J. Biochem. **143,** 377–383 (2008)
doi:10.1093/jb/mvm230

Quantitative Analysis of CUG-BP1 Binding to RNA Repeats

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Received November 12, 2007; accepted November 15, 2007; published online November 26, 2007

CUG-binding protein 1 (CUG-BP1) is a member of the CUG-BP1 and ETR-3-like factors (CELF) family of RNA-binding proteins, and is involved in myotonic dystrophy type 1 (DM1). Several mRNA targets of CUG-BP1 have been identified, including the insulin receptor, muscle chloride channel, and cardiac troponin T. On the other hand, CUG-BP1 has only a weak affinity for CUG repeats. We conducted quantitative-binding assays to assess CUG-BP1 affinities for several repeat RNAs by surface plasmon resonance (SPR). Although we detected interactions between CUG-BP1 and CUG repeats, other UG-rich sequences actually showed stronger interactions. Binding constants of CUG-BP1 for RNAs indicated that the affinity for UG repeats was far stronger than for CUG repeats. We also found that N-terminal deletion mutant of CUG-BP1 has UG repeat-binding activity in a yeast three-hybrid system, although C-terminal deletion mutant does not. Our data indicates that CUG-BP1 specifically recognized UG repeats, probably through cooperative binding of RNA recognition motifs at both ends of the protein. This is the first report of a binding constant for CUG-BP1 calculated *in vitro*.

Key words: binding constant, CUG-BP1, myotonic dystrophy, surface plasmon resonance, triplet-repeat.

Abbreviations: 3-AT, 3-amino triazole; CELF, CUG-BP and ETR3-like factor; DM, myotonic dystrophy; DMPK, DM protein kinase; RRM, RNA recognition motif.

CUG-BP and ETR3-like factor (CELF) proteins, also known as Bruno-like (BRUNOL) proteins, are a family of highly conserved RNA-binding proteins (*1*, *2*). All mammalian CELF proteins contain three RNA recognition motifs (RRMs; also referred to as RNP domains or consensus RNA-binding domains) and have a similar organization: two closely spaced N-terminal RRMs, a divergent hinge region of 60–90 residues, and a C-terminal RRM (*1*). RRM-containing proteins represent the largest family of RNA-binding proteins and perform various functions in post-transcriptional gene regulation. CUG-binding protein 1 (CUG-BP1) was the first discovered member of the CELF proteins and acts as a regulator of alternative splicing (*1*, *3–5*), translation (*6*, *7*) and deadenylation (*8*, *9*).

Several reports have shown the involvement of this protein in myotonic dystrophy (dystrophia myotonica or DM) type 1 (DM1) (3–6, 10 and 11 for review). As indicated by its name, the binding of CUG-BP1 to expanded CUG repeats was first demonstrated in the onset of DM (*12*, *13*). Indeed, CUG-BP1 was first identified as a protein binding to a $(CUG)_8$ probe in a gel retardation assay (*12*, *13*). However, whether this protein does actually bind expanded repeats is controversial. For example, it does not specifically co-localize with nuclear RNA foci, formed by expanded repeats in DM1 cells (*14*). Moreover, contrary to its name, CUG-BP1 actually appears to specifically bind to UG motifs, rather than to CUG repeats, in a yeast three-hybrid system (*15*, *16*).

Muscleblind-like proteins, another group of RNA-binding proteins, have recently emerged as more plausible proteins for the sequestration model (*14*, *16*). Nevertheless, CUG-BP1 is still an important factor in DM1, because reports have shown up-regulation of CUG-BP1 protein levels in DM1 cells by still unknown mechanisms, and its elevated activity is thought to cause abnormalities in DM1 (*4*).

CUG-BP1 may contribute to the aberrant splicing of multiple genes, a hallmark of DM1. Alternative splicing of the insulin receptor (IR), muscle chloride channel (CLCN1), cardiac troponin T (cTNT) and other genes is regulated by CUG-BP1, and the splicing patterns of these genes are altered in DM1 patients (*3–5*, *17*).

In addition to splicing defects, it has also been suggested that CUG-BP1 is involved in the altered translation of p21 and MEF2A in DM1 cells, and leads to defects in myogenic progression (*6*). Furthermore, over-expression of CUG-BP1 in mice recapitulated some abnormalities similar to those observed in DM1 patients, such as aberrant splicing and histological impairment of the muscle (*10*). It is also described that mice over-expressing normal DMPK 3′-untranslated region had increased levels of CUG-BP1 in skeletal muscle, as seen in individuals with DM1 (*18*). Thus, understanding CUG-BP function and its target genes is important for an understanding of DM pathogenesis.

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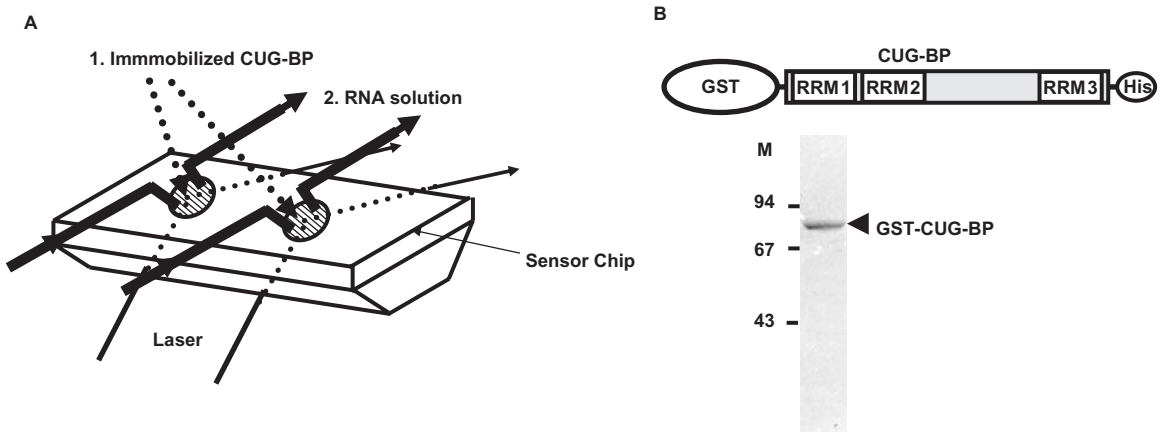

Fig. 1. **Design of SPR experiment and purified protein used in this study.** A: Schematic illustration of the SPR analysis. First, purified CUG-BP1 was immobilized on the gold layer of the sensor chip. RNA solution flowed through the sensor chip surface. CUG-BP1-RNA interactions were detected as the difference in resonance angle of the surface plasmon, elicited by a laser. B: Recombinant CUG-BP1. (Top) Structure of recombinant CUG-BP1. CUG-BP1 was fused with glutathione S-transferase at the N-terminus and a 6×His-tag at the C-terminus. (Bottom) Purified GST-CUG-BP1 used in this study. The purified fraction was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. Molecular weight is indicated by M.

Despite its importance, the RNA-binding specificity of CUG-BP1 remains to be determined, primarily because of the lack of biochemical analysis directly addressing the binding constants. In this article, we conducted an *in vitro* binding analysis between CUG-BP1 and CUG and other repetitive nucleotides to examine whether CUG-BP1 could interact with CUG repeats and to find specific target sequences of CUG-BP1.

MATERIALS AND METHODS

Synthesis of DNAs Containing Repetitive Sequences—DNA fragments of repetitive sequences such as $(CUG)_{140}$, $(UG)_{24}$, $(UG)_{41}$ and $(UAUG)_{14}$, were synthesized by a non-template polymerase chain reaction (PCR) method with Pfu Turbo polymerase (Stratagene) through 5–25 cycles of amplification (96°C for 1 min, 60°C for 30 s and 72°C for 90 s) after 10 rounds of 96°C for 1 min, 60°C for 30 s and 72°C for 1 min (*19*). DNAs for other short repeat fragments were purchased from Proligo, Japan.

In vitro Transcription—DNA fragments with repetitive sequences were ligated into the *Hin*cII site of a pBluescriptII SK(+)-derived vector containing an *Apa*I–*Eco*52I site deletion and a *Hin*cII site insertion. Using DNA fragments from these constructs as templates, RNAs were transcribed with the MEGAscript T7 or T3 kits (Ambion) in a 20 μL reaction. The quantity of the synthesized mRNAs was checked by the absorbance at 260 and 280 nm. Their quality was checked by denaturing agarose gel electrophoresis.

SPR Biosensor Analysis—In the SPR biosensor analysis, using SPR-MACS (Moritex), the sensor chip was coated with 4,4′-dithiodibuthyric acid (DDA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), according to the manufacturer's protocol. CUG-BP1 was immobilized on the sensor chip by amino-coupling, and RNAs were passed over the protein surface (Fig. 1A). The binding reaction was performed in a running buffer of 10 mM HEPES (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol (DTT), at a flow rate of 10 μl/min. Association and dissociation were measured in arbitrary units and displayed on a graph (sensorgram). The RNAs binding to the protein surface were released with 9 mM NaOH as a regeneration solution (Fig. 2). After this treatment, the next binding experiment was performed. Binding constants were calculated based on the sensorgram using Method 2 software (Moritex).

Bacterial Expression and Purification of Recombinant Double-Tagged CUG-BP1 and Mutants—An expression vector, human CUG-BP1 in pET-GX, has been previously described (*16*). BL21(DE3) competent cells (Stratagene) were transformed with this construct and used for protein expression. A single colony was grown overnight at 37°C in LB medium with 50 μg/ml ampicillin, diluted 1:20 in fresh medium, and then grown at 37°C until A_{600} reached 0.25–0.30. IPTG was then added to the culture to a final concentration of 0.1 mM and incubated for a further hour at 37°C. The cells were harvested as a pellet and were resuspended in 30 ml PBS containing 1/500 volume of proteinase inhibitor mixture (Wako), 1 mM PMSF and 1 mM DTT, and were lysed in a French press (Ohtake Works, Co.). The total cell lysate was centrifuged (15 min, 15,000*g*). The supernatant was subjected to affinity purification using GSTrap FF affinity columns (Amersham Pharmacia Biotech) with an FPLC system and 300 μl (bed volume) of BD Talon affinity resin (Clontech), according to the manufacturer's protocol. The protein sample was dialysed twice against HBS buffer (10 mM HEPES-NaOH pH 8.0, 150 mM NaCl, 1 mM DTT). The quantity and purity were checked on SDS-polyacrylamide electrophoresis gels, stained with Coomassie brilliant blue (CBB; Fig. 1B).

Yeast Three-Hybrid System—To make CUG-BP1 RRM mutants, conserved residues were changed by site-directed mutagenesis, K59Q and F63A in Mut1; R148Q and F152A in Mut2 and K438Q and F442A in Mut3.

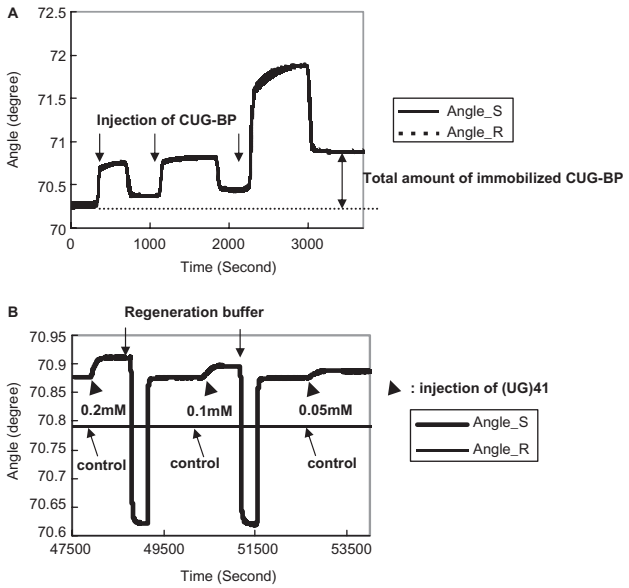

Fig. 2. **SPR measurement.** A: Immobilization of CUG-BP1. The signals of the resonance angle were monitored during the injection and immobilization of purified CUG-BP1 protein on the gold layer. Angle S and Angle R indicate the resonance angles at the measurement spots for the sample and the reference control, respectively. Arrows indicate the time when CUG-BP1 solution was injected. Multiple injections enhanced the amount of immobilized CUG-BP1. B: An example of SPR measurement of CUG-BP1 and $(UG)_{41}$ repeat. Arrows show the time point when $(UG)_{41}$ RNA solution at the concentration indicated was injected. Each time after measurement, regeneration buffer was injected to wash off RNAs bound to CUG-BP1. Injection of a higher concentration of $(UG)_{41}$ resulted in more rapid and larger changes in the signals.

Mut(1+2), Mut(2+3) and Mut(1+3) contained two combined RRM mutations. To make deletion mutants, fragments of CUG-BP1 (Fig. 4), were amplified by PCR. ΔRRM1, ΔRRM2, ΔRRM3 and Δlinker constructs have been described previously (*15*). In the *HIS3* assay, yeast strain L40-coat was transformed with pGAD and pIIIA/MS2-2 vectors encoding CUG-BP1 mutants and RNAs, respectively, and selected on plates lacking leucine and uracil. Yeast transformants were picked and spotted onto selection plates lacking leucine, uracil and histidine, with or without 0.5, 1, 2.5, 5 or 10 mM 3-amino triazole (3-AT) (*16*). The plates were incubated at 30°C for about 1 week and the viability of the yeast transformants was analysed. We classified the binding activity as (+++++), (++++), (+++), (++) and (+) when yeast growth was observed on the plates containing 10, 5, 2.5, 1 and 0.5 mM 3-AT, respectively; no growth of yeast transformants with empty vectors was observed on 0.5 mM 3-AT plates.

RESULTS

SPR Analysis—We used a recombinant CUG-BP1 protein, fused with glutathione S-transferase (GST) at the N-terminus and a histidine tag at the C-terminus of the protein (Fig. 1B). For measurements, CUG-BP1 was immobilized on the sensor chip and then RNA solution was passed over the chip (Fig. 1A). By detecting changes in the sensorgram, we could confirm the immobilization of CUG-BP1 (Fig. 2A) and the binding of RNAs to CUG-BP1 (an example is shown in Fig. 2B).

Comparative Analysis of CUG-BP1's RNA-Binding in an SPR Assay—First, we determined whether CUG-BP1 could bind to the $(CUG)_8$ or $(CUG)_{140}$ repeats. We detected weak binding of CUG-BP1 to $(CUG)_8$ and $(CUG)_{140}$ repeats, with K_d values of 27.2 μM and 6 μM, respectively, but detected no binding of CUG-BP1 to a $(CAG)_{10}$ repeat (Fig. 3, Table 1).

Next, we compared UG repeat sequences to CUG repeats, because the former interacted with CUG-BP1 in previous yeast three-hybrid experiments (*15*,*16*). We detected stronger binding of CUG-BP1 to UG repeats than to CUG repeats (Fig. 3, Table 1). Thus, CUG-BP1 actually bound tighter to a UG motif than to a CUG motif.

UG Dinucleotide is a Preferred Binding Motif of CUG-BP1—We next asked which was more important for the binding of CUG-BP1, the content of 'U' and 'G' nucleotides in the RNA sequence or that of 'UG' dinucleotides. Although $(UG)_{15}$ and $(UUGG)_7$ contain 15 Us and 15 Gs (Table 2), the binding of CUG-BP1 to $(UUGG)_7$ was weaker than to $(UG)_{15}$. Remarkably, another RNA $(UUUGGG)_5$, which has the same number of Us and Gs as $(UG)_{15}$ and $(UUGG)_7$, showed no detectable binding to CUG-BP1. These results suggest that the content of the UG dinucleotide motif, but not the number of U and G nucleotides, is important for recognition by CUG-BP1.

CUG-BP1 can bind to an RNA sequence, named embryonic deadenylation element (EDEN), which is composed of repetitive UAUG motifs (*20*). Thus, UA may be another recognition motif for CUG-BP1. To test this, we examined $(UAUG)_7$ as a probe. Compared to $(UG)_{15}$, $(UAUG)_7$ showed decreased binding to CUG-BP1, implying a weaker affinity for the UA motif than for a UG motif. Consistently, one substitution of U to A in one of the middle UG motifs in $(UG)_{15}$ (UG15mut, Table 2) doubled the value of K_d. Thus, UA is not as preferred a binding motif for CUG-BP1 as UG. Furthermore, we could detect no binding to a pure UA repeat, $(UA)_{15}$. This may reflect intramolecular or intermolecular base-pairing between UA repeats, which may affect the binding of CUG-BP1.

Finally, we examined the binding of CUG-BP1 to UGG (GUG/GGU) and UUG (UGU/GUU) motifs, because both GUG and UGU motifs can be found in a UG dinucleotide repeat. Also, repeats of both UGG/GUG/GGU and UUG/UGU/GUU motifs contain UG motifs. In both $(UGG)_{10}$ and $(UUG)_{10}$, the number of non-overlapping UG motifs was smaller than that of $(UG)_{15}$, while the number of GUG and UGU motifs, respectively, was larger than in $(UG)_{15}$. This comparison enabled the determination of whether the GUG or UGU motifs were more preferred than UG. Both $(UGG)_{10}$ and $(UUG)_{10}$ showed lower affinities than $(UG)_{15}$ (Table 2). From these results, we conclude that CUG-BP1 recognizes a UG (or GU) dinucleotide repeat as a binding motif, rather than other UG-rich repeats.

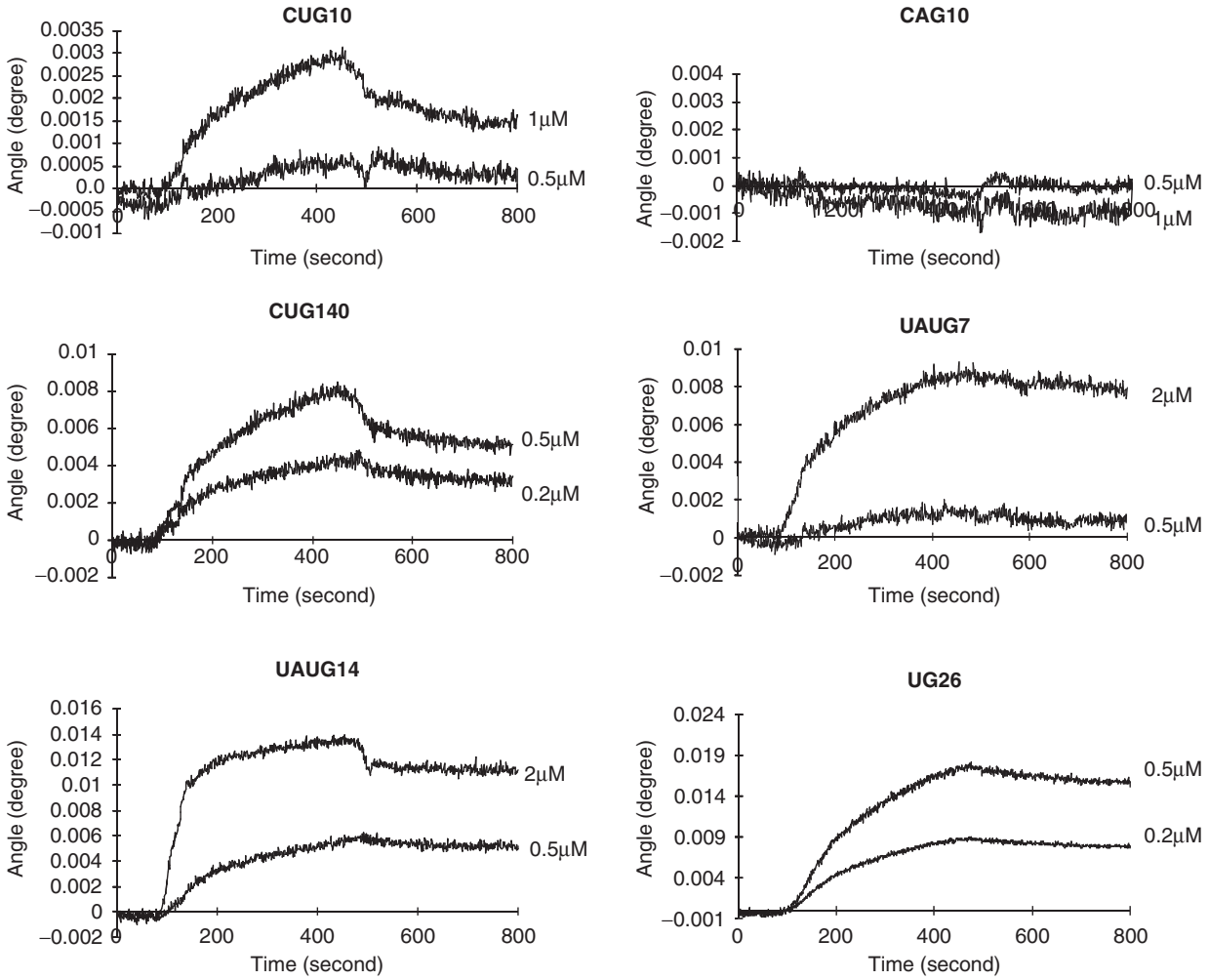

Fig. 3. **Binding of various repetitive RNAs in the SPR analysis.** SPR signals obtained with repetitive RNAs were monitored. Each RNA sequence is indicated above the panel. Concentrations (μM) represent that of injected RNA.

Table 1. **Binding constants of CUG-BP with di-, tri- and tetra-nucleotide repeats.**

Repeat	K_d (μM)	k_{ass} ($M^{-1}s^{-1}$)	k_{diss} (s^{-1})
(CA)15	–	–	–
(CAG)10	–	–	–
(CUG)10	27 ± 12	$4.6 \pm 2.5 \times 10^2$	$6.6 \pm 0.3 \times 10^{-3}$
(CUG)140	6.0 ± 2.0	$2.1 \pm 1.3 \times 10^3$	$9.2 \pm 0.9 \times 10^{-3}$
(UAUG)7	1.3 ± 0.1	$2.1 \pm 0.2 \times 10^3$	$2.6 \pm 0.2 \times 10^{-3}$
(UAUG)14	1.3 ± 0.3	$2.6 \pm 0.3 \times 10^3$	$3.4 \pm 1.1 \times 10^{-3}$
(UG)15	0.25 ± 0.10	$9.0 \pm 1.0 \times 10^3$	$2.3 \pm 1.1 \times 10^{-3}$
(UG)26	0.12 ± 0.05	$9.6 \pm 1.7 \times 10^3$	$1.1 \pm 0.03 \times 10^{-3}$
(UG)41	0.06 ± 0.02	$3.1 \pm 0.7 \times 10^4$	$1.9 \pm 0.7 \times 10^{-3}$

$N = 3$, mean ± SE.

$(UGG)_{10}$ and $(UUG)_{10}$ showed similar binding to CUG-BP1, while $(CUG)_{10}$, which contains the same number of UG motifs, showed a 7-fold increased K_d value. This apparently suggests that CUG is *not* an optimal binding motif for CUG-BP1, despite its name.

Mutation Analysis of CUG-BP1 in a Yeast Three-Hybrid System—Although CUG-BP1 has three RRM RNA-binding domains, it is still unknown which RRM is responsible for binding to a UG repeat. Previously, we reported that these three RRMs redundantly contributed to binding to a UG repeat (*15*). To determine the RNA-binding domain of CUG-BP1 more specifically, we conducted a yeast three-hybrid assay using several RRM mutants, in which two conserved residues in the respective RRM were disrupted. Mutation of one of any RRMs reduced binding of CUG-BP1 to UG repeats (Fig. 4). Double mutants did not have substantial-binding ability. On the other hand, the results of deletion mutants indicated that the C-terminal region of CUG-BP1 has an important function to bind to UG repeats. N1 and N2 (or N3) mutants did not show ability to bind to UG repeats. In contrast, a deletion mutant of both RRM1 and RRM2 (C2) still showed binding to a UG repeat. In addition, the experiment showed that a small portion of CUG-BP1 linker region was also essential for efficient binding to UG repeats.

Table 2. **K_d values of CUG-BP with di-, tri- and tetra-nucleotide repeats (30 nt.).**

Repeat	Sequence (30 nt)	K_d (μM)
(UG)15	UGUGUGUGUGUGUGUGUGUGUGUGUGUG	0.25 ± 0.1
(UG)15mut	UGUGUGUGUGUGUAUGUGUGUGUGUGUG	0.52 ± 0.05
(UAUG)7	UAUGUAUGUAUGUAUGUAUGUAUGUAUGUA	1.3 ± 0.1
(UUGG)7	GUUGGUUGGUUGGUUGGUUGGUUGGUUGGU	3.3 ± 0.4
(UUUGGG)5	UUUGGGUUUGGGUUUGGGUUUGGGUUUGGG	–
(UA)15	UAUAUAUAUAUAUAUAUAUAUAUAUAUA	–
(UGG)10	UGGUGGUGGUGGUGGUGGUGGUGGUGGUGG	4.1 ± 0.6
(UUG)10	UUGUUGUUGUUGUUGUUGUUGUUGUUGUUG	3.6 ± 0.1
(CUG)10	CUGCUGCUGCUGCUGCUGCUGCUGCUGCUG	27.2 ± 12
(CAG)10	CAGCAGCAGCAGCAGCAGCAGCAGCAGCAG	–

$N = 3$, mean ± SE. (UG)15mut has UA in the seventh UG repeat (underlined).

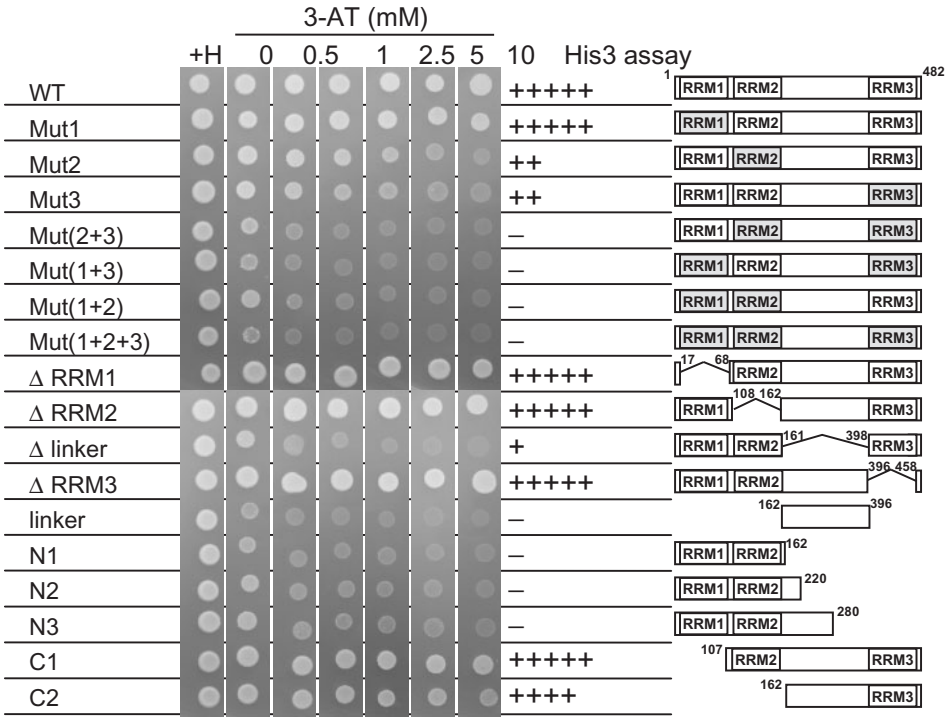

Fig. 4. **Yeast three-hybrid analysis of CUG-BP1 mutants.** Structures of CUG-BP1 mutants used in the assay are shown on the right. Mutated RRM motifs are indicated by hatched boxes. Results of the yeast three-hybrid HIS3 assay are shown in the middle. Wild-type or mutant CUG-BP1 constructs and the $(UG)_{24}$ expression vector were transformed into yeast cells. Transformants were seeded to test their viability on the selection plates, lacking histidine but containing 0.5, 1, 2.5, 5 or 10 mM 3-amino triazole (3-AT), a competitive inhibitor of histidine synthesis. Viability of yeast transformants was classified as follows: +++++, ++++, +++, ++, + means, viable with 10, 5, 2.5, 1 and 0.5 mM 3-AT, respectively and – means, not viable with 0.5 mM 3-AT.

DISCUSSION

CUG-BP1 Specifically Binds to (UG) Dinucleotide Repeat RNA in an SPR Assay—We previously reported that CUG-BP1 preferentially bound to UG dinucleotides in a yeast three-hybrid assay (*15*). In the current report, we used a SPR assay to determine actual binding constants of CUG-BP1 with RNAs *in vitro*. In the SPR assay, CUG-BP1 bound to $(CUG)_8$ and $(CUG)_{140}$ repeats, but not to a $(CAG)_{10}$ repeat. However, the dissociation constants obtained in our experiments between CUG-BP1 and $(CUG)_{10}$ or $(CUG)_{140}$ suggested that CUG-BP1 does not bind very strongly to the CUG triplet repeat *in vitro* or possibly under physiological conditions. The moderate binding between CUG-BP1 and CUG repeats in addition to differences in experimental systems may explain some of the previous controversial results.

In contrast to binding to the CUG repeat, CUG-BP1 bound strongly to UG dinucleotide repeats in the SPR assay. Subsequently, a comparative analysis using UG-like repetitive sequences (Table 2) revealed that CUG-BP1 prefers 'pure' UG dinucleotide repeats as binding targets, rather than other UG-rich sequences, such as $(UAUG)_7$ or $(UUG)_{10}$. Because longer UG repeats seemed to bind more strongly to CUG-BP1 (Table 1) and pure UG repeat tracts were sufficient for binding to CUG-BP1, we conclude that CUG-BP1 recognizes UG repeats as a

binding target. Importantly, a recent report showed that ETR-3, the closest CELF protein to CUG-BP1, recognized UG repeats as well as UGUU, which were identified from systematic evolution of ligands by exponential enrichment (SELEX) (*21*). These results suggest that these two CELF proteins have similar binding specificities. However, UGUU motifs were included in the $(UUG)_{10}$ repeat used in our study; binding was not as strong as to $(UG)_{15}$ in the assays. Thus, CUG-BP1 may bind more specifically to UG motifs than UGUU motifs. However, we cannot exclude that efficient recognition of UGUU motifs by CUG-BP1 requires a particular spacing of nucleotides between the UGUU motifs.

The Importance of RRMs in the Binding to UG Repeats—In our previous study, we examined the binding affinities of CUG-BP1 deletion mutants in a yeast three-hybrid assay and showed that no complete loss of RNA-binding ability of CUG-BP1 occurred when any of the three RRMs was singly deleted (*15*). This suggests redundant RNA recognition among the RRMs. We attempted to reveal more detail about the structural requirements of the RNA-binding abilities of CUG-BP1 using additional mutant proteins.

Deletion analyses indicated that the N-terminal fragment (containing RRM1 and RRM2) did not show ability to bind to UG repeats, although Mut3 had binding affinity to UG repeats. On the contrary, the C-terminal fragment (containing RRM3) of CUG-BP1 harbored UG repeat-binding abilities, although Mut(1 + 2) did not show any binding to UG repeats. These results indicate that a conformational change occurs in deletion mutants, and that a conformational and cooperative interaction of three RRMs is important for CUG-BP1 function.

Moreover, a deletion of linker (Δlinker) strongly reduced binding affinity to UG repeats. Taken that the linker region itself did not have RNA binding ability into account, this result indicates that the linker region is important for cooperative RNA-binding by both N- and C-terminus, possibly by modulating the conformation of the entire protein. Alternatively, it is conceivable that this linker region modulates the RNA-binding abilities or functions of CUG-BP1 by mediating multimer formation, as reported in a study of EDEN-BP, a CUG-BP1 ortholog in frogs (*22*).

Biological Implication of UG Binding of CUG-BP1—The characterization of the RNA-binding specificity of a protein is important in understanding its function or physiological role. In general, the presence of multiple binding motifs for an RNA-binding protein would enhance the probability of the protein binding to the RNA. From our results, it is predicted that the number of UG motifs in an RNA stretch will determine the affinity of CUG-BP1 for it. If this is so, the degree of influence on target RNAs of CUG-BP1 would be variable, depending on the content or length of a UG repeat. Indeed, we observed length-dependent binding of CUG-BP1 with UG repeats (Table 1).

Although there are many pure UG repeat-containing genes in the human genome (*15*), few of them have been analysed as targets of CUG-BP1. It would be of value to focus on such genes to obtain further insight into CUG-BP1 function. Several reports have described functional target sequences of CUG-BP1 (*3–5*). However, there is no previous example of a pure UG repeat as a functional target of CUG-BP1. Thus, it is of importance to examine the functional interaction between CUG-BP1 and UG-containing genes. Because CUG-BP1 may have pathogenic roles in DM, identification of its target genes may be a beneficial way to understand the molecular mechanism of this disease.

UG-binding ability is apparently a feature of CUG-BP1 (and ETR-3); another RNA-binding protein, TDP-43, also preferentially binds to a UG repeat (*23*). As TDP-43 is a splicing regulator, like CUG-BP1, there may be some functional connection, such as antagonism, between these proteins that bind to UG repeat-containing RNAs. In addition, functional competition or cooperation between CUG-BP1 and ETR-3 or other CELF proteins, which may share RNA substrates, would be an interesting area of future work toward understanding the importance of UG-binding proteins.

Yeast three-hybrid components were kindly provided by Dr Marvin Wickens. This work was supported in part by grants from the Ministry of Health, Labor and Welfare, Japan (17A-10) and the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES

1. Ladd, A.N., Charlet, N., and Cooper, T.A. (2001) The CELF family of RNA binding proteins is implicated in cell-specific and developmentally regulated alternative splicing. *Mol. Cell. Biol.* **21**, 1285–1296
2. Good, P.J., Chen, Q., Warner, S.J., and Herring, D.C. (2000) A family of human RNA-binding proteins related to the Drosophila Bruno translational regulator. *J. Biol. Chem.* **275**, 28583–28592
3. 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
4. Savkur, R.S., Philips, A.V., and Cooper, T.A. (2001) Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy. *Nat. Genet.* **29**, 40–47
5. Charlet, B.N., Savkur, R.S., Singh, G., Philips, A.V., Grice, E.A., and Cooper, T.A. (2002) Loss of the muscle-specific chloride channel in type 1 myotonic dystrophy due to misregulated alternative splicing. *Mol. Cell.* **10**, 45–53
6. Timchenko, N.A., Iakova, P., Cai, Z.J., Smith, J.R., and Timchenko, L.T. (2001) Molecular basis for impaired muscle differentiation in myotonic dystrophy. *Mol. Cell. Biol.* **21**, 6927–6938
7. Timchenko, N.A., Welm, A.L., Lu, X., and Timchenko, L.T. (1999) CUG repeat binding protein (CUGBP1) interacts with the 5' region of C/EBPbeta mRNA and regulates translation of C/EBPbeta isoforms. *Nucleic Acids Res.* **27**, 4517–4525
8. Paillard, L., Legagneu, X V., and Osborne, H.B. (2003) A functional deadenylation assay identifies human CUG-BP as a deadenylation factor. *Biol. Cell* **95**, 107–113
9. Moraes, K.C., Wilusz, C.J., and Wilusz, J. (2006) CUG-BP binds to RNA substrates and recruits PARN deadenylase. *RNA* **12**, 1084–1091
10. Ho, T.H., Bundman, D., Armstrong, D.L., and Cooper, T.A. (2005) Transgenic mice expressing CUG-BP1 reproduce splicing mis-regulation observed in myotonic dystrophy. *Hum. Mol. Genet.* **14**, 1539–1547

11. Ranum, L. and Cooper, TA. (2006) RNA-mediated neuromuscular disorders. *Annu. Rev. Neurosci.* **29**, 259–277
12. Timchenko, L.T., Timchenko, N.A., Caskey, C.T., and Roberts, R. (1996) Novel proteins with binding specificity for DNA CTG repeats and RNA CUG repeats: implications for myotonic dystrophy. *Hum. Mol. Genet.* **5**, 115–121
13. 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 repeat RNA-binding protein and its expression in myotonic dystrophy. *Nucleic Acids Res.* **24**, 4407–4414
14. Fardaei, M., Rogers, M.T., Thorpe, H.M., Larkin, K., Hamshere, M.G., Harper, P.S., and Brook, J.D. (2002) Three proteins, MBNL, MBLL and MBXL, co-localize in vivo with nuclear foci of expanded-repeat transcripts in DM1 and DM2 cells. *Hum. Mol. Genet.* **11**, 805–814
15. 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
16. Kino, Y., Oma, Y., Sasagawa, N., and Ishiura, S. (2004) Muscleblind protein, MBNL1/EXP, binds specifically to CHHG repeats. *Hum. Mol. Genet.* **13**, 495–507
17. Nezu, Y., Kino, Y., Sasagawa, N., Nishino, I., and Ishiura, S. (2007) Expression of MBNL and CELF mRNA transcripts in muscles with myotonic dystrophy. *Neuromuscular Disord.* **17**, 306–312
18. Mahadevan, M.S., Yadava, R.S., Yu, Q., Balijepalli, S., Frenzel-McCardell, C.D., Bourne, T.D., and Phillips, L.H. (2006) Reversible model of RNA toxicity and cardiac conduction defects in myotonic dystrophy. *Nat. Genet.* **38**, 1066–1070
19. Takahashi, N., Sasagawa, N., Suzuki, K., and Ishiura, S. (1999) Synthesis of long trinucleotide repeats in vitro. *Neurosci.Lett.* **262**, 45–48
20. 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
21. Faustino, N.A. and Cooper, T.A. (2005) Identification of putative new splicing targets for ETR-3 using sequences identified by systematic evolution of ligands by exponential enrichment. *Mol. Cell Biol.* **25**, 879–887
22. Bonnet-Corven, S., Audic, Y., Omilli, F., and Osborne, H.B. (2002) An analysis of the sequence requirements of EDEN-BP for specific RNA binding. *Nucleic Acids Res.* **30**, 4667–4674
23. Buratti, E., Dork, T., Zuccato, E., Pagani, F., Romano, M., and Baralle, F.E. (2001) Nuclear factor TDP-43 and SR proteins promote in vitro and in vivo CFTR exon 9 skipping. *EMBO J.* **20**, 1774–1784