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Julien Marquis, Luc Paillard, Yann Audic, Bertrand Cosson, Olivier Danos, et al.. CUG-BP1/CELF1 requires UGU-rich sequences for high-affinity binding.. Biochemical Journal, Portland Press, 2006, 400 (2), pp.291-301. 10.1042/BJ20060490 . inserm-00292923

HAL Id: inserm-00292923

<https://www.hal.inserm.fr/inserm-00292923>

Submitted on 3 Jul 2008

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CUG-BP1/CELF1 requires UGU-rich sequences for high affinity binding

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Running title: CUG-BP1 and mRNA binding

Keywords: RNA-binding protein, SELEX, deadenylation, EDEN-BP, MAP kinase phosphatase - XCI100.

Abbreviations used: ARE, AU-rich element; BRUNOL, Bruno-like; CELF, CUG-BP1 and ETR3 Like Factors; CUG-BP1, CUG-binding protein 1; EDEN-BP, Embryo Deadenylation ElemeNt-binding protein; EMSA, Electrophoretic Mobility Shift assay; SELEX, Systematic Evolution of Ligand by EXponential enrichment; SPR, Surface Plasmon Resonance.

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ABSTRACT

CUG-BP1 (CUG-binding protein 1 also called CELF1) is a human RNA-binding protein that has been implicated in the control of splicing and mRNA translation. The *Xenopus* homologue (EDEN-BP) is required for rapid deadenylation of certain maternal mRNAs just after fertilisation. A variety of sequence elements have been described as target sites for these two proteins but their binding specificity is still controversial. Using a SELEX procedure and recombinant CUG-BP1 we selected two families of aptamers. Surface Plasmon Resonance and Electrophoretic Mobility Shift Assays showed that these two families differed in their ability to bind CUG-BP1. Furthermore; the selected high affinity aptamers form two complexes with CUG-BP1 in electrophoretic mobility assays whereas those that bind with low affinity only form one. The validity of the distinction between the two families of aptamers was confirmed by a functional *in vivo* deadenylation assay. Only those aptamers that bound CUG-BP1 with high affinity conferred deadenylation on a reporter mRNA. These high affinity RNAs are characterised by a richness in UGU motifs. Using these binding site characteristics we identified the *Xenopus* maternal mRNA encoding the MAP kinase phosphatase (XCI100) as a substrate for EDEN-BP. In conclusion, high affinity CUG-BP1 binding sites are sequence elements at least 30 nucleotides in length that are enriched in combinations U and G nucleotides and contain at least 4 UGU trinucleotide motifs. Such sequence elements are functionally competent to target a RNA for deadenylation *in vivo*.

INTRODUCTION

CUG-BP1 (CUG-binding protein 1) is a human RNA-binding protein that was first identified by its capacity to bind to a (CUG)₈ probe [1, 2]. It was thereby implicated in type 1 Myotonic Dystrophy (DM1, MIM #160900) which is a neuromuscular disease associated with an unstable CUG triplet expansion in the 3' untranslated region (3'UTR) of the myotonin protein kinase gene (DMPK). A variety of functions have been described for CUG-BP1 (reviewed in [3]). In the nucleus, it was demonstrated to control the alternative splicing of certain pre-mRNAs [4-10]. In the cytoplasm, it stimulates the translation of p21 mRNA [11], or controls the choice of translation initiation codon of C/EBP mRNA [12]. The *Xenopus* equivalent of CUG-BP1, EDEN-BP (Embryo Deadenylation Element-binding protein), is responsible for the rapid cytoplasmic deadenylation (poly(A) tail shortening) of certain maternal mRNAs [13], that is correlated with translational repression [14]. In *Xenopus* egg extracts the human CUG-BP1 and *Xenopus* EDEN-BP are interchangeable [15] and recently, CUG-BP1 was reported to act as a deadenylation factor in human cells by recruiting PARN deadenylase to target mRNA [16]. Lastly, CUG-BP1 is one of the founding members of the CELF (CUG-BP1 and ETR-3 Like Factors) family of RNA-binding proteins [17] that are key molecular actors which determine the fate of a large number of mRNA (reviewed in [3]).

Despite the importance of CUG-BP1 and related proteins in the post-transcriptional regulation of gene expression, the sequence requirements for high affinity CUG-BP1 binding are still ambiguous. This protein was initially reported to cross-link DMPK 3'UTR CUG expansion [1]. Other natural sequences described for their capacity to bind to CUG-BP1 include U/G-rich intronic motifs [4, 18, 19], 3'UTR U/G-rich motifs such as the c-jun AU-rich element (ARE) [20], c-mos EDEN [15], and GCN-rich coding regions [12, 21, 22]. However, the capacity of CUG-BP1 to bind to the CUG expansion has been questioned [23, 24]. In two independent triple hybrid assays using synthetic probes, it was observed that CUG-BP1 had a low affinity for CUG repeats but interacted very efficiently with (UG)_n probes [24, 25]. In addition, CUG-BP1 cross links to a synthetic (UG)₁₅ probe [26]. The sequence requirement for CUG-BP2/CELF2/ETR-3, that shares 75% identity with CUG-BP1 (and almost 100% identity in the consensus RNA binding domains [3]), is also ambiguous. RNA aptamers selected against this protein resulted in UG-rich sequences and in particular UGUU motifs [27], but this protein was also shown to

interact with cyclooxygenase 2 mRNA ARE [28]. This ARE is not UG-rich but rather contains several AUUUA motifs.

This variety in the sequences reported to be recognised by CUG-BP1, and the ambiguity of autonomous binding, highlights the need for a systematic, non-biased (without prior limitations), examination of the binding specificities of this protein. In this work, we have used a SELEX procedure (Systematic Evolution of Ligand by EXponential enrichment) [29, 30] to select RNA aptamers from a library of random sequences on the basis only of their autonomous association with CUG-BP1. Analysis of the binding affinities and the functionality of the selected aptamers showed that a minimal high affinity CUG-BP1 binding site could be defined as a relatively short RNA (around 36 nucleotides) highly enriched in UG repeats with a particular importance of the UGU motif. These criteria was used to identify a biologically important maternal *Xenopus* mRNA MAP kinase phosphatase (XCI100 alpha) that is a target for the *Xenopus* homologue of CUG-BP1.

EXPERIMENTAL .

Recombinant protein production

CUG-BP1 open reading frame was cloned in the pTRC-His plasmid (Invitrogen). His-tagged protein was produced by overnight induction at 25°C with 5 mM IPTG and purified on Ni-NTA agarose beads (Qiagen) following the manufacturer's instructions. Denaturing electrophoresis and Coomassie blue staining was used to check the purity of recombinant CUG-BP1 and to measure its concentration as compared with several BSA concentrations.

Synthesis of the initial random RNA library

The initial random oligonucleotide was purchased from IBA GmbH/Göttingen. The sequence is 5'-CCACTAAACCAGCCTCAAGGGTACCGCTCTAGA(35N)GCTAGCGTATCTGCTCCTAATAAAAAGAGGATCCCC-3' where N represents the randomised part and the underlined sequences are XbaI and NheI restriction sites respectively. This oligonucleotide was PCR amplified (4 cycles) using the Forward (5'-AGTAATACGACTCACTATAGGGCCACTAAACCAGCCTCAAGG-3') and Reverse (5'-GGGGATCCTCTTTTATTAGG-3') primers to generate a double stranded DNA template with a 5'-end T7 promoter (underlined in the Forward primer). Each 100 µl PCR reaction contained 8

pmol of random oligonucleotide template and 2.5 U of Amplitaq DNA polymerase (Perkin Elmer). The PCR products were phenol/chloroform extracted, ethanol precipitated and further purified on Microcon YM30 columns (Amicon). The initial RNA pool was synthesised from 240 pmol of PCR DNA with 2400 U of T7 RNA polymerase (New England BioLabs) in a 6 ml reaction mixture supplemented with 24 U of inorganic pyrophosphatase (Sigma) and 300 U of porcine RNase inhibitor (Amersham Biosciences). After 4 h at 37°C, the reaction was treated (30 min at 37°C) with 700 U of DNase I (Invitrogen). The RNA mixture was extracted with phenol/chloroform and chloroform (twice), ethanol precipitated and further purified on YM30 Microcon columns. The RNA was eluted by using the SELEX binding buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM DTT and 0.1 mM CaCl₂).

SELEX procedure

RNA (300 pmol) was refolded by heating at 85°C and slowly cooled to room temperature before counter selection through a HAWP filter (Amicon). RNA was then incubated with CUG-BP1 (30 pmol) for 80 min (first round) or 30 min (subsequent rounds) in the presence of 1.2 nmol of tRNA (Ambion). Partition was achieved by filtration through a HAWP filter that was washed three times with 200 µl of binding buffer. The filter was incubated 5 min at 85°C in the elution buffer (7 M urea, 100 mM sodium citrate and 3 mM EDTA) to recover the CUG-BP1 associated RNA species that were isopropanol precipitated. The RNA was refolded in binding buffer and subjected to a second counter selection as described above. The eluted RNA was ethanol-precipitated and resuspended in nuclease free water.

After each round of selection the RNA was hybridised with the Reverse primer, reverse transcribed with 15 U of AMV reverse transcriptase (QBiogen) during 50 min at 42°C and PCR amplified with 30 U of Amplitaq DNA Polymerase as described for the initial RNA pool. The PCR products were purified on a YM30 Microcon column and 18 pmol of DNA were *in vitro* transcribed with 200 U of T7 RNA polymerase for 3 h at 37°C. Finally, the RNA was treated with 140 U of DNase I for 20 min at 37°C, and then purified and quantified with Ribogreen (Molecular Probes).

Sequence analysis

Individual aptamers were cloned from a retro-transcribed RNA pool using TA cloning procedure (Invitrogen) with pCR2.1 TOPO plasmid as described by the manufacturer. The DNA of interest was PCR amplified from individual positive bacteria colonies using M13-Forward and Reverse primers and sequenced. The random sequences were extracted and analysed using the Clustal W algorithm [31] and Emboss web facility.

Evaluation of RNA affinity

Individual or pooled clones were PCR amplified using the Forward (that contains a T7 promoter) and Reverse primers and Amplitaq DNA polymerase. The PCR products were purified and quantified. The RNAs were transcribed in the presence (for Electrophoretic Mobility Shift assay, EMSA) or the absence (for Surface Plasmon Resonance, SPR) of $\alpha^{32}\text{P}$ -UTP. The plasmids encoding the s3'Eg5 and s3'Eg5C6 RNAs have been described [13] and were used as transcription templates as above. EMSA was performed as described by Cosson et al [32]. The SPR experiments were performed using a BIAcore 2000 biosensor system (BIAcore) at 25°C according to the manufacturer's instruction. CUG-BP1 was immobilised directly on a CM5 sensor chip (BIAcore) using amine coupling kit (BIAcore). This was preferred to attachment via a tag because two of the three RNA Recognition Motifs in CUG-BP1 are very close to the N- and C-termini and immobilisation via a N- or C-terminal tag could interfere with the overall CUG-BP1 RNA recognition specificity. A blank flow cell (without protein) was used to evaluate non-specific interactions. RNA was diluted to 100 nM in the running buffer (10 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM MgCl₂, 0.1 mM CaCl₂ and 0.05% Tween 20) supplemented with 0.2 U/ μl SuperaIn (Ambion), and injected for 2 min followed by a 2 min dissociation step at a flow rate of 20 $\mu\text{l}/\text{min}$. Regeneration was achieved with a 6 s pulse of 100% ethylene glycol at a flow rate of 50 $\mu\text{l}/\text{min}$. Each RNA sample was injected once before performing the second injection (serial duplicates procedure). The sensorgrams were analysed using the BIAevaluation software (version 3.2, BIAcore).

***In vivo* deadenylation assay and analysis of maternal Xenopus mRNA.**

DNA corresponding to the RNA aptamers were digested by NheI and XbaI, and cloned into pGbORF/mosEDEN plasmid in place of the minimal c-mos EDEN [13]. The 3'UTR of

XCI100 alpha maternal mRNA was amplified by PCR (forward primer (5'CTAGCTAGCGGTACTGAGCAAACAGAC; reverse primer 5'CGGGATCCCAGTACAAATATCATAATTTA) and cloned into the *NheI* and *BamHI* restriction sites of pGbORF [33]. The globin reading frame was then deleted by restriction by *NheI* (blunt ended) and *SacI* and religation. All constructs were sequenced.

Capped, polyadenylated radiolabelled RNA were microinjected in *Xenopus* 2-cell embryos and the deadenylation assays were performed as already described [13]. Briefly, RNAs were extracted with Tri-Reagent (Euromedex) and separated by electrophoresis on 4% polyacrylamide gels containing urea. The dried gels were analysed using a Phosphorimager (STORM 840, Molecular Dynamics). The proportion of fully deadenylated RNA was quantified, using the ImageQuant software (Version 5.4, Molecular Dynamics), by determining the signal in a rectangle at the position of the fully deadenylated transcript (A)₀ relative to the total signal of this transcript (fully adenylated, partially and totally deadenylated transcripts).

To analyse *Xenopus* maternal mRNAs, total RNA was extracted from embryos using Tri-Reagent (Euromedex) and separated into poly(A)⁺ and poly(A)⁻ population by oligodT cellulose chromatography (Promega). The RNAs were analysed by Northern blotting. XCI100 alpha mRNA was revealed using ³²P-labelled cDNA probes corresponding to the coding sequence (plasmid obtained from Dr J.E. Fenell Jr. Sanford, Ca, USA; Accession number AJ320158). Immunoprecipitations and UV-induced cross-linking, using the indicated ³²P-labelled RNAs, were performed as previously described [13]. The XCI100 alpha RNA corresponded to the UGU rich region was amplified by PCR (Forward primer, CTGGTACTGTAATTCCTGTG; Reverse primer, AAATCATAGCATAGCATACAA).

RESULTS

The SELEX procedure against CUG-BP1

A SELEX procedure was developed that could generate fully functional CUG-BP1 RNA aptamers. An initial SELEX RNA library of 1.8×10^{14} distinct RNA sequences, randomised on 35 positions, was used (see Experimental). The 35 nucleotide random sequence was flanked by invariant 5' and 3' regions derived from the β -globin 3'UTR (Figure 1A). After an initial counter selection to remove non-specific RNA species, the RNA pool was incubated with CUG-BP1 and loaded onto the filter. Bound RNA was eluted from the filter and submitted to a second counter

selection. The RNA was then amplified by RT-PCR, transcribed into a new RNA library and then applied to the next round of selection/amplification. This selection/amplification procedure was repeated 7 times but with a reduced incubation time to increase stringency. Throughout the experiments, the same preparation of recombinant, bacterially-produced CUG-BP1 protein was used. The integrity and purity of this protein were verified by SDS-PAGE (Figure 1B).

Evaluation of the enrichment using a SPR biosensor and EMSA.

To evaluate the quality of the SELEX enrichment after 8 rounds of selection, two analytical methods were used, a SPR assay using a BIAcoreTM 2000 instrument and EMSA. The SPR assay was used for screening as it is rapid and can be automated. However, a high level of CUG-BP1 immobilisation (about 10000 Resonance Units) was necessary to detect a clear association signal with c-mos EDEN RNA (positive control, see Figure 2B). A high immobilisation level on the chip causes mass transport limitations in the proximity of the sensor chip surface [34] which prevents analysis of the kinetics of RNA:protein associations. Therefore, we used a ranking analysis which consisted of comparing the overall qualitative shape of specific binding figures (sensorgrams) to determine the end-point association level (in RU) reached after injection of each analyte [35, 36]. When all the samples are injected under the same conditions (concentration, volume, flow rate, temperature), the endpoint value should be directly correlated with RNA affinity. The validity of this SPR method for the present case was demonstrated by the close correlation between the SPR and EMSA results (see below).

First, the specificity and the sensitivity of both assays were assessed by running individual RNA sequences. Except where indicated, EMSA assays were performed with 4-fold serial dilutions of CUG-BP1, between 0.5 and 2000 nM. The S0(1) RNA was randomly taken from the cDNAs cloned from the starting pool of unselected aptamers. No association of this RNA with CUG-BP1 was detected by either the SPR assay or EMSA (Figure 2A). The c-mos EDEN, previously described as a good CUG-BP1 target in a UV cross-linking assay [15], was used as a positive control. This RNA presented a strong association in the SPR assay, reaching an endpoint value around 45 RU (Figure 2B, left panel). In the EMSA (Figure 2B, right panel) increasing amounts of CUG-BP1 shifted this RNA from the free state to a first (C1), and then a second (C2) complex. The affinity of CUG-BP1 for c-mos EDEN was estimated from the

concentration of protein that bound 50% of the RNA probe. This value, which would be the K_d if only one complex was formed, was 25 ± 6 nM (average of three independent experiments).

Pools of RNA were next tested. In both SPR and EMSA, the initial RNA library (S0 pool) behaved very similarly to the S0(1) RNA, demonstrating that the S0 pool had no overall affinity for CUG-BP1 (Figure 2C). In contrast, the enriched RNA pool (S8 pool) gave an association signal in the SPR assay, and this pool of RNA was shifted to two different complexes by increasing concentrations of CUG-BP1 in the EMSA (Figure 2D). The "fuzzy" appearance of the shifted complexes in the EMSA using the S8 pool (Figure 2D, right panel) may be due to the heterogeneity of the RNA population. The different sequences forming this pool may have different conformations and hence have different electrophoretic mobilities. This dispersion of the bands also reduces the precision of the quantification of the affinity of this pool of RNA for CUG-BP1 (115 ± 27 nM). Irrespective of these considerations, the results in Figure 2 show that the SELEX procedure had successfully allowed the evolution of the large random RNA library into an enriched RNA population whose global affinity was similar to that of the c-mos EDEN. Four additional SELEX rounds did not increase the overall affinity of the RNA library (data not shown).

Characterisation of individual aptamers after round 8

The cDNA libraries corresponding to RNA aptamers (S0 pool and S8 pool) were cloned and randomly selected clones were sequenced. As expected, no sequence similarity was detected in 41 clones from the S0 pool. The sequences of 101 clones from the S8 pool are given in the Supplementary data (Supplementary Figure 1). For these S8 clones, although no consensus sequence or obvious conservation was detected, the Clustal algorithm (ClustalW) identified two families of aptamers. In family 1, containing 63 clones, the aptamers were enriched in uridine and depleted in adenosine and cytosine nucleotides relative to the 41 clones from the S0 pool. The nucleotide composition of the second family (38 clones) was not significantly different from that of the S0 pool. Within the 38 clones of family 2, a subpopulation of 10 clones containing 6 different sequences (S8(2) is present in 5 copies) displayed a conserved motif in the 3' region (GUUCUGCUAGUUU(U/C)GGUU) (see Supplementary Figure 1).

SPR and EMSA analyses were performed on seven individual aptamers from the S8 pool. In EMSA assays, two of them, S8(12) and S8(13), that both belong to family 2, were not shifted

by CUG-BP1 (Figure 3). In the SPR assay, the same RNAs gave no association signal (see Table 1). In contrast, S8(5), S8(6), S8(7) and S8(8), that all belong to family 1 and S8(2) that belongs to family 2, showed association with CUG-BP1 in EMSA (Figure 3) and SPR (Table 1) assays. However, there was a noticeable difference between the EMSA patterns of these RNAs. S8(5), S8(6) and S8(7) produced the slower mobility complex, C2, (complex C1 was also observed at low CUG-BP1 concentrations) but only the fast mobility complex, C1, was observed for RNAs S8(2) and S8(8). These differences in EMSA pattern were reflected in the affinity of the individual RNAs for CUG-BP1 as estimated from the EMSA or the SPR assay (see Figure 3 and Table 1). The three RNAs that produced the C2 complex had an affinity for CUG-BP1 comparable to that of *c-mos* EDEN. For the two RNAs, S8(2) and S8(8), which only formed the C1 complex, at least 20 fold more protein was required to shift 50% of the probe.

The results shown in Figures 2 and 3 and Table 1 demonstrate an excellent agreement between the SPR and EMSA results obtained with the individual RNAs. Hence, 6 additional aptamers from the S8 pool were analysed by SPR alone; 2 were from family 1 and 4 from family 2 of which 3 came from the subpopulation with the conserved motif. Based on the end-point values, the two RNAs from family 1, S8(9) and S8(10), showed a high affinity for CUG-BP1 whereas none of the RNAs from family 2 displayed any binding to CUG-BP1 (see Table 1). This result indicates that the sequence present in several RNAs of family 2 is not implicated in binding to CUG-BP1 as determined by the assays used here.

Functionality of SELEX aptamers

The SPR and EMSA assays allowed us to discriminate between the two aptamer families initially defined on a basis of ClustalW alignment. To biologically confirm this discrimination we used an *in vivo* test. In *Xenopus* embryos, EDEN-BP, the equivalent of human CUG-BP1, acts as a sequence specific factor that directs target RNA for deadenylation [13]. For this process EDEN-BP can be replaced by the orthologous CUG-BP1 [15]. Therefore we used a deadenylation assay to evaluate aptamer functionality. The sequences to be tested were cloned in the 3'UTR of a β -globin cDNA upstream of a synthetic polyA tail (A)₆₅. The corresponding radiolabelled RNA was injected into *Xenopus* embryos and samples were taken at hourly intervals to follow deadenylation kinetics. The deadenylation-targeting activities of the tested aptamers were

compared to that of a similar mRNA but containing the c-mos EDEN in the 3'UTR (c-mos EDEN).

As already reported, c-mos EDEN RNA (Figure 4A, top panel) was deadenylated by the EDEN pathway with the characteristic accumulation of the deadenylated ((A)₀) mRNA within 1 to 2 hours while fully adenylated ((A)₆₅) mRNA persists [13]. In the experiment shown in Figure 4A, top panel, a completely deadenylated form of this transcript ((A)₀) was detected as soon as 1 hour after injection (lane 2) and, 4 hours after injection, the (A)₀ band was very abundant (lane 5). In *Xenopus* embryos, the (A)₀ mRNAs are stable for at least 6 h [33, 37]. The deadenylation pattern observed for the control c-mos EDEN RNA was compared with those conferred by one aptamer from family 1 that bound CUG-BP1 with high affinity, S8(7), one aptamer from family 2 with low affinity, S8(2) and one aptamer from family 2 for which no CUG-BP1 binding had been detected, S8(12) (See Table 1). The deadenylation of the RNA containing the S8(7) aptamer (Figure 4A, middle panel) presented the characteristics of EDEN-dependent deadenylation, with a (A)₀ band easily detectable 2h after injection and abundant after 4h (lane 5). In contrast, the mRNA containing the S8(12) and S8(2) sequences (Figure 4A, two bottom panels) were not or only very slowly deadenylated. No deadenylated molecules (A)₀ could be detected before 4h of incubation (Figure 4A). Quantification of these experiments (Figure 4B) showed that the deadenylation kinetics of S8(7) and c-mos EDEN mRNAs were similar whereas that of S8(2) and S8(12) was about 5 times slower.

A threshold number of UGU for efficient CUG-BP1 binding

To better characterise the RNA sequence requirements for CUG-BP1 binding, the nucleotide composition of the sequenced RNAs from family 1 and 2 were analysed and compared to that of the 41 sequenced RNAs from the S0 pool. The graphical presentation of the proportions of mono-, di-, tri and tetra-nucleotides in the sequenced RNAs from family 1 and 2 and the S0 pool are given in the supplementary data (Supplementary Figure 2). The most notable features of these analyses were an enrichment in the family 1 RNAs of U mono-nucleotides and certain di and tri nucleotides containing different combinations of U and G nucleotides. In particular, UGU tri-nucleotides represented nearly 20% of all the tri-nucleotides in the family 1 RNAs.

In Table 1 is reported the number of UGU tri-nucleotides for the RNAs tested in the EMSA and SPR experiments described above. Sequences with 0 to 2 UGU do not bind, or bind

only weakly, to CUG-BP1, whereas sequences with 8 to 10 motifs bind strongly. Analysis of the number of UGU in the individual RNA sequences (Figure 5) showed that the RNAs in the S0 pool and family 2 contain 2 or less UGU while those of family 1 contain 4 or more UGU. Importantly, more sequences are found with 6 UGU than with 4 UGU suggesting that aptamers with 6 UGU were more efficiently selected during the SELEX procedure than aptamers with 4 UGU. In contrast, more than 6 UGU does not appear to confer a selection advantage on an aptamer relative to those with 6 UGU. This suggests that efficient binding sites for CUG-BP1 consist of sequences containing at least 4 to 6 UGU. Accordingly, a sequence containing 5 UGU was tested. The EDEN motif in the 3' portion of the *Xenopus* Eg5 mRNA (s3'Eg5) contains 5 UGU [13] (Table 1). EMSA analysis demonstrated that the affinity of the s3'Eg5 RNA is comparable to that of the c-mos, S8(5), S8(6) and S8(7) RNA (Figure 3, Table 1), confirming that a threshold for efficient binding occurs between 4 and 5 UGUs. The binding affinity of a mutant RNA (s3'Eg5C6), in which three UGU trinucleotides were changed to cytosines, was next determined. This mutation reduces the number of UGU's from 5 to 2 (Table 1), which is below the proposed threshold. No retarded complex was observed for the s3'Eg5C6 RNA by EMSA, demonstrating a much reduced affinity (Figure 3, Table 1). Together, these data indicate that the main sequence requirement for affine binding to CUG-BP1 is the presence of at least 5 UGU within a 35 nucleotides window.

Identification of a maternal *Xenopus* mRNA containing a CUG-BP1/EDEN-BP binding site.

In *Xenopus* embryos the majority of maternal mRNAs that are known functional targets for EDEN-BP, encode kinases important for the cell cycle. Therefore, to relate the sequence requirements for CUG-BP1/EDEN-BP binding to a biologically important event we searched 3'UTR sequences of *Xenopus* mRNAs encoding kinases or phosphatase for a potential novel target using the criteria defined above. This analysis identified the MAP kinase phosphatase XCI100 alpha [38] whose 3'UTR contains 12 UGU tri-nucleotides within a 35 nucleotide stretch (Accession number of mRNA, AJ320158) making it a potential target for EDEN-BP, which would limit the expression of this protein after fertilisation. This mRNA is of particular interest to the present study as the over expression of XCI100 alpha mRNA in embryos causes severe defects in gastrulation and posterior development [39] (and unpublished data).

To ensure that the endogenous XC1100 alpha mRNA is a potential target for CUG-BP1/EDEN-BP the adenylation behaviour of the maternal mRNA was determined. Total mRNA extracted from embryos at 0h, 2h and 4h after fertilisation were fractionated into poly(A)⁺ and poly(A)⁻ populations and analysed by Northern blot (Figure 6A). As already observed for a number of maternal *Xenopus* mRNAs [40] the amount of XC1100 alpha mRNA in the total population did not vary over this 4h period. In contrast, there was a reciprocal exchange of this mRNA between the poly(A)⁺ and poly(A)⁻ populations. The majority of XC1100 alpha mRNA, that is in the poly(A)⁺ population at fertilisation (0h), rapidly becomes deadenylated.

Next, a radiolabelled RNA corresponding to the 180 nt portion of the XC1100 alpha 3'UTR that encompasses the UGU rich motif was synthesised and used in a uv-induced cross linking assay with *Xenopus* egg extracts (Figure 6B). In parallel with this RNA, the RNA GbORF and GbORF c-mos were included as negative and positive controls respectively. Both the XC1100 alpha and GbORF c-mos RNA produced a strong radioactive signal for a protein migrating as wide band between 54 and 56 kDa which is characteristic of *Xenopus* EDEN-BP [13]. The identity of this protein was confirmed by immunoprecipitation of the radiolabelled protein with anti-EDEN-BP antibodies (data not shown).

To verify that the 3'UTR of XC1100 alpha could confer the deadenylation behaviour observed for the maternal mRNA we analysed the adenylation behaviour of a reporter mRNA containing this sequence after injection into *Xenopus* embryos (Figure 6C). This reporter mRNA was rapidly deadenylated with kinetics similar to those already described for the reporter mRNA containing the c-mos EDEN (compare Figure 4A and 6C). Together these results show that the 3'UTR of XC1100 alpha targets an mRNA for rapid EDEN-dependent deadenylation in *Xenopus* embryos and therefore further validate the UGU-richness of the 3'UTR as a pertinent criterion to identify novel CUG-BP1/EDEN-BP mRNA targets.

DISCUSSION

A population of RNA (S8 pool) with a global affinity for CUG-BP1 equivalent to that of the c-mos EDEN was obtained after 8 rounds of SELEX. Sequencing of 101 clones from this S8 pool did not identify any consensus motif, but allowed the clustering of these sequences into 2 families. The affinity of 13 individual aptamers was measured which showed that the majority (5/6) of tested family 1 RNAs had a high affinity for CUG-BP1 and produced two shifted

complexes (C1 and C2) in EMSA. Family 2 RNAs had no or a low (1/7) affinity for CUG-BP1 and the low affinity aptamers produced only the faster mobility complex (C1). In an *in vivo* deadenylation assay the family 1, but not the family 2, aptamers conferred efficient deadenylation on a reporter mRNA.

Analysis of the sequenced aptamers showed, for family 1, that UGU triplets represented approximately 20% of all the trinucleotides, and that at least 4 non-overlapping UGU are found in these aptamers. The number of UGU trinucleotides in the family 2 aptamers was not significantly different from that in the S0 pool. The importance of the UGU trinucleotide for CUG-BP1 binding was demonstrated by EMSA using the Eg5 EDEN and a mutant form that decreased the number of UGU trinucleotides from 5 to 2. This mutation drastically reduced the affinity of the RNA for CUG-BP1.

These data indicate that most functional CUG-BP1 binding sites will probably be sequences of more than 30 nucleotides containing at least 4 non-overlapping UGU motifs. Using this information we identified the *Xenopus* maternal mRNA encoding the MAP kinase phosphatase XCI100 alpha as a substrate for the deadenylation process targeted by CUG-BP1/EDEN-BP. Gotoh et al [39] have previously shown that the over expression of XCI100 alpha in *Xenopus* embryos caused severe defects during gastrulation and in posterior development. These and our present data imply that the post-fertilisation deadenylation of the maternal mRNA, directed by the deadenylation factors CUG-BP1/EDEN-BP, is necessary in order to limit the amount of XCI100 alpha expressed in the embryo. Furthermore, it shows that the criteria defined for autonomous binding of CUG-BP1 or the *Xenopus* homologue EDEN-BP can be used to identify functionally important mRNAs.

All but one of the sequenced aptamers of family 1 fulfil the criteria stated above (Supplementary data) and this is also the case for natural sequences previously identified by their capacity to bind CUG-BP1. These include the cTNT [4] and the Clc-1 [19] mRNA precursors, the BRE element [41] and the c-jun ARE [20]. Moreover, we noted that mutations in the cTNT, Clc-1 and BRE sequences that abolished the interaction with CUG-BP1 [4, 19, 41] also disrupted these UGU motifs. CUG-BP1 was also shown to efficiently bind synthetic (UG)_n repeats probes in UV cross link experiments [26] and triple hybrid assays [24, 25].

Although the SELEX procedure produced a population of aptamers, S8 pool, that had overall affinity for CUG-BP1, this population contained a large proportion (about 38%) of

aptamers with low or no affinity for this protein. It should be noted that the SELEX procedure and the SPR and EMSA analyses do not use equivalent criteria or conditions. The binding and selection steps in the SELEX procedure were performed in conditions that selected aptamers with a range of affinities and also a number of false positive aptamers. Although the SPR and EMSA analyses discriminated between the high and low affinity sites, we can not formally exclude that the family 2 aptamers may represent binding sites that require auxiliary factors or are only used in certain cellular contexts.

The family 1 UGU-rich aptamers, tested for their ability to interact with CUG-BP1 (S8(5), S8(6), S8(7) and c-mos EDEN) form two complexes, C1 and C2, in EMSA. In contrast, the two aptamers with a low affinity for CUG-BP1 that were tested, S8(2) and S8(8), only formed the first complex, C1. The different complexes could correspond to different conformations of the RNA probes associated with a single CUG-BP1 protein. However, this explanation would mean that, as the CUG-BP1 concentration increased, all the family 1 RNAs tested (including those in the S8 pool) but not the RNAs from family 2 would be able to take up two different conformations; we consider this unlikely. An alternative explanation is that the protein components of the two complexes are different. Although we can not exclude that the minor contaminants in the recombinant CUG-BP1 preparation partake in these complexes we think that this is improbable. We previously showed that EDEN-BP could form a homodimer in a yeast two-hybrid assay [42] and we have now demonstrated that this can occur *in vivo* [32] Therefore, we prefer the explanation that the two complexes correspond to different oligomerisation states of CUG-BP1 bound to the RNA probe.

What are the RNA binding specificities of proteins close to human CUG-BP1? In human, CUG-BP1 is a member of the Bruno-like (BRUNOL) or CELF family of RNA-binding proteins [17, 41] reviewed in [3]. The best known member of this family, with CUG-BP1, is ETR-3 (also named CUG-BP2 or BRUNOL3), that shares 75% identity with CUG-BP1. This protein was shown to bind to an AU-Rich element in Cyclooxygenase-2 mRNA that is not enriched in UGU trinucleotide [28], which suggests a different binding specificity between CUG-BP1 and ETR-3. However, an ETR-3 SELEX selected sequences enriched in UG and UGUU [27], which is closer to the sequence requirement for CUG-BP1. In addition, Suzuki et al [7] showed using UV-crosslinking that CUG-BP1 and ETR-3 could bind to UG-rich sequences. Binding to motifs

enriched in UGU may therefore be a general property of at least these two BRUNOL/CELF proteins.

The proportion of CUG and GCN sequences decreased during the SELEX procedure (see Supplementary data) in contradiction with previously published data for CUG-BP1 targets [2, 12, 21, 22]. Other authors also failed to find any affinity between CUG-BP1 and these sequences in three-hybrid assays [24, 25]. Moreover, CUG expansions form double-stranded RNA that is unable to bind CUG-BP1 [23]. This does not exclude that CUG-BP1 could bind to non-UGU rich elements when associated with particular co-factors. For instance, the association of CUG-BP1 with another RNA-BP could significantly modify the characteristics of the element to which the complex is bound. For example, hnRNPE1 is involved both in the silencing of 15-lipoxygenase mRNA and the stabilisation of β -globin mRNA during erythroid differentiation, and these properties are mediated by binding to different sequences [43, 44]. Also, hnRNPD/AUF1 is involved in AU-rich element mediated mRNA degradation [45, 46] but belongs to stabilizing complexes that are associated with the coding region of c-fos mRNA [47] or the 3'UTR of α -globin mRNA [48]. Again, the binding sites for AUF1 in these opposing functions are different.

ACKNOWLEDGEMENTS

We thank D. To Van (Biacore) for advices and helpful discussions during the SPR experiments, Agnès Méreau (UMR 6061) for help in designing bandshifts and Valérie Allo (Généthon) who provided technical help for the constructs. This work was supported by the Association Française contre les Myopathies (to CLB), by grants from ARC 4791 and Ministère de la Jeunesse, de l'Education Nationale et de la Recherche ACI BCMS314 (to LP), European Union DG XII biotechnology program contract QLK3-CT-2000-00721 (to HBO). JM was financed by doctoral fellowships from the CNRS and Généthon and BC by postdoctoral fellowships from the CNRS and Rennes Métropole.

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FIGURE LEGENDS**Table 1: CUG-BP1 affinity of individual aptamers**

The indicated cloned RNA aptamers were analysed by SPR and EMSA assays as described in the legend to Figure 2. The sequence of the aptamers and the number of UGU tri-nucleotides are indicated. The aptamers are ordered according to the SPR values that are the averages from two separate injections. The data for the EMSA assay give the concentration of CUG-BP1 (nM) required to shift 50% of the RNA probe (averages from three separate experiments). The families are defined on the basis of sequence criteria. The *Xenopus* EDEN sequences and that from the S0 pool (S0(1)) are not clustered into a family. nd. Not determined.

Figure 1: Overview of the SELEX procedure

A, The 36 nucleotides invariant sequences of the random RNA library were those flanking the *c-mos* EDEN in the GbORF/*mos*EDEN RNA. The Forward and Reverse primers, used for the PCR amplification steps, hybridise to approximately 20 nt of these invariant sequences. The Forward primer has a T7 promoter overhang (underlined). **B**, Coomassie blue staining of a sample of the recombinant CUG-BP1 used in the experiments (lane 2). Lane 1, molecular weight markers, sizes in kDa are given on the left.

Figure 2: SPR and EMSA evaluations of SELEX enrichment

The negative control (S0(1) RNA) (**A**), the positive control (*c-mos* EDEN RNA) (**B**), the initial random RNA population (S0 pool) (**C**) and the enriched RNA population (S8 pool) (**D**) were tested for their capacity to interact with recombinant CUG-BP1 by SPR (left panels) and EMSA (right panels) assays. For SPR, RNAs were injected at 100 nM over a sensor chip containing approximately 11000 Resonance Unit (RU) of immobilised CUG-BP1 (corresponding to 11 ng of CUG-BP1/mm²). The grey and the black curves represent the first and the second injection of each RNA sample. The end point values (in RU) correspond to the response signal reached at the end of the RNA injections ($t = 120$ s). For EMSA, uniformly labelled RNA probe was incubated with the following concentrations of CUG-BP1 (nM): lane 1, 0; lane 2, 0.5; lane 3, 2; lane 4, 8; lane 5, 31; lane 6, 125; lane 7, 500; lane 8, 2000. The free probe (F) and two complexes (C1 and C2) were resolved by native electrophoresis. The gel was dried and analysed using a phosphoimager and the ImageQuant software. The concentrations (nM) of CUG-BP1 required to

achieve 50% of shifted probe are indicated on the top of each gel (mean \pm sd of three independent experiments).

Figure 3: EMSA analysis of individual aptamers

The indicated aptamers were analysed by EMSA for their capacity to bind CUG-BP1 as described in Figure 2. For the EMSA with the s3'Eg5 and s3'Eg5C6 RNAs the protein concentrations were (nM): lane 1, 0; lane 2, 1; lane 3, 2; lane 4, 4; lane 5, 8; lane 6, 17; lane 7, 33; lane 8, 67; lane 9, 133 and lane 10, 266. The concentrations (nM) of CUG-BP1 required to achieve 50% of shifted probe are indicated at the top of each gel (mean \pm sd of three independent experiments). The positions of the free probe (F) and the complexes C1 and C2 are indicated on the right of each panel.

Figure 4: *In vitro* deadenylation assay of selected aptamers

A, SELEX sequences (S8(2), S8(7), and S8(12)) and c-mos EDEN (mosEDEN) were cloned upstream of a synthetic (A)₆₅ tail in the 3'UTR of a β -globin reporter gene. Capped, *in vitro* synthesised radiolabeled transcripts were micro-injected into *Xenopus* embryos. At different time points, total RNA was extracted from batches of embryos and analysed by electrophoresis on a denaturing polyacrylamide gel. The positions of the fully adenylated (A)₆₅ and the fully deadenylated (A)₀ forms of the transcripts are indicated on the left of each panel. **B**, Quantification of the experiments shown in **A**. The percentage of (A)₀ RNA at each time point was calculated as described in the Materials and Methods.

Figure 5: CUG-BP1 binding sites are enriched in UGU tri-nucleotides.

The number of UGU tri-nucleotides in the individual sequenced aptamers of the S0 pool and family 1 and 2 are shown as a histogram. Family 1, grey bars; family 2, black bars; S0 pool, white bars.

Figure 6: XCI100 alpha maternal mRNA is a target for EDEN-BP and is deadenylated after fertilisation.

A) Maternal RNA isolated from unfertilised eggs (0h) or 2h and 4h after fertilisation was separated into poly(A⁺) and poly(A⁻) populations and analysed by Northern blotting. XCI100

alpha was revealed using a ^{32}P -labelled probe as described in the Experimental. RNA loading was 1 and 11 embryos equivalents for total RNA and poly(A^+) or poly(A^-) RNAs respectively.

B) ^{32}P -labelled RNAs containing the globin ORF with or without the c-mos EDEN, respectively gbORF and gbORFmos, and the UGU rich region of XC1100 alpha 3'UTR were incubated in *Xenopus* egg extracts and processed for uv-induced cross linking. After separation by SDS-PAGE the proteins that had become radiolabelled were revealed by phosphoimager analysis. The positions of molecular weight markers (kDa) and of EDEN-BP are indicated on the right.

C) Two cell *Xenopus* embryos were injected with a reporter mRNA containing the XC1100 alpha 3'UTR. At the indicated times samples were taken for extraction and analysis of the RNA. After separation by electrophoresis on denaturing gels the radiolabelled RNAs were revealed by phosphoimager analysis. The position of the fully adenylated (A^+) and deadenylated (A^-) RNAs are indicated on the left. Lane M, RNA markers of 888 and 715 nt; lane A^- the reporter RNA containing the XC1100 alpha 3'UTR synthesised devoid of a poly(A) tail.

Table 1.

Clone	Family	Sequence	SPR end point	EMSA	Number of UGU
S8(7)	1	UAUGUUGUGUGUGUGUUGUUUUUGUGUUUUUGUUUU	203	18	10
S8(6)	1	UUUUUUUUUUUUUUUUUUUGUGUUUGUGUUUUUGUGUU	151	6.4	8
S8(5)	1	CCUGUGUCUUGUGUGUGUGUUUUUGUUUCUGGUCUA	104	16	8
S8(10)	1	CUAGCAGUUUGUGUGUGUGUGUGUGUUUAUGCUA	70	nd	9
c-mos EDEN	none	GUAUAUGUAUGUGUUGUUUUUUUUUGUGUGUGUGUGU	51	25	10
S8(9)	1	UGUGUGUGUGUUUUUUUGUUCGUGUUUUUGUGAUCUA	48	nd	8
s3'Eg5	none	UAUAUAUGUGUGUCUAUCGUCACUUGUAUGUCAAUAU U	nd	34	5
S8(2)	2	CCCGGUUACCGUGUUUGUUCUGCUAGAUUUUGGUU	24	980	2
S8(8)	1	AGCCGUCGCUAGUUUGAUUCCGUGUGUUAGUGGUU	19	510	2
S0(1)	none	AGUACUCGACGGGACGGCUGCUGUGAACGACUACG	0	>2000	
S8(12)	2	GUGAUCCAAACCGUUUUUUUCUGUCUCUCCAGUUUG	0	>2000	1
S8(13)	2	UCGUAAAAGGCGUCGCUAAACUGAUUGCCGCUAGA	0	>2000	0
S8(3)	2	GGCUGUACGCCUAUACGUUCUGCUAGAUUUUCGGUU	0	nd	1
S8(4)	2	GGUUUUAAACCUACAUUUGUUCUGCUAGAUUUUGGUU	0	nd	1
S8(1)	2	UAAUCGUACUAUUUUUUUGUUCUGCUAGAUUUUGGUU	0	nd	1
S8(11)	2	UGAAGACGUAAAACCGGUUCUUCACGAUGUGGGUGU	0	nd	2
S3'Eg5C6	none	UAUAUAUCCCCCUAUCGUCACUUGUAUGUCAAUAUU	nd	>500	2

Figure 1 Marquis et al

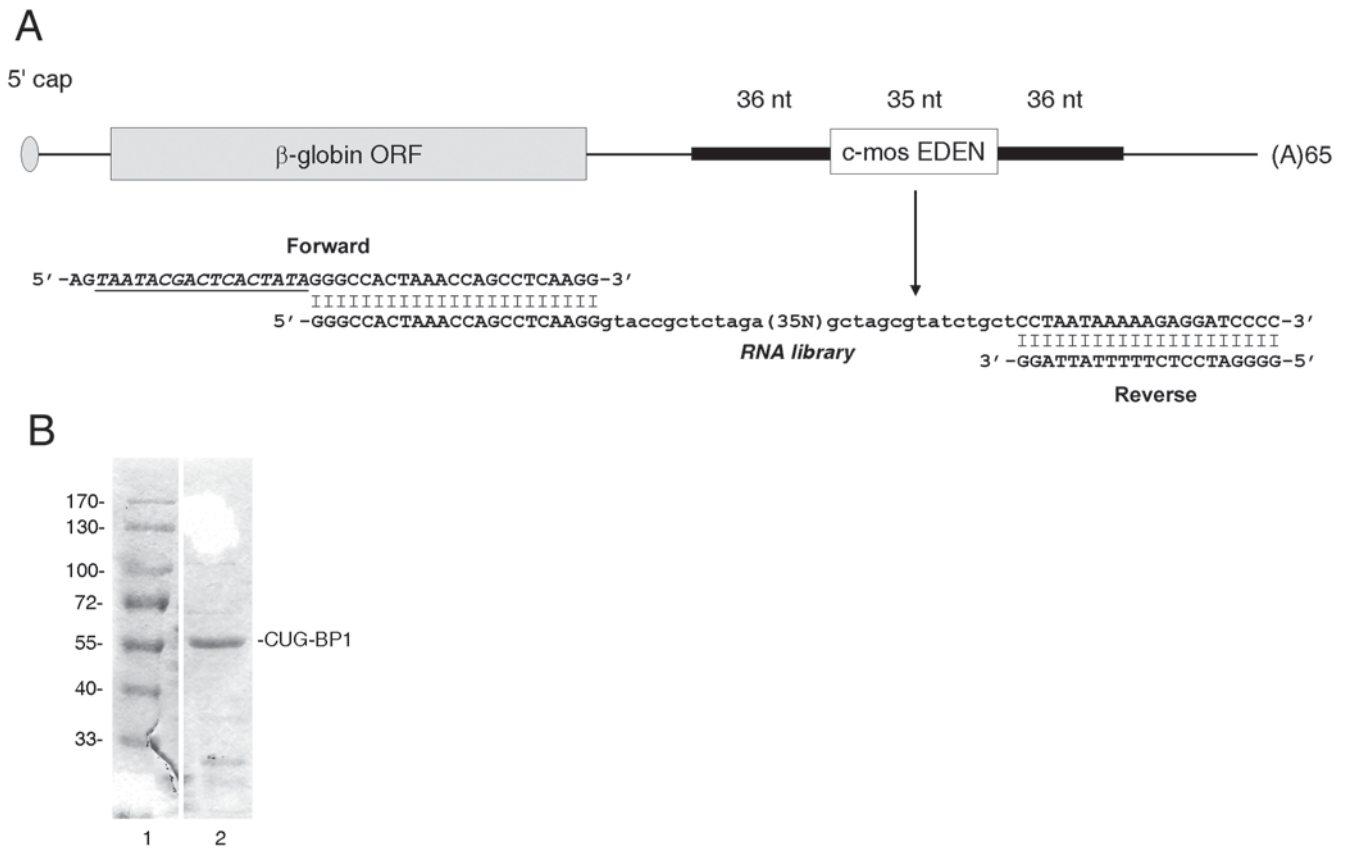
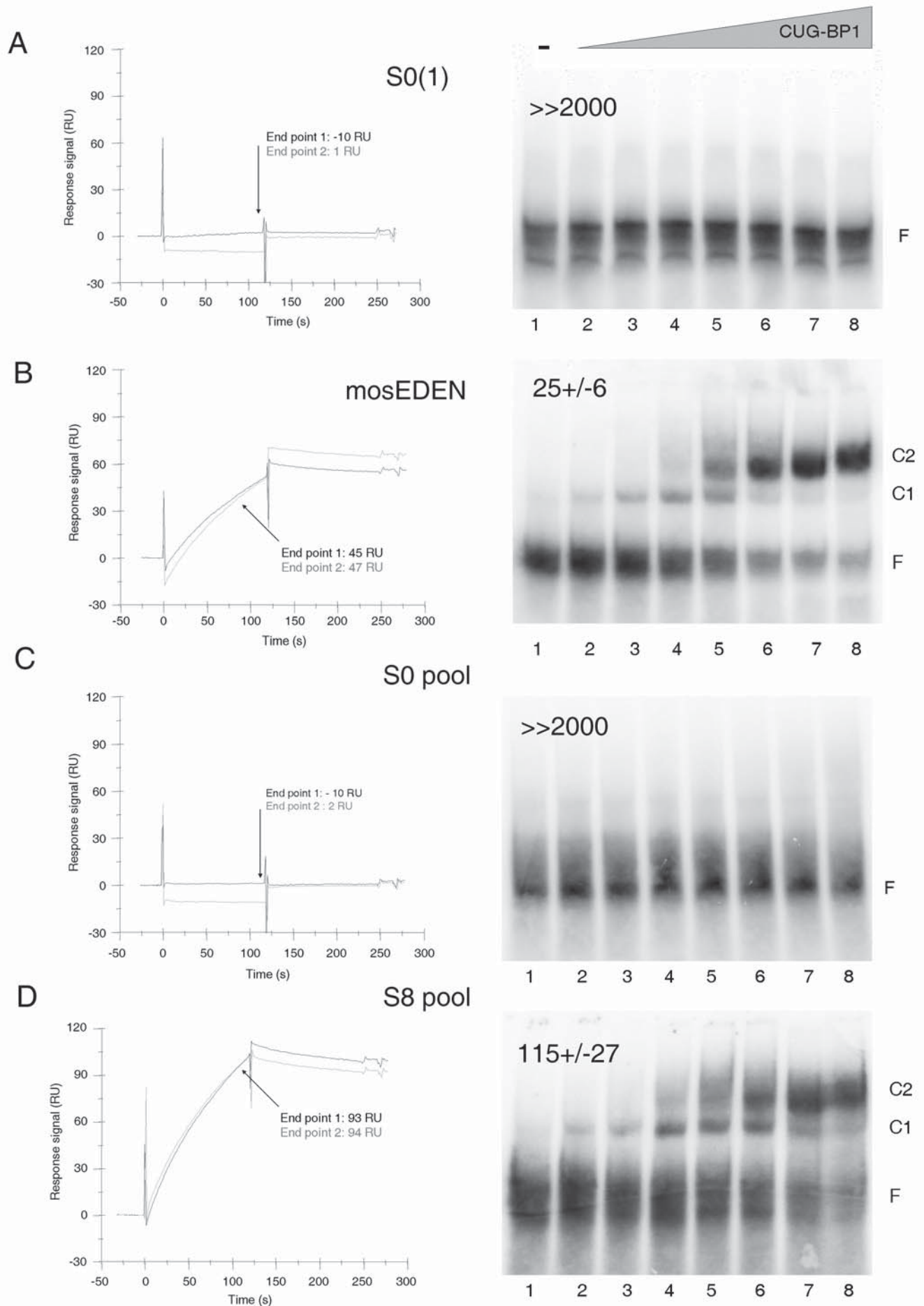
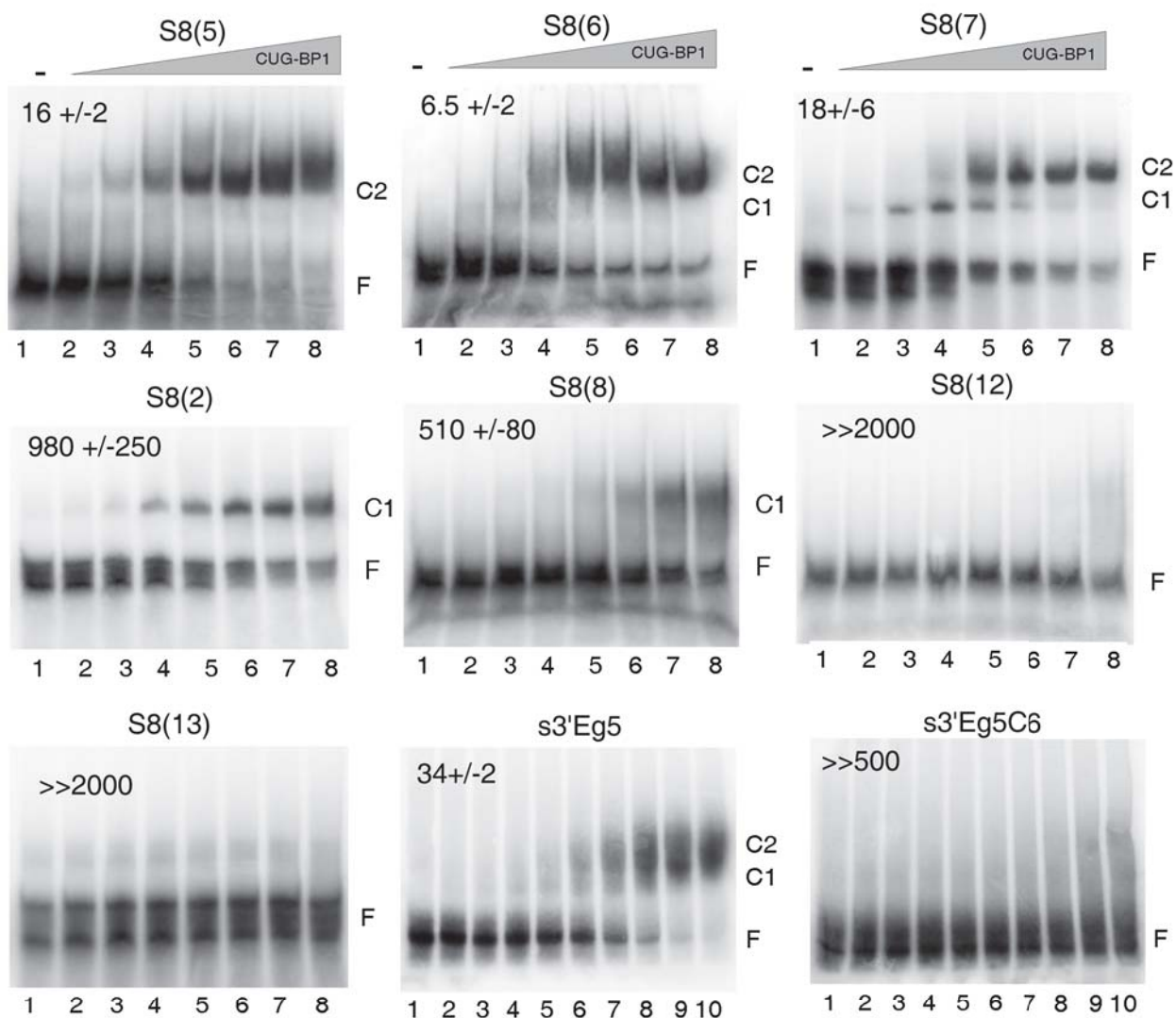


Figure 2 Marquis et al

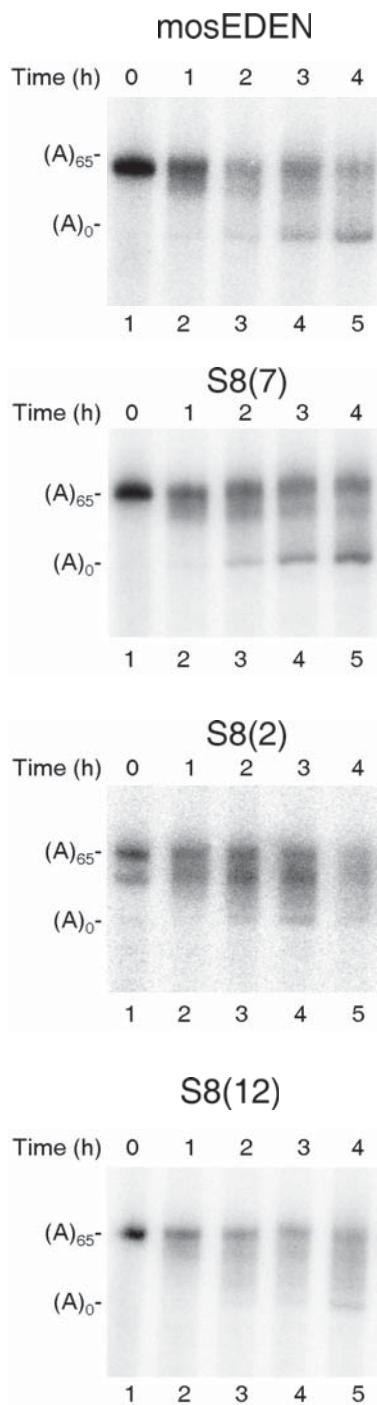




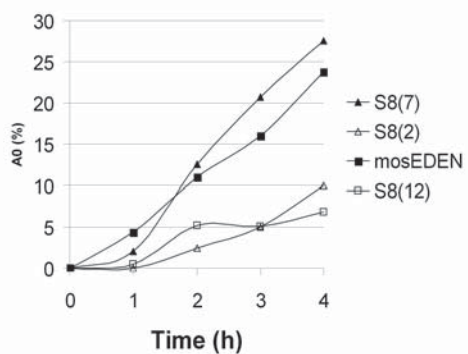
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Figure 4 Marquis et al

A



B



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Figure 5 Marquis et al.

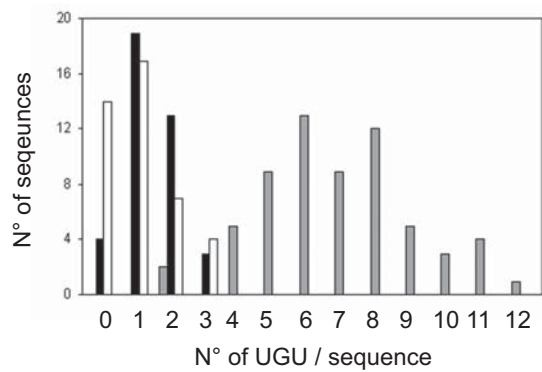
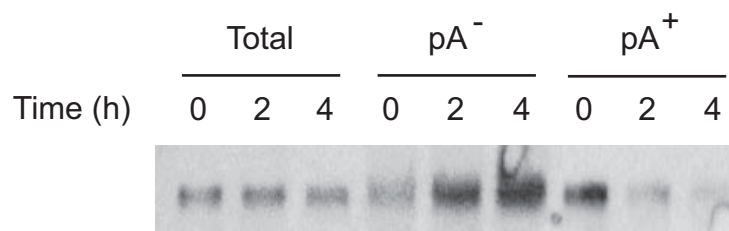
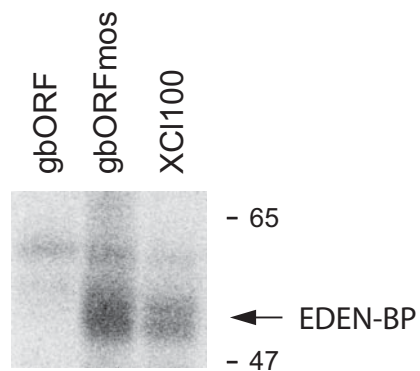


Figure 6 Marquis et al.
The final Version of Record is available at www.biochemj.org

A



B



C

