厚生労働科学研究研究費補助金

こころの健康科学研究事業

スプライシングを利用した筋強直性ジストロフィーの治療

平成20年度　総括研究報告書

主任研究者　石浦　章一

平成21(2009)年　4月

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厚生労働科学研究費補助金（こころの健康科学研究事業）
総括研究報告書

スプライシングを利用した筋強直性ジストロフィーの治療

研究代表者　石浦　章一　　東京大学・大学院総合文化研究科・教授

研究要旨

筋強直性ジストロフィーの治療戦略として、スプライシングの正常化にアンチセンス法を導入するとともに、患者のQOLに最も重要な筋力低下に関係する遺伝子のスクリーニングを行い、いくつかの有力遺伝子候補を得た。

研究分担者　西野一三・
国立精神神経センター神経研究所・部長

A. 研究目的

筋強直性ジストロフィー（DM）は遺伝子が伸長することによる全身性疾患で、遺伝的にDM1とDM2の2種類がある。DM1はDMPK遺伝子の非翻訳領域のCTGリピートが伸長し、DM2はZNF9遺伝子のイントロン1にあるCCTGリピートが伸長する。症状は、長く伸びたRNAのCUG3塩基リピートまたはCCUG4塩基リピートにMBNL1やCUG-binding protein（CUG-BP）などのRNA結合タンパク質がトラップされ、正常の機能であるスプライシングが異常になることで生じ、そのために「RNA病」であると言われている。そのため、スプライシングを正常化することにより治療が可能になるのではないかと考えた。

そのための方策は2つある。1つはRNAリピートに結合するスプライシング調節タンパク質の発現を加減することによって、正常化スプライシングを導くという戦略である。もう1つは、薬物添加やアンチセンスの手法を用いてスプライシング自体を変化させるものである。本研究では、後者の方法を検討した。また、分担研究者の西野の協力の下、DM患者筋を用いた新しいスプライシング異常の検出を行った。

B. 研究方法

塩素チャネルミニ遺伝子（エキソン6、7A、7）を用いたスプライシング・アッセイ系を用いてアンチセンスの効果を見た。私たちは以前の研究より、イントロン6－エキソン7Aの境界部位とエキソン7A内部にスプライシング因子であるMBNL1結合配列を同定した。そこで、この配列に対するアンチセンスを用いてスプライシング調節機能があるかどうかを検討した。

次に、DM3検体と非DM3検体の筋よりmRNAを抽出し、エキソンアレイを用いてスプライシング異常がないかどうかを検討した。

（倫理面配慮）

全てのDM検体の使用について、国立精神・神経センター倫理委員会で研究の承認を受けた。

C. 研究結果

まず、私たちが同定した配列に対するアンチセンスSオリゴを試したが、スプライシング調節機能は認められなかった。そこで、2'-O-methyl-phosphothioateに変えたところ、エキソン7Aのスキッピングが促進され、非活性型の割合が減り、活性型塩素チャネル遺伝子が多くなることが確認できた。

次に、エキソンアレイを用いて、DM筋でスプライシングが異常になっている遺伝子を探索した結果、myomesinのエキソン18のスプライシング

異常が観察された。

D. 考察

エキソン 7A のスキッピングにより成熟型の塩素チャネルが作られることが分かっており、いかにして効率良く行うかが問題となっていた。平成20年度の研究により、MBNL1 結合配列を標的としたエキソンスキッピングを行うことが重要であることが明らかになった。今後は、モルフォリノオリゴを用いることにより、より in vivo に近い条件でのエキソンスキッピングを行うことを目標にしたい。

ミオトニアの治療は塩素チャネル遺伝子のスプライシング正常化で行う可能性が出てきたが、筋力低下を反映する遺伝子の探索は、以前からの問題となっていた。今回、エキソンアレイを用いた解析により fibronectin、titin など十数個の候補が挙がったが、その中で myomesin は M 線の構成タンパク質であり、筋収縮に関係あると考えられる。今後は、筋力との関係を明らかにしたい。

E. 結論

エキソンスキッピングは、DM の効果的治療になるのではないかと結論づけられた。また、筋力低下に関係するのではないかと考えられる遺伝子も発見された。

F. 健康危険情報

特になし

G. 研究発表

1．論文発表

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4) Sasagawa, N., Ohno, E., Kino, Y., Watanabe, Y. & Ishiura, S. (2008) Identification of *Caenorhabditis elegans* K02H8.1 (CeMBL) as a functional ortholog of the mammalian MBNL proteins. J. Neurosci. Res. 87, 1090-1097

H. 知的財産権の出願・登録状況(予定を含む)

1．特許取得

特になし

2．実用新案登録

特になし

3．その他

特になし

厚生労働科学研究費補助金（こころの健康科学研究事業）
分担研究報告書

先天性筋強直性筋ジストロフィーとミオチュブラーミオパチーの病理学的鑑別に関する研究

研究分担者　西野　一三　　国立精神・神経センター神経研究所部長

筋強直性ジストロフィーは、筋病理学的に多彩な所見を呈することが知られている。特に、先天性筋強直性筋ジストロフィー（CDM）では、乳児重症型ミオチュブラーミオパチーや先天性筋線維タイプ不均等症（CFTD）との鑑別が困難なことがある。CFTD と病理学的に診断された 104 例を対象に *DMPK* 遺伝子の繰り返し配列伸長の有無を検討したところ、13 例（12.5%）が CDM（CFTD_CDM）であった。この 13 例の筋線維タイプ不均等度（%FSD）は、17-42%（平均 29.4±7.4%）であった。この結果は、*TPM3* 変異による CFTD（CFTD_TPM3）よりも優位に低かった。タイプ 1 線維の頻度は 55.0±4.7%であり、やはり CFTD_TPM3 よりも明らかに低頻度であった。タイプ 2C 線維の頻度は 11.4±11.9%で、CFTD_CDM で優位に高かった。%FSD が 50%以下であり、タイプ 2C 線維の多い例では、CDM の可能性を検討する必要がある。

A. 研究目的

先天性筋強直性ジストロフィーは、病理学的に乳児重症型のミオチュブラーミオパチーや先天性筋線維タイプ不均等症（CFTD）との鑑別が困難であるとされている。CFTD と診断された例の中に CDM 例がないかを検討するとともに、遺伝学的に CDM と確認された例（CFTD_CDM）の病理学的特徴を明らかにすることを目的とした。

B. 研究方法

対象は、1978 年～2007 年までに国立精神･神経センター生検筋レポジトリーに登録された凍結筋検体の内、CFTD と病理診断された 104 例（男 47 例、女 57 例、年齢 9.3±14.7 歳）を対象とした。DNA を抽出し、triplet repeat PCR 法により *DMPK* 遺伝子内の CTG リピート伸張の有無を確認した。また、CTG リピート伸張のなかった例を対象に *TPM3* 遺伝子のシークエンス解析を行った。先天性筋強直性ジストロフィーと確認された 13 例（CTFD_CDM）を対象に、*TPM3* 変異による CFTD（CTFD_TPM3）例（自験例 2 例＋文献例 8 例）の組織所見と比較検討した。

（倫理面配慮）

全ての検体について、国立精神・神経センター倫理委員会で承認を受けた「診断と検体の研究使用に関する承諾書」を用い、患者からのインフォームド・コンセントを取得している。

C. 研究結果

CTG リピート伸張は 13 例（12.5%）に認められた。この 13 例における筋線維タイプ不均等度（%FSD）は、17-42%（平均 29.4±7.4%）であった。一方、

CFTD_TPM3 では、45-76%（平均 59.7±9.9%）であり、CFTD_CDM で優位に低かった。タイプ 1 線維の頻度は 55.0±4.7%であったが、CFTD_TPM3 では 76.4±13.0%であり、CFTD_TPM3 でよりタイプ 1 線維優位が明らかであった。タイプ 2C 線維の頻度は 11.4±11.9%に対して、CFTD_TPM3 では 0.8±1.0%と、CFTD_CDM で優位に高かった。

D. 考察

CFTD は%FSD が 12%以上であり、他に構造的異常が認められない筋疾患と定義されている。Clarke NF ら（Ann Neurol 2008）によれば、CFTD では *TPM3* 変異を原因とする例（CFTD_TPM3）が最も頻度が高い。今回の結果から、病理学的に CFTD と診断される例のうち 10%以上が CFTD_CDM であることが明らかとなった。CFTD_CDM は、CFTD_TPM3 と比較して明らかに%FSD が低く、CFTD の定義をより高い%FSD に設定することで、CFTD_CDM を除外することが技術的に可能であろうと考えられる。また、タイプ 2C 線維が高頻度で認められたことは、CDM が筋線維の何らかの未熟性を病態とすることを示唆するものと考えられた。診断的には、%FSD が 50%以下であり、タイプ 2C 線維の多い例では、CDM の可能性を検討する必要があることが示唆された。

E. 結論

%FSD が 50%以下であり、タイプ 2C 線維の多い例では、CDM の可能性を検討する必要がある。

F. 健康危険情報

特になし

G. 研究発表

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H. 知的財産権の出願・登録状況(予定を含む)

1．特許取得

特になし

2．実用新案登録

特になし

3．その他

特になし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Dhaenens , M-C., Schraen-Maschke , S., Tran, H., Vingtdeux , V., Ghanem-Le-Dizes, D., Leroy, O., Delplanque, J., Vanbrussel, E., Delacourte, A., Vermersch, P., Maurage, C-A., Gruffat, H., Sergeant, A., Mahadevan, M., Ishiura, S., Buee, L., Cooper, T., Caillet-Boudin, M-L., Charlet-Berguerand, N., Sablonniere B. & Sergeant, N.	Overexpression of MBNL1 fetal isoforms and modified splicing of tau in the DM1 brain: two individual consequences of CUG trinucleotide repeats.	Exp. Neurol.	210	467-478	2008
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Sasagawa, N., Ohno, E., Kino, Y., Watanabe, Y. & Ishiura, S.	Identification of *Caenorhabditis elegans* K02H8.1 (CeMBL) as a functional ortholog of the mammalian MBNL proteins.	J.Neurosci. Res.	87	1090-1097	2009

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
石浦章一	筋強直性ジストロフィーの原因と治療戦略	こころの健康科学研究	10月号	8-9	2008
大西隼、石浦章一	筋強直性ジストロフィー発症に関わるRNA結合タンパク質ファミリーの新しい機能	実験医学	26	1596-1601	2008
石浦章一	筋強直性ジストロフィー	Clinical Neuroscience	26	178-180	2009
石浦章一	筋強直性ジストロフィーの治療戦略	医学のあゆみ	226	447-451	2009

研究成果の刊行に関する一覧表

雑誌

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Ⅳ. 研究成果の刊行物・別刷

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

Quantitative Analysis of CUG-BP1 Binding to RNA Repeats

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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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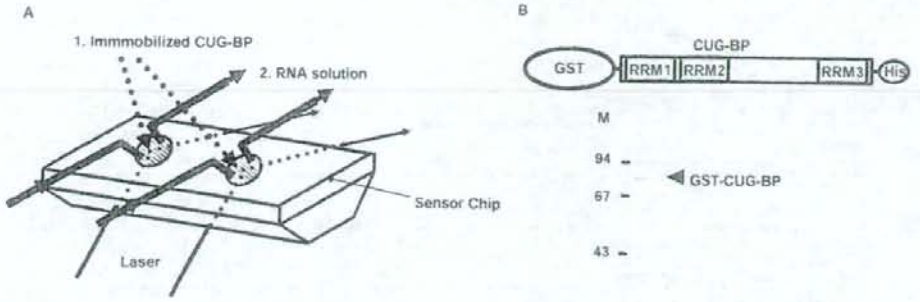

Fig. 1. Design of SPR experiment and purified protein used in this study. A: Schematic illustration of the SPR analysis. First, purified CUG-BP1 was immobilized on the gold layer of the sensor chip. RNA solution flowed through the sensor chip surface. CUG-BP1-RNA interactions were detected as the difference 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 HincII site of a pBluescriptII SK(+)-derived vector containing an ApaI–Eco52I site deletion and a HincII site insertion. Using DNA fragments from these constructs as templates, RNAs were transcribed with the MEGAscript T7 or T3 kits (Ambion) in a 20 mL 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 ml/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 mg/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 ml (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.

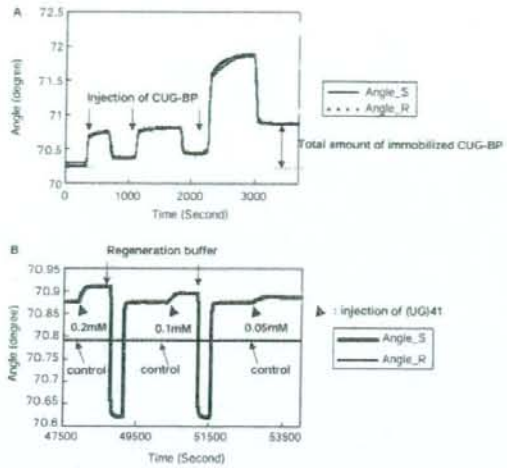

Fig. 2. SPR measurement. A: Immobilization of CUG-BP1. The signals of the resonance angle were monitored during the injection and immobilization of purified CUG-BP1 protein on the gold layer. Angle S and Angle R indicate the resonance angles at the measurement spots for the sample and the reference control, 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. • RRM1, • RRM2, • RRM3 and • linker 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 308C 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 mM and 6 mM, 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.

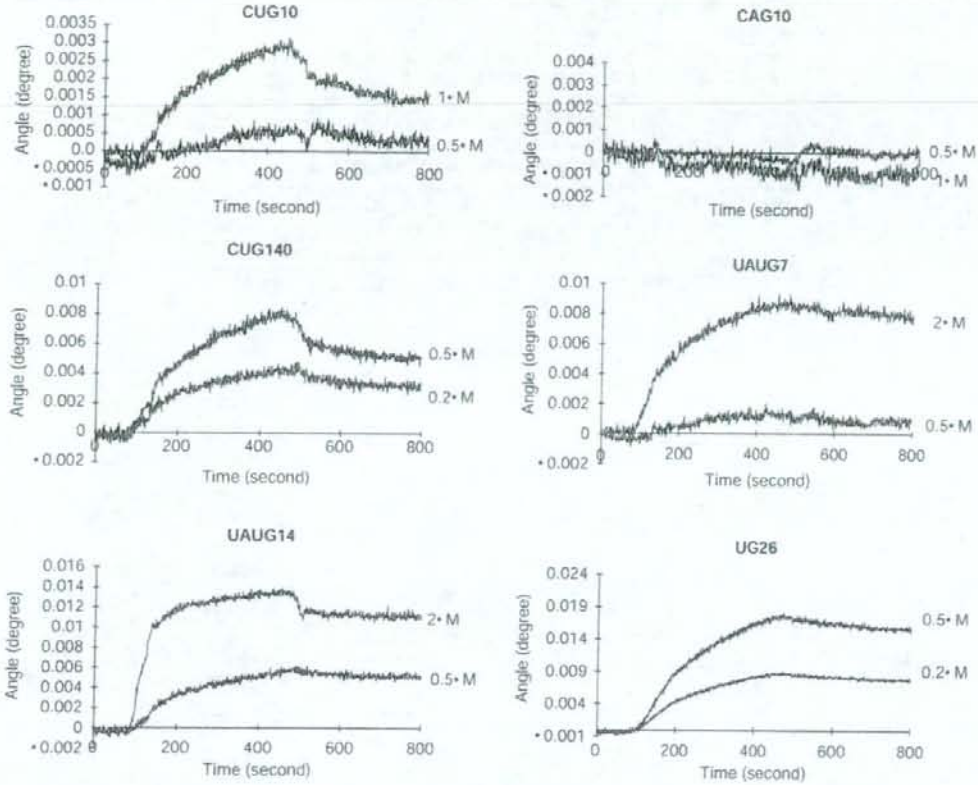

Fig. 3. Binding of various repetitive RNAs in the SPR analysis. SPR signals obtained with repetitive RNAs were monitored. Each RNA sequence is indicated above the panel. Concentrations (mM) represent that of injected RNA.

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

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

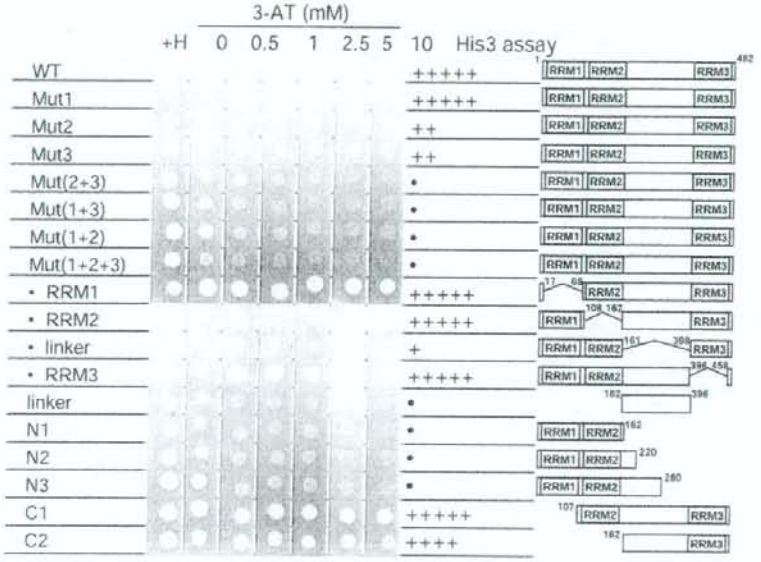

Fig. 4. Yeast three-hybrid analysis of CUG-BP1 mutants. Structures of CUG-BP1 mutants used in the assay are shown on the right. Mutated RRM motifs are indicated by hatched boxes. Results of the yeast three-hybrid HIS3 assay are shown in the middle. Wild-type or mutant CUG-BP1 constructs and the $(UG)_{24}$ 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 (• linker) 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.

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Overexpression of MBNL1 fetal isoforms and modified splicing of Tau in the DM1 brain: Two individual consequences of CUG trinucleotide repeats

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Abstract

Neurofibrillary degeneration is often observed in the brain of patients with type 1 myotonic dystrophy (DM1). It consists principally of the aggregation of Tau isoforms that lack exon 2/3 encoded sequences, and is the consequence of the modified splicing of Tau pre-mRNA. In experimental models of DM1, the splicing of several transcripts is modified due to the loss of Muscleblind-like 1 (MBNL1) function. In the present study, we demonstrate that the MBNL1 protein is also present in the human brain, and consists of several isoforms, as shown by RT-PCR and sequencing. In comparison with controls, we show that the adult DM1 brain exhibits modifications in the splicing of MBNL1, with the preferential expression of long MBNL1 isoforms — a splicing pattern similar to that seen in the fetal human brain. In cultured HeLa cells, the presence of long CUG repeats, such as those found in the DM1 mutation, leads to similar changes in the splicing pattern of MBNL1, and the localization of MBNL1 in nuclear RNA foci. Long CUG repeats also reproduce the repression of Tau exon 2/3 inclusion, as in the human disease, suggesting that their effect on MBNL1 expression may lead to changes in Tau splicing. However, while an overall reduction in the expression of MBNL1 mimics the effect of the DM1 mutation, none of the MBNL1 isoforms tested so far modulates the endogenous splicing of Tau. The modified splicing of Tau thus results from a possibly CUG-mediated loss of function of MBNL1, but not from changes in the MBNL1 expression pattern.

Keywords: Neuromuscular disease; Neurodegeneration; Microtubule-associated protein Tau; Splicing; Muscleblind-like protein

Introduction

Type 1 myotonic dystrophy (DM1) is an autosomal dominant multisystemic disorder caused by the unstable expansion of CTG trinucleotide repeats located in the 3′ untranslated region (UTR) of the gene encoding the myotonic dystrophy protein kinase (DMPK). The number of CTG repeats varies from 50 to

Abbreviations. DM, Myotonic dystrophy; IR, Insulin receptor; DMPK, Myotonic dystrophy protein kinase; MBNL, Muscleblind-like; PCR, Polymerase chain reaction; TNT, Troponin T; UTR, Untranslated region.

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