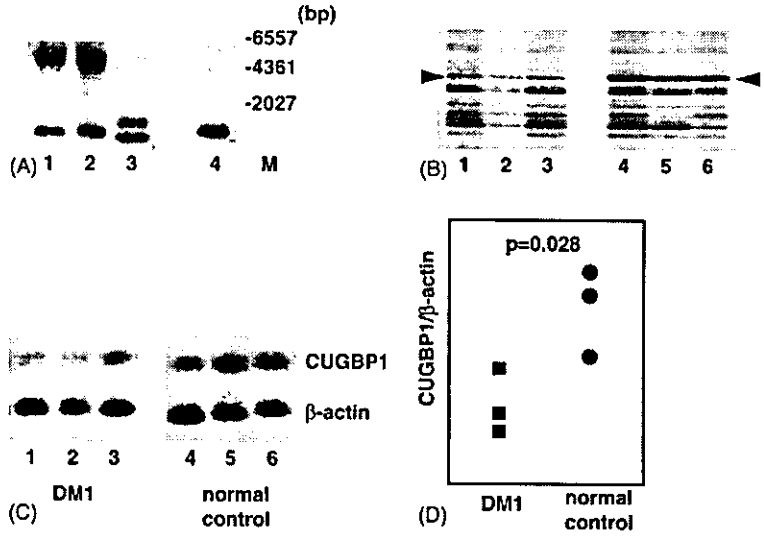

Fig. 1. (A) Measurement of the size of the CTG expansion. The DNA probe, which was 316 bp in size, was labeled with [^{32}P] dCTP and was used for Southern blot analysis. The BamHI fragment from the normal allele is 1376 bp in size (lane 4), while an additional larger band, varying between approximately 2500 and 11 000 bp, is identified in DM patients (lanes 1–3). M: size marker. (B) Differentially expressed clones. Lanes 1–3: DM patients; lanes 4–6: normal controls. The band indicated by the arrowhead is amplified to a lesser degree in DM patients than in the controls. This PCR product was excised from the gel, subcloned into a plasmid vector and then sequenced. The sequence of the subclone was 100% identical to that of CUGBP1. (C, D) Northern blot analysis of the CUG binding protein 1 mRNA. Lanes 1–3: DM patients; lanes 4–6: normal controls. The differentially amplified PCR product, identified as CUGBP1 mRNA, was labeled and hybridized with total RNA (15 μg loaded/lane) from biopsied muscle samples. β-Actin mRNA is included at the bottom of the figure to control for RNA loading (C). The data show that the quantity of CUGBP1 mRNA in the controls is approximately 113% greater than that in DM patients (D).

position almost identical to that of (CAG)12 or (CUG)12. Next, we generated CUGBP1 sense and anti-sense RNAs and incubated these with the CUG repeats. Fig. 3C shows that CUGBP1 sense RNA hybridized with (CUG)60 RNA, while no band shift was found after the incubation of CUGBP1 anti-sense RNA with (CUG)60 RNA. In addition, β-actin RNA containing no CAG repeats did not interact with (CUG)60 RNA. To identify the binding site, three truncated forms of CUGBP1 RNA were incubated with (CUG)60. Fig. 3D shows that R2 region, which contained the CAG rich region (see Fig. 3A), produced a band shift. To confirm that these band shifts were produced by the formation of double strand RNA (dsRNA), the incubations were processed by Rnase III diluted to either 0.0001, 0.001 or 0.01 U. Fig. 3E shows that the band shifts disappeared as the concentrations of Rnase III were increased. Finally, we diluted the CUGBP1 RNA to 20, 40, 60, 80, 100 and 120 pM with RNase free water, and added 20 pM of (CUG)60 RNA. Fig. 3F shows that an 80 pM concentration of CUGBP1 RNA was required for the band of (CUG)60 RNA to disappear.

4. Discussion

In this study, we performed differential display analysis to find mRNAs whose levels of expression are altered in muscle tissue of DM1 patients. We showed that CUGBP1 mRNA levels were lower in DM1 patients compared to those in normal individuals and myopathic disease controls. Furthermore, the amount of the mRNA was demonstrated to negatively correlate with the number of CTG repeats. From these results, we speculated that the mutant expansion may be involved in the alteration of CUGBP1 mRNA expression.

The repeat expansion has been suggested to affect neighboring DNAs located on chromosome 19q 13.3, such as the DMAHP gene, which is present in the 3′ untranslated region of the DMPK gene (Thornton et al., 1997; Klesert et al., 1997), or *dmwd*, a gene upstream of the DMPK start site (Eriksson et al., 1999). However, these observations may not be identical to our results, since the CUGBP1 gene is located in chromosome 11p11.

The CTG expansion has been reported to alter the accumulation of poly(A) + RNA in *trans* in DM1 muscle biopsies (Wang et al., 1995). Several studies have demonstrated that the expression of specific mRNAs is affected in DM1 patients. For example, insulin receptor mRNA is reduced in DM1 biopsy specimens (Morrone et al., 1997). In addition, the expression of ion channel mRNAs is altered in DM1 muscle (Kimura et al., 2000), and the expression of muscle-specific chloride channel mRNA is altered in a transgenic mouse model (Charlet et al., 2002) and in DM1 patients (Charlet et al., 2002; Mankodi et al., 2002). The mechanism by which the expression of particular genes is affected in DM1 patients remains unknown. It was reported that mutant CUG expansion has base-pairing interaction with other mRNAs that have expanded CAG repeats in vitro (Sasagawa et al., 1999). The authors reported that the RNA–RNA complex forms when the number of CUG and

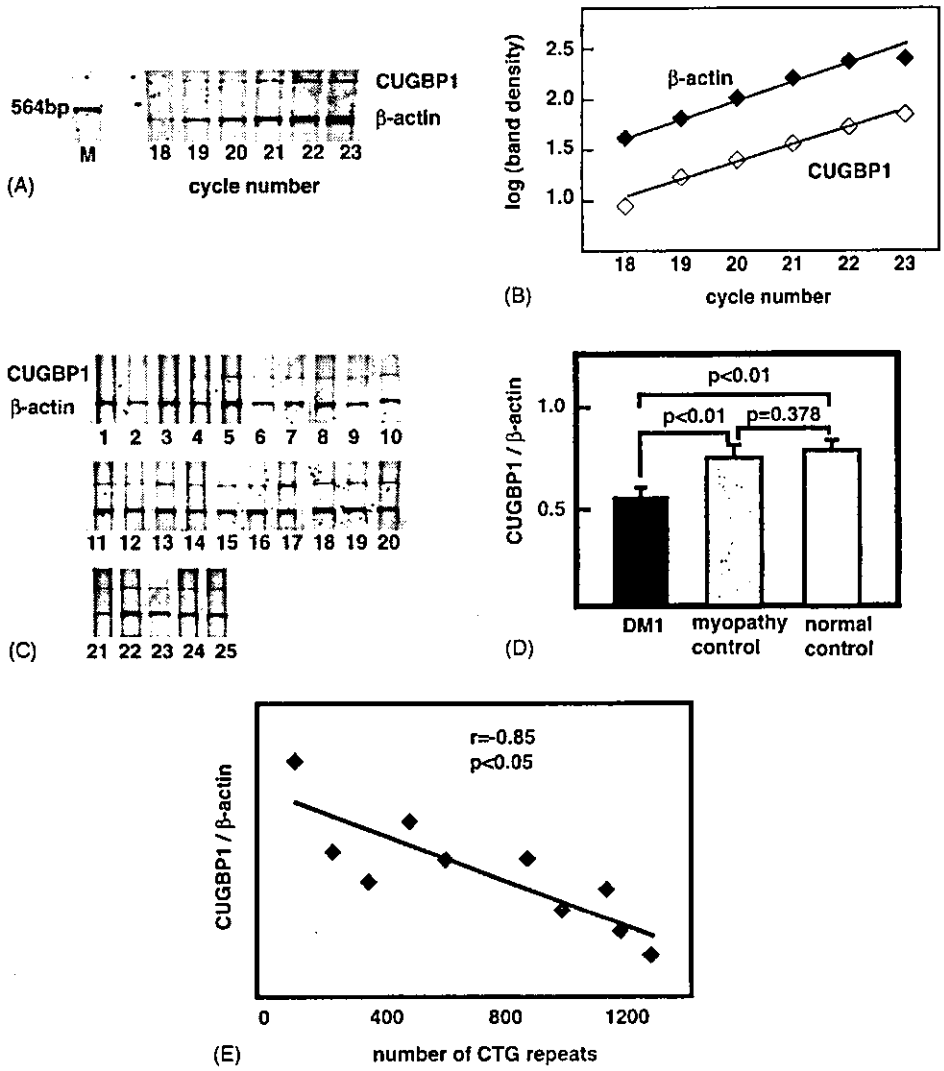

Fig. 2. Measurement of the CUGBP1 mRNA by semi-quantitative multiplex PCR assay. Reactions were sampled at every cycle of reaction, resolved by electrophoresis on 5% polyacrylamide sequencing gel and stained with SYBR Green I (A). Reaction cycle-intensity curves are fitted to the linear portion of a semi-logarithmic graph between 18 and 23 cycles, and it is assumed that both PCR products would be amplified with comparable efficiency under those conditions (B). (C) Gel images of the multiplex PCR with tissue expression levels of β-actin and CUGBP1 mRNAs. The numbers below the gel photograph are 1–10: DM1 patients; 11–20: myopathic controls; and 21–25: normal controls. (D) Differences in the ratio of CUGBP1 to β-actin band density between DM1 and controls. (E) Correlation of the number of CTG repeats with the relative band density in DM1 patients.

CAG repeats is over 140 and 35, respectively. However, in present study, (CUG)60 hybridized with (CAG)12, as well as with (CAG) These discrepancies may be caused by differences in the experimental procedures. In the previous study, RNAs were generated in a same tube and hybridized without heating, while, in the present study, we generated RNAs in separate tubes and heated the samples, followed by hybridization (see methods). We demonstrated that CUGBP1 RNA, which has short CAG repeats sequences, hybridized with (CUG)60, despite the fact that it did not bind to (CUG) These results suggest that the expanded CUG repeat may interact with complementary CAG repeat sequences within the CUGBP1 mRNA. This possibility is supported by the present data that the CUG expansion did not bind to β-actin RNA and CUGBP1 anti-sense RNA, which have no CAG repeats within their PCR-amplified sequences.

Fig. 3B showed slight band shifts in all lanes that contain more than one RNA in addition to those indicated by closed arrow heads. Since relatively smaller band shifts do not necessarily indicate that they have weaker interaction, we did negative control experiments using (CUG)12 RNA probe for Figs. 3E and F. To clarify whether this band shift is derived from formation of (CUG)12-CUGBP1 complex, we performed RNase III protection assay. We found that band pattern including the band shift was not changed after incubation with 0.01 Unit RNase III at 37 °C for 15 min, although intensities of each band corresponding to CUGBP1 and (CUG)12 were slightly increased (data not shown). Next, to examine the dose-dependent synthesis of RNA–RNA

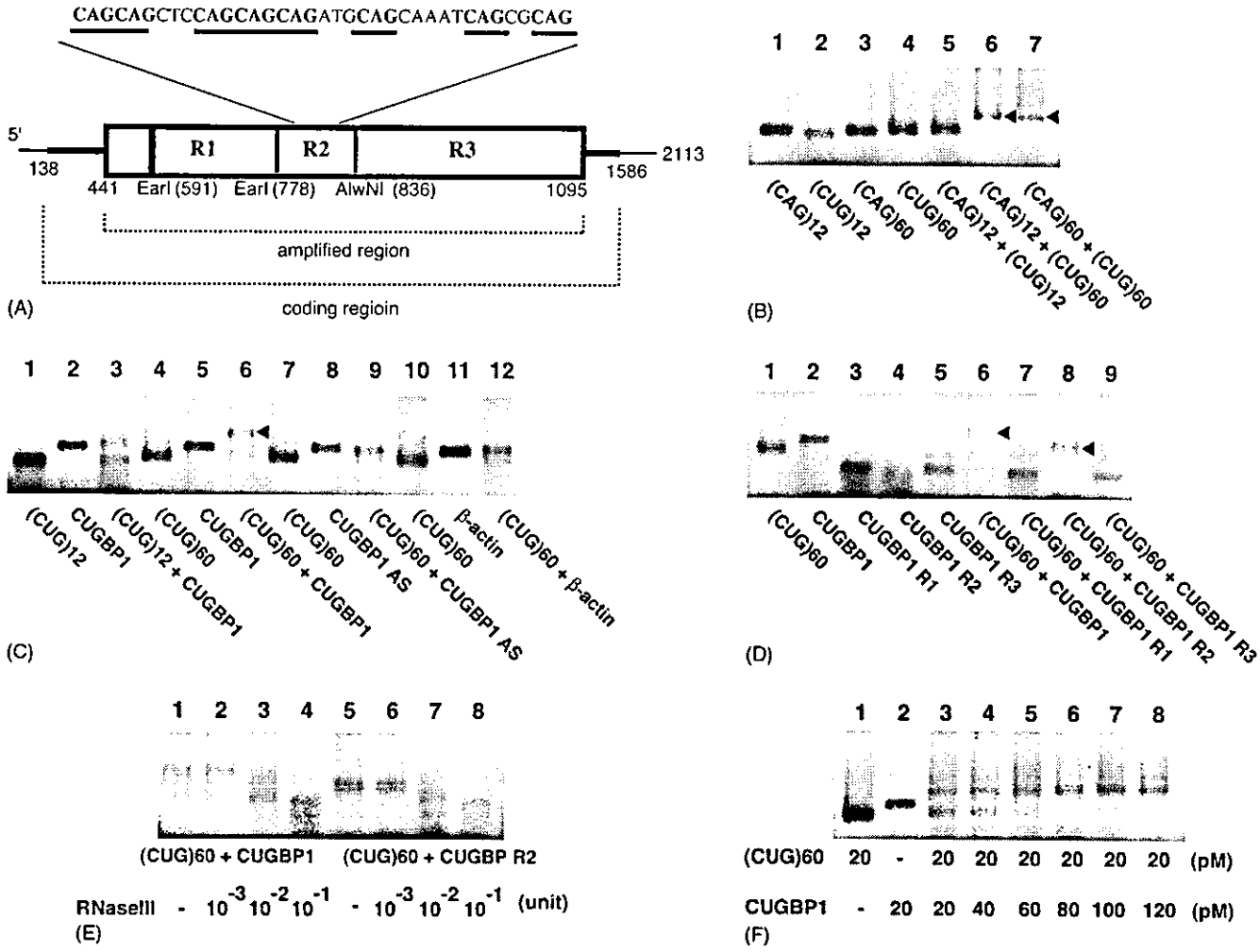

Fig. 3. In vitro binding of the RNA CUG repeat to CUGBP1 mRNA. (A) Whole molecule of CUGBP1 cDNA (Accession no. U63289), its coding sequence (138–1586nt) and PCR-amplified 655 bp product (441–1095nt). The product was cleaved with Ear I (position 591 and 778) and AlwNI (position 836) into three fragments, named R1 (591–778nt), R2 (778–836nt) and R3 (836–1095nt). CAG trinucleotides in R2 are underlined. (B) Antisense binding of CUG repeats to CAG repeats. Lane1, (CAG)12; lane2, (CUG)12; lane3, (CAG)60; lane4, (CUG)60; lane5, (CAG)12 + (CUG)12; lane6, (CAG)12 + (CUG)60; and lane7, (CAG)60 + (CUG)60. Band shifts are marked by an arrowhead. (C) Binding of CUG repeats to CUGBP1 RNA. Lane1, (CUG)12; lane2 and 5, CUGBP1 RNA; lane4, 7 and 10, (CUG)60; lane8, CUGBP1 anti-sense (AS) RNA; lane11, β-actin RNA; lane3, (CUG)12 + CUGBP1 RNA; lane6, (CUG)60 + CUGBP1 RNA; lane9, (CUG)60 + CUGBP1 anti-sense RNA; and lane12 (CUG)60 + β-actin RNA. Band shift is produced by the hybridization of (CUG)60 with CUGBP1 RNA (lane6, arrow head). (D) Binding of CUG repeats to the CAG rich region within CUGBP1 RNA. Lane1, (CUG)60; lane2, CUGBP1 RNA; lane3, CUGBP1 R1; lane4, CUGBP1 R2; lane5, CUGBP1 R3; lane6, (CUG)60 + CUGBP1 RNA; lane7, (CUG)60 + R1; lane8, (CUG)60 + R2; and lane9, (CUG)60 + R3. Band shift is seen in lane 8, as well as lane 6 (arrow head). (E) Degradation of hybrid RNAs by Rnase III. Lanes 1–4, (CUG)60 + CUGBP1 RNA; and lanes 5–8, (CUG)60 + CUGBP1 R2. Lanes 2–4 and 6–8 are incubations with RNase III at the indicated concentration. (F) Dose dependent synthesis in RNA–RNA complex. Lane1, (CUG)60; lane2, CUGBP1 RNA; and lane 3–8, (CUG)60 + CUGBP1 RNA. The concentrations of CUGBP1 RNAs in lanes 3–7 are 20, 40, 60, 80, 100 and 120 pM, respectively, and those of (CUG)60 were all 20 pM.

complex, we added 20 pM of (CUG)12 to 5, 10, 20, 30, 40 and 50 pM of CUGBP1 RNA, although we did not find significant change in the band intensity (data not shown). From these results, we suggested that CUGBP1 and (CUG)12 did not produce RNA–RNA complex. We suggest that a slight band shift may be largely caused by *cis*-effect within RNA. We also did the negative control studies using other RNA combinations, which were (CAG)12-(CUG)12, (CUG)60-CUGBP1(AS), (CUG)60-β-actin, (CUG)60-CUGBP1(R1) and (CUG)60-CUGBP1(R3), and we obtained the same results as that of (CUG)12-CUGBP1.

We showed that 20 pM of (CUG)60 RNA combined with 80 pM of CUGBP1 RNA in vitro. These observations suggest that as the size of the CUG expansion increases, it is able to interact with an increasing number of CUGBP1 mRNA molecules, which may explain the in vivo results that the expression of CUGBP1 mRNA correlated inversely with the number of CTG repeats in DM1 patients.

The biological effect of the RNA–RNA complex has not been fully clarified. Sasagawa et al. speculated that CUG/CAG double strand RNA may be recognized by a specific RNase and digested (Sasagawa et al., 1999). Our

results showed that the (CUG)60/CUGBP1 RNA complex was degenerated by Rnase III, which digests long dsRNA to short dsRNA (Lamontagne et al., 2001). Short double stranded RNAs of 20- to 25-nt can trigger the sequence specific degradation of homologous mRNA, which is termed RNA interference (RNAi) (Sharp, 2001).

Some proteins that bind to DMPK mRNA have been reported to play a role in DM1 pathogenesis. The CUG expansion in DMPK mRNA is a binding site for the CUGBP1 protein and the required level of CUGBP1 protein is increased in DM1 cells (Timchenko et al., 1996; Roberts et al., 1997). CUGBP1 mRNA levels were reported as unchanged in cells expressing expanded CUG repeats (Timchenko et al., 2001), although few in vivo results have been demonstrated. We also examined the muscle tissue levels of CUGBP1 protein by western blot analysis. However, our polyclonal antibody was unable to detect a definite band of CUGBP1 protein (data not shown).

In summary, our results indicate that the expansion mutation may cause alterations in CUGBP1 mRNA expression. We suggested that the CUG expansion and CUGBP1 mRNA formed a direct RNA–RNA contact in vitro. Previous studies demonstrated that mutant DMPK transcripts are retained within the nuclei of DM1 patient myoblasts (Taneja et al., 1995) and in muscle tissue from transgenic mice expressing an expanded CUG repeat in the DMPK gene (Mankodi et al., 2000). These observations suggest that the CUG repeats may have a greater chance to encounter other RNAs, which contain complementary sequences, in DM1 myonuclei, although the CUG/CAG complex formation has yet to be demonstrated in DM1 tissue. Further studies are required to confirm that such a molecular interaction is involved in DM1 pathogenesis.

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