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CUG repeat binding protein (CUGBP1) interacts with the 5′ region of C/EBPβ mRNA and regulates translation of C/EBPβ isoforms

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ABSTRACT

The transcription factor CCAAT/enhancer binding protein β, C/EBPβ, plays a significant role in the regulation of hepatocyte growth and differentiation. A single mRNA coding for C/EBPβ produces several protein isoforms. Two pathways for generation of low molecular weight C/EBPβ isoforms have been described: specific proteolytic cleavage and initiation of translation from different AUG codons of C/EBPβ mRNA. A truncated C/EBPβ isoform, LIP, is induced in rat livers in response to partial hepatectomy (PH) via the alternative translation mechanism. Here we present evidence that CUG repeat binding protein, CUGBP1, interacts with the 5′ region of C/EBPβ mRNA and regulates translation of C/EBPβ isoforms. Two binding sites for CUGBP1 are located side by side between the first and second AUG codons of C/EBPβ mRNA. One binding site is observed in an out of frame short open reading frame (sORF) that has been previously shown to regulate initiation of translation from different AUG codons of C/EBPβ mRNA. Analysis of cytoplasmic and polysomal proteins from rat liver after PH showed that CUGBP1 is associated with polysomes that translate low molecular weight isoforms of C/EBPβ. The binding activity of CUGBP1 to the 5′ region of C/EBPβ mRNA shows increased association with these polysomal fractions after PH. Addition of CUGBP1 into a cell-free translation system leads to increased translation of low molecular weight isoforms of C/EBPβ. Our data demonstrate that CUGBP1 protein is an important component for the regulation of initiation from different AUG codons of C/EBPβ mRNA.

INTRODUCTION

The CCAAT/enhancer binding protein (C/EBP) family members C/EBPα and C/EBPβ are intronless genes (1,2). However, a single mRNA species of each gene can produce several protein isoforms that display distinct biological functions (3–7). The mechanisms for regulation of these isoforms are not well understood. High levels of C/EBPβ are observed in several tissues including liver. C/EBPβ plays a role in the regulation of liver development, hepatocyte proliferation and the acute phase response to inflammation (8–14). The role of C/EBPβ in hepatocyte proliferation was recently demonstrated by Greenbaum *et al.* (14). Using a liver regeneration model, the authors showed that DNA synthesis is reduced and delayed in mice that contain a targeted disruption of the C/EBPβ gene (14). Significant changes in the expression of cell cycle associated proteins were also observed in C/EBPβ knockout mice after partial hepatectomy (14).

It has been described that a single C/EBPβ mRNA directs production of four isoforms: full-length (FL) C/EBPβ (38 kDa), 35 kDa LAP (liver-enriched transcriptional activating protein), 20 kDa LIP (liver-enriched transcriptional inhibitory protein) and a smaller 16 kDa isoform of C/EBPβ (4,6). Several laboratories demonstrated that low molecular weight C/EBPβ isoforms are induced in response to partial hepatectomy (15–17). Because both LIP and the 16 kDa isoform lack most or all of the activation domain, respectively, it has been suggested that these proteins may function as dominant negative regulators of other C/EBP proteins (18,19). The precise mechanisms that regulate translational control of C/EBPβ mRNA are not known. Descombes and Schibler have proposed that C/EBPβ isoforms are generated by a leaky ribosome scanning mechanism (6). According to this putative mechanism, ribosomes scan C/EBPβ mRNA until they meet the first AUG codon and start translation of the protein. However, a subset of the ribosomes may ignore the first AUG codon and continue scanning, leading to initiation from the next AUG codon. The 5′ region of C/EBPβ mRNA contains a short out of frame open reading frame (sORF) that codes for a putative 9 amino acid peptide. Several lines of evidence showed that this region is involved in the regulation of initiation from methionine codons of C/EBPβ mRNA (3,20). According to the leaky ribosome scanning model, initiation from the sORF AUG codon would prevent initiation at the LAP AUG codon due to close proximity. It would, in turn, lead to increased initiation at the downstream LIP and 16 kDa AUG codons. Although the role of the sORF sequence in translational control of C/EBPβ has been described, the mechanisms that regulate usage of different AUG codons in C/EBPβ mRNA are unknown. Recently, an alternative pathway for the generation of LIP has been proposed. We found that specific proteolytic

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cleavage is activated in the liver during prenatal development and in newborn animals. This pathway is regulated by another member of the C/EBP family, C/EBPα (21,22). Although some proteolytic cleavage of C/EBPβ is observed in cultured cells, translational regulation seems to be the major mechanism for generation of LIP in cultured cells as well as during liver regeneration (22). To further investigate the mechanisms responsible for translational control of C/EBPβ isoforms, we studied the binding of liver proteins to the 5′ region of C/EBPβ mRNA. Data from this paper show that CUG triplet repeat binding protein, CUGBP1, binds to the 5′ region of C/EBPβ mRNA and is involved in the regulation of C/EBPβ isoform production in liver and in a cell-free translation system.

CUGBP1 protein was originally identified as an RNA binding protein that specifically interacts with CUG repeats located in the 3′ untranslated region (UTR) of the myotonin protein kinase (DMPK) gene (23,24). CUGBP1 has also been cloned as the protein interacting with yeast Nab2 factor that is important for transport and polyadenylation of mRNA (24). CUGBP1 is located in both nuclei and cytoplasm (24). One nuclear function of CUGBP1 seems to be the regulation of splicing of CUG-repeat containing RNAs (25). In this paper, we describe a cytoplasmic function of CUGBP1—regulation of C/EBPβ isoforms through alternative translation. Results from this paper show that the C/EBPβ mRNA contains two CUGBP1 binding sites in the 5′ region that has been shown to be important for the regulation of C/EBPβ isoforms. CUGBP1 binds to this region of C/EBPβ mRNA and this binding correlates with an alteration of C/EBPβ isoform production during liver regeneration. Additionally, rat CUGBP1 induces translation of low molecular weight C/EBPβ isoforms when it is added to a cell-free translation system.

MATERIALS AND METHODS

RNA oligonucleotides

RNA oligonucleotides $(CUG)_8$, $(CCG)_8$, LAP (AUGCACCG-CCUGCUGGCCUGGGAC) and ORF (AUGCCUCCCCGC-CGCCGCCCGCCGCCUUAG) were synthesized by Oligo's Etc. Co. RNA oligonucleotides were purified by gel electrophoresis and labeled by T4 kinase with [γ-^{32}P]ATP.

Protein extracts

Whole cell protein extracts from HeLa cells, as well as cytoplasmic and nuclear extracts from cultured fibroblasts were isolated as described (24,26). Isolation of nuclear proteins from rat liver after partial hepatectomy was described previously (27). Fractionation of cytoplasmic proteins by DEAE chromatography was described in our earlier papers (23,24). DEAE elution fractions were analyzed by gel-shift assay and by western blotting with monoclonal or polyclonal antibodies to CUGBP1 as described below.

Generation of truncated CUGBP1 proteins

Sets of primers specific for RBD1 (5′-CCAGACCAACCAG-ATCTTGATGCT and 5′-AGGTTTCATCTGTATAGGGTG-ATG), RBD2 (5′-GAGAAGAACAATGCAGTGGAAGAC and 5′-TTTTACCACCATGGGTGATGAGCAACC) and RBD3 (5′-AGCCAGAAGGAAGGTCCAGAGGGA and 5′-CAAA-CGTTTGAGCTGCACTTTAAGCCG), were synthesized in the Core Facility of Baylor College of Medicine. DNA fragments containing RBD1, 2 and 3 were synthesized by PCR with specific primers shown above using plasmid DNA containing the full-length CUGBP1 cDNA. The resulting DNA fragments were cloned into the *Stu*I site of pMALc vector and the orientation of CUGBP1 was verified by sequencing. To generate RBD1+2, CUGBP1 plasmid DNA (24) was digested with *Spe*I and *Eco*RI and cloned into *Eco*RI/*Hin*dIII of pMALc vector. *Escherichia coli* TB1 cells were transformed with recombinant plasmids containing full-length or truncated CUGBP1 cDNAs. After addition of 2 mM IPTG, the maltose binding protein (MBP) fusion proteins were purified by chromatography on amylose resin and analyzed by polyacrylamide gel electrophoresis with SDS.

Electrophoretic mobility shift assay

The conditions for gel-shift assay are described in our previous papers (23,24). In experiments with whole cell extracts, 50 μg of protein were used. In experiments with cytoplasmic and nuclear extracts, 20 μg of proteins were incubated with ^{32}P-labeled RNAs under conditions described earlier. RNA–protein complexes were analyzed by 8% polyacrylamide gel electrophoresis in TBE buffer. After electrophoresis, gel was dried and exposed to X-ray film.

UV cross-link assay

RNA oligos CUG_8, CCG_8, LAP and sORF were labeled with [γ-^{32}P]ATP and T4 kinase. Purified full-length and truncated CUGBP1 proteins were incubated with ^{32}P-labeled RNA probes, treated with UV light for 30 min, and analyzed by polyacrylamide gel electrophoresis with SDS. Where indicated, unlabeled RNA competitors (100 ng) were added prior to protein addition. Proteins were separated by electrophoresis, transferred onto nitrocellulose membranes, and subjected to autoradiography. To verify the concentration of proteins used in UV cross-link assays, the membrane was stained with Coomassie blue.

Western analysis

Protein (50 or 100 μg) was loaded on a 10–12% polyacrylamide gel and transferred onto a nitrocellulose filter (Bio-Rad). The filter was blocked with 10% dry milk/2%BSA prepared with TTBS buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.1% Tween-20) for 1 h at room temperature. Primary antibodies to CUGBP1 (monoclonal or polyclonal) or to C/EBPβ (C-19, Santa Cruz Biotechnology) were added and the filter was incubated for 1 h, washed, and incubated with secondary antibody for 1 h. Immunoreactive proteins were detected using ECL (Amersham). To normalize protein loading, a preliminary gel was stained with Coomassie blue. After detection of the protein of interest, the membrane was stripped and re-probed with anti-β-actin antibodies as a loading control. Monoclonal and polyclonal antibodies to CUGBP1 were described in our earlier publications (24,28). In experiments with partial hepatectomy, protein extracts from three animals for each time point were analyzed. Data presented in the paper were obtained by three-five repeats of western analysis with each isolate.

Preparation of polysomes

Liver was homogenized in polysomal buffer (PSB) containing 25 mM Tris–HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 0.25 M

sucrose and RNase inhibitor. Nuclei and mitochondria were collected by centrifugation at 13 000 r.p.m. for 30 min. The supernatant (cytoplasm) was incubated with 1% Triton X-100 for 1 h on ice, loaded onto a 2 M sucrose solution prepared with PSB and centrifuged at 50 000 r.p.m. in a SW60 rotor (Beckman) for 2 h. The pellet (polysomes) was dissolved in the standard sample buffer for protein gel electrophoresis and analyzed by western assay. Protein concentration was determined by Bio-Rad protein assay. Data presented in the paper were obtained with different isolates from three animals for each time point after PH.

Analysis of C/EBPβ isoforms in reticulocyte lysate (RL) and in wheat germ (WG) systems

To examine translational regulation of C/EBPβ isoforms by CUGBP1, cell-free coupled transcription/translation systems in rabbit RL and in WG extracts (Promega) were used. The conditions for this assay are described in our previous publication (22). Briefly, C/EBPβ was translated in RL or in WG containing ^{35}S-methionine from a wild type construct or from mutant constructs containing ATG→TTG mutations of initiation AUG codons for full-length (ATG-1), LAP (ATG-2) and LIP (ATG-3) C/EBPβ isoforms (22). Partially purified rat liver CUGBP1 or immunoprecipitated CUGBP1 were added to the mixture before addition of constructs. After incubation, C/EBPβ isoforms were immunoprecipitated with polyclonal antibodies (C-19, Santa Cruz Biotechnology) and analyzed by electrophoresis. Densitometric analysis was carried out using a Molecular Dynamics Personal Densitometer and ImageQuant Software.

RESULTS

CUGBP1 binds to CUG repeats located within the 5′ region of C/EBPβ mRNA

We have previously found that CUGBP1 is located in both nuclei and cytoplasm (23,24). In nuclei, CUGBP1 is involved in the regulation of splicing of CUG-repeat containing RNAs (25). The cytoplasmic function of CUGBP1 is unknown. To address this function, we searched for sequences in mRNAs that contain CUG repeats in regulatory regions. A number of different cDNAs were identified that contain on average 5–25 CTG repeats. These repeats were located mostly in the ORFs (data not shown). One of these mRNAs, C/EBPβ mRNA, is encoded by an intronless gene that produces several protein isoforms, presumably through alternative usage of AUG codons (3,6) and through specific cleavage of full-length protein (22). The sequence of the 5′ region of C/EBPβ mRNA is shown in Figure 1. CUG repeats in C/EBPβ mRNA are located in the 5′ region close to the out of frame sORF sequence which has been shown to regulate C/EBPβ isoform production (3). Analysis of the sORF sequence revealed the presence of CCG repeats in this sequence. Although CUGBP1 was originally found to bind to CUG repeats, we showed that CUGBP1 is also able to bind to CCG repeats in RNA (see below). Therefore, we investigated the interaction of bacterially expressed purified maltose binding protein/CUGBP1 (MBP–CUGBP1) fusion protein with both LAP and sORF sequences (Fig. 1). CUGBP1 contains three RNA binding domains (24) that may potentially possess different specificity for the interaction

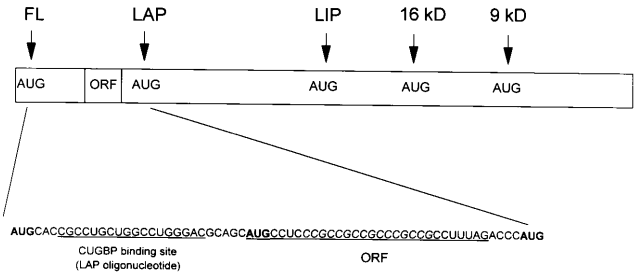

Figure 1. Distribution of AUG codons within C/EBPβ mRNA. Nucleotide sequence of the 5′ region of C/EBPβ mRNA is shown. Molecular weights of C/EBPβ isoforms 16 and 9 kDa are based on the electrophoretical mobilities of the proteins. CUGBP1 binding site containing CUG repeats (LAP oligomer) and sORF sequences are underlined.

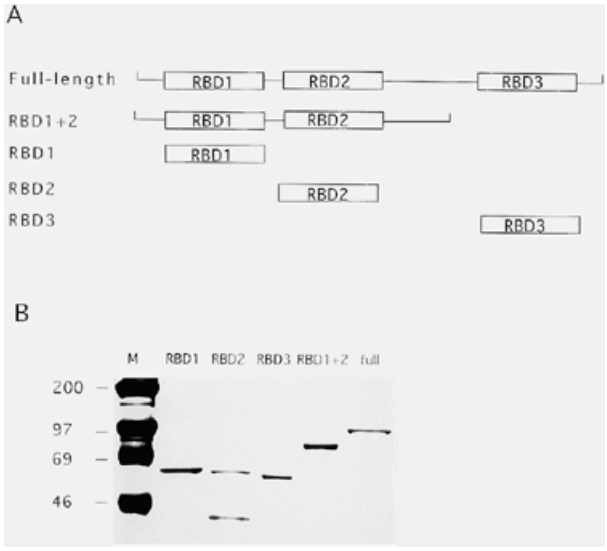

Figure 2. (**A**) A diagram showing truncated CUGBP1 proteins used in these studies. (**B**) Electrophoretical analysis of bacterially expressed, purified full-length and truncated CUGBP1 proteins. The gel was stained with Coomassie blue.

with RNA consensuses. To investigate the interaction of CUGBP1 with the 5′ region of C/EBPβ mRNA, a number of CUGBP1 constructs were generated. Figure 2A shows the constructs used in these studies. cDNA for each construct was generated by PCR with specific primers (Materials and Methods). The coding regions for full-length, RBD1+2, RBD1, RBD2 and RBD3 were fused to MBP and isolated from bacteria as described (28). Each construct was verified by sequencing. Figure 2B shows electrophoretical analysis of full-length and truncated CUGBP1 proteins after purification. The preparations represent highly purified proteins. The RBD2 preparation contains an additional, faster migrating band that presumably is a degradation product since it interacts with antibodies to CUGBP1 (data not shown). Initially, we studied

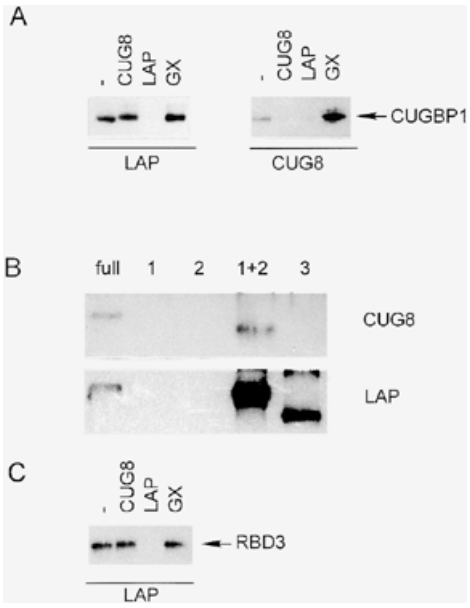

Figure 3. Interaction of CUGBP1 with the 5′ region of C/EBPβ mRNA containing CUG repeats (LAP oligomer). (**A**) Binding of bacterially expressed, purified full-length CUGBP1 to the LAP and CUG_8 oligomers. CUGBP1 was incubated with LAP and CUG_8 probes and analyzed by UV cross-link assay as described in Materials and Methods. Unlabeled competitors (shown at the top) were added to the reaction before probe addition. A representative result of three independent experiments is shown. (**B**) Binding of the RBDs of CUGBP1 to CUG_8 and LAP oligomers. Proteins were incubated with RNA probes and analyzed by UV cross-link assay. None of the single domains bind to CUG_8. RBD3, however, interacts with the LAP oligomer. (**C**) UV cross-link analysis of RBD3 interaction with the LAP oligomer. Cold competitors (indicated at the top) were added to the reaction before probe addition.

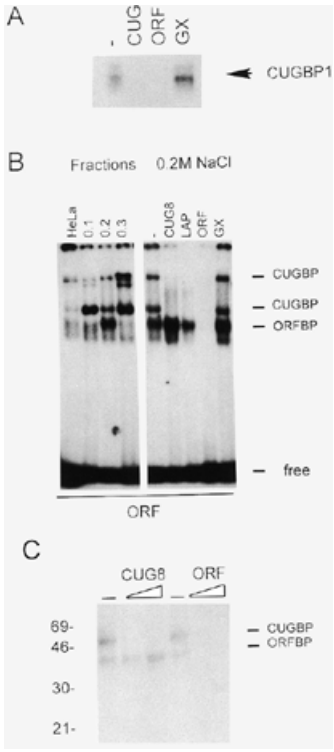

Figure 4. CUGBP1 binds to the out of frame sORF sequence. (**A**) Bacterially expressed, purified CUGBP1 interacts with the sORF oligomer. The sORF probe was incubated with the full-length CUGBP1 protein and analyzed by UV cross-link assay as described in Materials and Methods. Cold competitors (shown at the top) were added before probe addition. (**B**) Binding of cytoplasmic proteins from HeLa cells to the sORF sequence. The left part (fractions) shows analysis of fractions eluted from DEAE–Sepharose with 0.1, 0.2 and 0.3 M NaCl. Gel-shift analysis was performed with the sORF probe. The right part (0.2 M NaCl) shows a gel-shift/competition assay of the 0.2 M NaCl fraction. Cold competitors are indicated at the top. Positions of RNA–protein complexes that are formed by CUG binding proteins (CUGBP) are shown. ORFBP, a complex that is competed by sORF oligomer only. (**C**) UV cross-link analysis of the 0.2 M NaCl elution fraction. Binding of the 0.2 M NaCl fraction to the sORF oligomer was examined in the presence of increasing amounts of cold CUG_8 and sORF competitors (shown at the top). Molecular weight markers are shown to the left.

the binding of full-length and truncated CUGBP1 proteins to the region of C/EBPβ containing CUG repeats (LAP oligomer). Figure 3A shows UV cross-link analysis of the binding of CUGBP1 to LAP oligo. This interaction is specific since incorporation of cold LAP abolished the binding. Competition with non-specific (GX) or CUG_8 oligomers did not abolish the binding of CUGBP1 to LAP (left panel); however, LAP oligo competes for binding of CUGBP1 to CUG8 probe (right panel). Because CUG_8 oligomer does not compete for the binding of CUGBP1 to LAP sequence, we suggested that different RBDs of CUGBP1 might have different sequence specificities. Therefore, we studied the interaction of each RNA binding domain with CUG repeats and with the LAP oligomer. RNA-binding activities of each RBD to both CUG_8 and the LAP oligomers were analyzed by UV cross-link assay. Figure 3B shows that full-length CUGBP1 protein binds specifically to both CUG_8 and LAP. RBD1 and RBD2 separately do not bind to any of the analyzed RNAs. When linked together, they bind to both CUG_8 and LAP. RBD3, on the contrary, binds exclusively to the LAP oligomer. To further study the binding of RBD3 to LAP sequence, UV cross-link/competition experiments were carried out. The RBD3 domain was incubated with LAP probe in the presence of CUG_8, LAP and GX (non-specific) competitors. Only LAP could inhibit binding of RBD3 to the LAP oligomer (Fig. 3C). Thus, our data show that both RBD1 and 2 are necessary for specific binding to CUG repeats. These data also show that the RBD3 domain of CUGBP1 protein binds specifically to the LAP oligomer and that this binding may not involve CUG repeats.

CUGBP1 also interacts with the out of frame sORF sequence of C/EBPβ mRNA

Because the sORF sequence has been shown to regulate C/EBPβ isoform production, we examined whether CUGBP1 is able to bind to this sequence. Although the sORF sequence does not contain CUG repeats, several CCG repeats are observed within the sORF. The sORF probe was incubated with bacterially expressed CUGBP1 and analyzed by UV cross-link assay. As one can see in Figure 4A, CUGBP1 specifically interacts with sORF sequence. Cold CUG_8 and ORF oligomers, but not GX oligomer, abolished the binding. Competition by cold CUG_8

shows that the binding of CUGBP1 to sORF sequence occurs via domains of CUGBP1 that interact with CUG repeats.

CUGBP1 was originally isolated from cytoplasm of HeLa cells expressing high levels of C/EBPβ mRNA (24). To examine whether CUGBP1 binds to the 5′ region of C/EBPβ mRNA under conditions consistent with a biological environment, we investigated the interaction of cytoplasmic proteins from HeLa cells with the 5′ region of C/EBPβ mRNA. Gel-shift analysis of cytoplasmic HeLa proteins with the sORF probe showed a quite complex pattern of binding (Fig. 4B, lane 1). To separate proteins that interact with sORF sequence, cytoplasm from HeLa cells was fractionated by DEAE–Sepharose chromatography and analyzed by gel-shift/competition assay. Figure 4B shows the binding activity of different elution fractions. Although only four RNA–protein complexes are detected in the cytoplasm, fractionation of proteins over DEAE–Sepharose revealed additional RNA–protein complexes that migrate with similar mobility, despite being separated by DEAE fractionation. We have previously shown that CUGBP1 is eluted from DEAE–Sepharose in the 0.2 M NaCl fraction (24). Therefore, we examined the 0.2 M NaCl fraction for interaction with sORF sequence using gel-shift and UV cross-link assays. Incorporation of cold competitors into binding reactions showed that two of the complexes are formed by CUG binding proteins, since addition of cold CUG_8 abolished the binding. One RNA–protein complex (labeled ORFBP) is not competed with CUG_8. This complex is formed by a specific interaction with sORF, since addition of cold sORF oligomer competes for the binding. To determine the molecular weight of proteins contained within the 0.2 M NaCl fraction that interact with the sORF oligomer, UV cross-link assay was carried out. Figure 4C shows that sORF interacts with two proteins from the 0.2 M NaCl fraction. Competition with CUG_8 oligomer shows that a slow migrating protein (~50 kDa) binds to CUG repeats, while a faster migrating protein (ORFBP, molecular weight ~38 kDa) does not interact with the CUG repeats. Addition of cold sORF oligomer leads to competition with both proteins indicating specific interactions. Because gel-shift assay showed two RNA–protein complexes that are competed by cold CUG_8 oligomer, we suggest that the upper sORF–protein complex may be formed by CUGBP1 (51 kDa) associated with another protein. Thus, our studies showed that bacterially expressed, purified CUGBP1 as well as endogenous CUG binding protein(s) from HeLa cells bind to the sORF sequence located in the 5′ region of C/EBPβ mRNA.

CCG repeat within sORF sequence interacts with CUGBP1 protein

Interaction of the sORF with CUGBP1 protein is observed with both bacterially expressed purified CUGBP1 and endogenous CUG binding proteins from HeLa cytoplasmic extracts. We next determined the binding motif for CUGBP1 within the sORF sequence. The sORF oligomer contains five CCG repeats that potentially might be bound by CUGBP1. To test this possibility, we examined the interaction of bacterially expressed, purified CUGBP1 with CCG_8 RNA oligomer using UV cross-link assay. Our data showed that full-length CUGBP1 interacts with CCG repeats (Fig. 5A). To further examine the interaction of CUGBP1 with RNA CCG repeats, rat liver CUGBP1 was partially purified by DEAE chromatography and used for gel-shift assay. Figure 5B shows interactions of

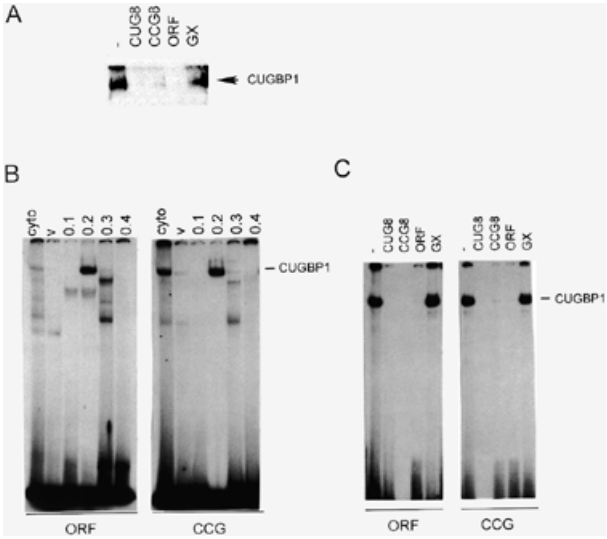

Figure 5. CCG repeats within the sORF sequence interact with CUGBP1. (**A**) Binding of bacterially expressed, purified CUGBP1 to the CCG_8 oligomer was analyzed by UV cross-link as described in the legend to Figure 3. Cold competitors were added before probe addition. (**B**) Fractionation of rat liver cytoplasm by DEAE–Sepharose chromatography. Elution fractions were analyzed by gel-shift assay with sORF (left) and CCG_8 (right) probes. V, void volume fraction. (**C**) Interaction of the 0.2 M NaCl DEAE fraction with the sORF and CCG_8 oligomers. Proteins were incubated with sORF or CCG_8 probes and separated by gel electrophoresis. Cold competitors (shown at the top) were incorporated into the binding reaction.

fractionated proteins with sORF and CCG_8 oligomers. At least six different complexes are detected in rat liver DEAE elution fractions with both probes. We have previously shown that human CUGBP1 is eluted with 0.2 M NaCl (24). As one can see in Figure 5B, the 0.2 M NaCl fraction from rat liver contains a single protein that strongly interacts with both probes. Western analysis of DEAE fractions showed that CUGBP1 was eluted in this fraction (data not shown). Therefore, the 0.2 M NaCl elution fraction was used in gel-shift competition experiments with both sORF and CCG_8 probes. These studies confirmed that the RNA–protein complex in the 0.2 M NaCl fraction is formed by CUG repeat binding protein (CUGBP1), since it is competed by CUG_8 cold oligomer (Fig. 5C). This complex is also competed by addition of cold CCG_8 and sORF oligomers. These data demonstrate that CUGBP1 binds to both CUG and CCG repeats and that CCG repeats within sORF sequence are involved in the interaction with CUGBP1. Thus, investigations of the binding pattern of cytoplasmic proteins to the 5′ region of C/EBPβ mRNA showed the presence of two specific binding sites for CUGBP1 that are located side by side between the first and second AUG codons.

CUGBP1 is associated with polysomes during rat liver regeneration

We recently demonstrated the existence of two different pathways for generation of low molecular weight C/EBPβ isoforms: specific proteolytic cleavage and alternative translation initiation (22). Our results showed that LIP is generated during liver regeneration by the translational mechanism (22). We

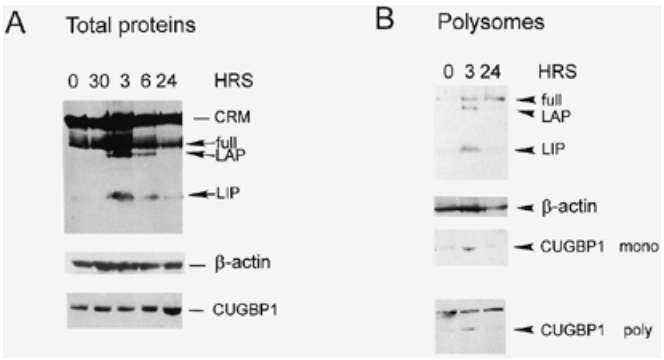

Figure 6. CUGBP1 is associated with polysomes that translate LIP. (**A**) Western analysis of C/EBPβ and CUGBP1 expression after partial hepatectomy. C/EBPβ isoforms LAP and LIP are induced 3 and 6 h after partial hepatectomy. The same membrane was reprobed with antibodies to β-actin. CUGBP1 was detected by western analysis with monoclonal antibodies. CRM, cross reactive molecule; full, full-length C/EBPβ. (**B**) Association of CUGBP1 with polysomes correlates with the induction of LIP 3 h after PH. Polysomal proteins were analyzed by western blotting. The membrane was probed with antibodies against C/EBPβ and antibodies to β-actin to verify protein loading. CUGBP1 was detected with monoclonal (mono) and polyclonal (poly) antibodies against CUGBP1. These results were obtained with different isolates from three animals per time point.

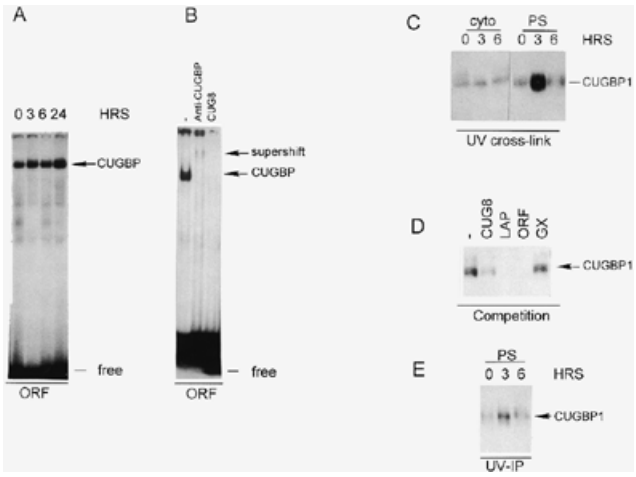

Figure 7. Binding activity of CUGBP1 is induced when associated with polysomes that translate LIP. (**A**) Gel-shift analysis of cytoplasmic proteins at different time points after PH. Ten micrograms of rat liver cytoplasmic proteins were incubated with sORF probe and analyzed by native acrylamide gel. (**B**) Rat CUGBP1 binds to the sORF sequence. Polyclonal antibodies to CUGBP1 or cold CUG8 oligomer (100 excess) were incorporated in the binding reaction before probe addition. (**C**) UV cross-link analysis of polysomal fractions from regenerating liver. Cytoplasmic proteins (cyto, 20 μg) or polysomal fractions (PS, 20 μg) were incubated with the sORF probe, treated with UV and fractionated by 10% polyacrylamide gel. Time points after PH are shown at the top. (**D**) UV cross-link/competition experiments. The polysomal fraction (PH, 3 h) was incubated with the sORF probe in the presence of cold competitors (indicated at the top) and analyzed as described above. (**E**) Binding activity of CUGBP1 is associated with the polysomes. Polysomal fractions were incubated with the sORF probe, treated with UV and immunoprecipitated (IP) with monoclonal antibodies to CUGBP1. IPs were separated by gel electrophoresis. The results presented in this figure were obtained with two different isolates from three animals for each time point.

suggested that CUGBP1 protein might be involved in the translational regulation of LIP during liver regeneration. To investigate this, we first examined the expression of CUGBP1 during rat liver regeneration. Total proteins were isolated 0, 30 min, 3, 6 and 24 h after PH as described in the Materials and Methods and analyzed by western blotting with monoclonal antibodies against CUGBP1 and with polyclonal antibodies to C/EBPβ. In agreement with previously published observations, production of LAP and LIP isoforms is induced at 3 and 6 h after PH (15). However, the concentration of CUGBP1 in whole cell extracts was not altered in response to PH (Fig. 6A). Analysis of CUGBP1 levels as a ratio to β-actin in three animals per each time point showed some variability between animals (1.2- to 2-fold). To examine the intracellular distribution of CUGBP1, nuclear extract, cytoplasm and polysomal fractions were isolated from rat liver after PH and analyzed by western blotting. The filter was probed with antibodies to C/EBPβ and CUGBP1, as well as with antibodies to β-actin to verify protein loading. Figure 6B shows that the low molecular weight isoform of C/EBPβ, LIP, is detected in the polysomal fraction isolated from rat livers 3 h after PH. The same filter was probed with monoclonal antibodies to CUGBP1. The elevation of CUGBP1 in polysomal fractions correlates with induction of LIP expression. A similar result was observed with polyclonal antibodies to CUGBP1 (bottom part of Fig. 6B). The increased association of CUGBP1 with polysomes that translate LIP during liver regeneration, together with the observation that CUGBP1 binds to C/EBPβ mRNA suggests that CUGBP1 may be involved in the regulation of C/EBPβ isoform translation.

Binding activity of CUGBP1 to sORF is increased when associated with polysomes

Because alterations in C/EBPβ isoform production occur at 3–6 h after PH, we studied interactions of liver proteins with sORF during this time period. Cytoplasmic proteins and polysomes were isolated 0, 3, 6 and 24 h after PH and used for gel-shift and UV cross-link assays. Gel-shift analysis of cytoplasmic proteins with the sORF probe did not reveal a significant change in the binding pattern during liver regeneration (Fig. 7A). To confirm that CUGBP1 is involved in the binding to the 5′ region of C/EBPβ mRNA, specific antibodies to CUGBP1 were incorporated in the binding reaction with cytoplasmic proteins. Figure 7B shows that antibodies to CUGBP1 supershifted the major sORF binding protein of liver cytoplasm. This RNA–protein complex is also competed by incorporation of cold CUG_8 oligomer (Fig. 7B). Thus, gel-shift/supershift analysis showed that CUGBP1 is a major sORF binding protein in cytoplasm and that its binding activity is not changed in cytoplasm during rat liver regeneration. To determine whether CUGBP1 binding activity is altered in the polysomal fractions, UV cross-link with the sORF probe was carried out. The results are shown in Figure 7C. One major protein (51 kDa) that binds to sORF is observed in both the cytoplasmic and polysomal fractions. The binding activity of this protein is not altered in cytoplasm during liver regeneration; however, its binding activity is significantly induced in the polysomal fraction 3 h after PH. To examine the specificity of the binding, cold CUG_8, LAP, ORF and GX (non-specific control) oligomers were added to the binding reaction

containing proteins from the 3 h polysomal fraction. Figure 7D shows that CUG_8, LAP and sORF oligomers compete for the binding with this protein. Incorporation of non-specific sequence (GX) did not affect the binding. Since CUGBP1 is a 51 kDa protein, we suggested that CUGBP1 accumulates on polysomes at 3 h after PH and binds specifically to the sORF. To determine if this protein was in fact CUGBP1, polysomal proteins bound to sORF were immunoprecipitated with monoclonal antibodies to CUGBP1 after UV treatment and separated by gel electrophoresis. As one can see in Figure 7E, monoclonal antibodies to CUGBP1 immunoprecipitated the polysomal protein (51 kDa) that specifically binds to sORF sequence. The binding activity of this protein accumulates on polysomes that translate low molecular weight C/EBPβ isoforms 3 h after PH. Taken together, these data show that CUGBP1 protein and its C/EBPβ RNA binding activity are increased in polysomes at 3 h after PH. This increase correlates with translation of LIP and is consistent with the hypothesis that CUGBP1 regulates translation of C/EBPβ isoforms through the interaction with C/EBPβ mRNA.

CUGBP1 induces translation of LIP in a cell-free translation system

To directly examine the effect of CUGBP1 on translation of C/EBPβ isoforms, we analyzed two cell-free translation systems, rabbit RL and WG extract. Translation of C/EBPβ mRNA in RL has been shown to produce several isoforms via differential usage of AUG codons (6,22). We have previously shown that the production of these isoforms in RL depends on different AUG codons, because mutation of each internal AUG codon abolished the production of the isoforms (22). We compared the expression of CUGBP1 in both of these systems, and found that WG does not contain detectable amounts of CUGBP1 (data not shown), while CUGBP1 protein is a highly abundant component of RL (Fig. 8A). UV cross-link/competition assay confirmed these data and revealed that the CUGBP1 in RL is able to bind to the sORF sequence of C/EBPβ mRNA (Fig. 8A). Comparison of C/EBPβ isoforms produced in the RL and WG systems showed that although the same three C/EBPβ isoforms are generated, the full-length protein represents the majority of C/EBPβ products in WG, whereas the RL system produces a majority of LAP protein (Fig. 8B). This difference is consistent with the high levels of CUGBP1 in RL and with the suggestion that CUGBP1 regulates C/EBPβ isoform production. Because WG does not contain CUGBP1 immunological proteins, we further characterized the production of C/EBPβ isoforms in the WG system and found that, as in the RL system, the production of the three isoforms of C/EBPβ in WG is dependent on internal AUG codons, because mutation of each ATG codon (ATG→TTG) abolished the production of corresponding isoform (Fig. 8C). To examine the effect of CUGBP1 on C/EBPβ isoform production in WG, partially purified rat liver CUGBP1 (0.2 M DEAE fraction, as in Fig. 5) was incorporated into the WG system. Figure 8D shows that CUGBP1 induces translation of the LIP isoform. Densitometric analysis of C/EBPβ isoforms showed a 7- to 8-fold induction of the LIP/LAP ratio by the fraction enriched for CUGBP1 (Fig. 8E). To verify the effect of CUGBP1 on translation of C/EBPβ isoforms, CUGBP1 was immunoprecipitated with polyclonal Abs from liver cytoplasm and added to the WG system programmed with wild type or ATG3 mutant C/EBPβ constructs. Figure 8F shows that immunoprecipitated CUGBP1 induces production of LIP from wild type C/EBPβ mRNA, but not from mRNA that contains a mutation at the third AUG codon. The specificity of this effect is indicated by

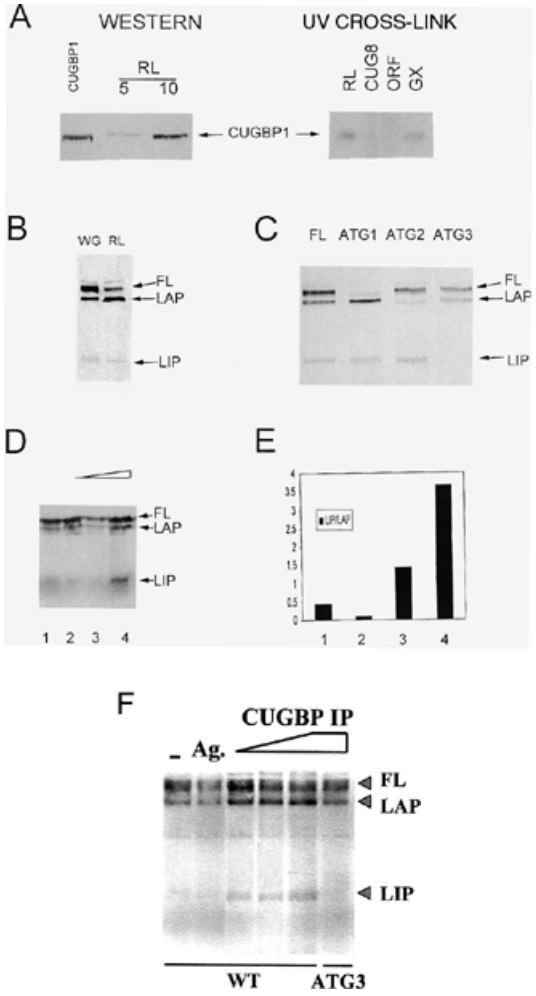

Figure 8. Translation of C/EBPβ isoforms is regulated by CUGBP1 in WG extract. (**A**) RL contains high levels of CUGBP1. Western, 5 and 10 µg of total protein from RL were analyzed by western assay with monoclonal antibodies to CUGBP1. For comparison, 100 ng of purified CUGBP1 is loaded in the first lane. UV cross-link, 10 µg of RL protein were incubated with sORF probe, treated with UV and separated by gel electrophoresis. Cold competitors (indicated at the top) were added into the reaction before probe addition. (**B**) Comparison of C/EBPβ isoforms generated in WG extract versus RL. (**C**) Generation of C/EBPβ isoforms in WG is dependent on AUG codons. ATG1, ATG2 and ATG3 mutant C/EBPβ constructs were translated in WG. FL indicates wild type C/EBPβ. Following translation, C/EBPβ was immunoprecipitated with antibodies specific for the C-terminus and analyzed by gel electrophoresis. (**D**) A rat liver fraction enriched for CUGBP1 induces translation of LIP in the WG system. Increasing amounts of partially purified rat liver CUGBP1 were added to WG (lanes 2–4). Lane 1, no CUGBP1 added. (**E**) Effect of CUGBP1 on LIP production was calculated by densitometry as a ratio of LIP/LAP. This is a representative example of three independent experiments with similar results. (**F**) CUGBP1 was immunoprecipitated (IP) from liver cytoplasm and increasing amounts of IP were added to WG system translating wild type (WT) or ATG3 mutant C/EBPβ. Immunoprecipitate with agarose alone (Ag) serves as a control for non-specific absorption. Positions of FL, LAP and LIP isoforms are shown on the right.

incorporation of a mock immunoprecipitation agarose control (Ag). These data demonstrate that addition of CUGBP1 into cell-free translation system leads to increased initiation of translation from the third AUG codon of C/EBPβ mRNA. Thus, examination of the effect of CUGBP1 on translation of C/EBPβ isoforms in WG showed that CUGBP1 regulates production of the truncated LIP isoform.

DISCUSSION

Proliferation of liver in response to PH is a highly organized process that involves coordinate expression of many genes (29). One of these genes, C/EBPβ, is induced in the earlier stages of regeneration (15,16) and is an important component of the normal proliferative response to PH (14). Induction of C/EBPβ expression after PH is accompanied by alterations in the ratio of LIP/LAP (15,22). Although we showed that production of LIP during liver regeneration depends on the third AUG codon in C/EBPβ mRNA, the mechanisms that regulate this initiation are unknown. In this paper, we present evidence that CUGBP1 is involved in the translational regulation of C/EBPβ isoforms in the liver and in a cell-free translation system. CUGBP1 protein has been identified as a protein that is affected by CUG repeats in myotonic dystrophy (DM) (24). Expansion of CTG repeats in DM leads to accumulation of RNA transcripts containing long CUG repeats in the 3′ region of DMPK mRNA. The increased number of binding sites for CUG binding proteins affects the expression and binding activity of CUGBP1 (24). CUGBP1 has been shown to be located in both nuclei and cytoplasm (24). Previous data have demonstrated that, in nuclei, CUGBP1 is involved in the regulation of splicing for CUG containing transcripts (25). Because a significant portion of CUGBP1 is located in cytoplasm (23,24), we suggested that CUGBP1 may also be involved in the regulation of translation of CUG containing mRNAs. A search for mRNAs containing CUG repeats in regulatory regions showed that the mRNA coding for C/EBPβ contains several CUG repeats in the 5′ region (Fig. 1). Experimental results from this paper show the presence of two CUGBP1 binding sites in the 5′ region of C/EBPβ mRNA between the first and second AUG codons. One binding site contains CUG repeats (LAP oligomer), the other binding site contains CCG repeats in the sORF sequence, which has previously been shown to be involved in regulation of alternative translation of C/EBPβ mRNA (3). Although CUGBP1 binds to both sequences, different RNA binding domains of CUGBP1 are involved in the binding to each sequence. Our *in vitro* experiments showed that the N-terminal portion of CUGBP1 containing RBDs 1 and 2 binds to both LAP and sORF sequences. However, the C-terminal region of CUGBP1, RBD3, binds only to the LAP oligomer. Theoretically, one molecule of CUGBP1 would be able to bind to two regions of C/EBPβ mRNA: RBD1+2 could interact with sORF, while RBD3 can be bound to the upstream LAP region. Taking into account the binding of CUGBP1 to the 5′ region of C/EBPβ mRNA and the resultant generation of low molecular weight C/EBPβ isoforms, it is possible that this interaction may stabilize a structure that favors translational initiation at downstream AUG codons. It is interesting to note that separately RBD1 and RBD2 do not bind specifically to any tested RNA. However, when linked together these two domains show a high level of specific binding to CUG repeat containing RNAs.

Expression of LIP is induced in rat livers in response to partial hepatectomy (15,16). We showed that the major pathway for generation of LIP in response to PH is due to alternative translation initiation (22). To further address this issue, we investigated the interaction of cytoplasmic proteins with the 5′ region of C/EBPβ mRNA at different stages of liver regeneration. Although several proteins (including CUGBP1) interact with the 5′ region of C/EBPβ mRNA, gel-shift analysis with cytoplasmic proteins showed only minor changes that correlate with induction of LIP translation and CUGBP1 binding activity. However, we found that CUGBP1 is associated with polysomes, and that both CUGBP1 levels and CUGBP1 binding activity are increased in polysomal fractions in response to PH. The mechanism of CUGBP1 association with the polysomes is unknown. It is possible that the induction of CUGBP1 protein and binding activity on the polysomes are due to direct interaction with C/EBPβ mRNA. Another mechanism of the association with polysomes might be due to possible interaction of CUGBP1 with ribosomal proteins. Under either of these situations, the increased levels of CUGBP1 on the polysomes that translate LIP suggest that CUGBP1 may regulate translation of C/EBPβ isoforms during liver regeneration. It is interesting to note that in heart and liver tissues of DM patients, where CUGBP1 binding activity is elevated, expression of C/EBPβ isoforms is also altered (N.A.Timchenko, X.Lu, T.Ashizawa and L.T.Timchenko, submitted). The role of CUGBP1 in alternative translation of C/EBPβ isoforms was examined in a cell-free translation system, WG extract, which does not have detectable endogenous CUGBP1. In this system, addition of rat liver CUGBP1 leads to a 7-fold increase in LIP production. These data clearly demonstrate that CUGBP1 regulates alternative translation of C/EBPβ isoforms. Additional studies also showed that the ability of CUGBP1 to regulate translation of C/EBPβ isoforms might be regulated by phosphorylation, because the treatment of CUGBP1 by phosphatase leads to the loss of its capability to regulate the C/EBPβ isoforms (A.L.Welm and N.A.Timchenko, unpublished data). It is also possible that CUGBP1-dependent regulation of C/EBPβ isoforms requires cooperation of CUGBP1 with other proteins. This suggestion is consistent with the observation that several other cytoplasmic proteins bind to the 5′ region of C/EBPβ mRNA (Figs 4 and 6). In addition, western analysis of rat cytoplasmic proteins with polyclonal antibodies to CUGBP1 showed several additional immunoreactive proteins (data not shown) that may also bind to C/EBPβ mRNA and may potentially be involved in the regulation of C/EBPβ isoforms.

Our results are consistent with observations by Calkhoven and co-authors showing that the sORF region controls translation of C/EBPβ isoforms (3). However, it is also necessary to note that the role of sORF in the regulation of C/EBPβ isoforms is the subject of contradictory observations. Lincoln *et al.* have recently shown that the sORF inhibits translation of C/EBPα and C/EBPβ mRNAs, but it does not play a role in generating truncated forms of these proteins (30). Our data demonstrate that a portion of cytoplasmic CUGBP1 is associated with polysomes and that the cell-free translation system (RL) contains high levels of CUGBP1. These observations suggest that cytoplasmic CUGBP1 may be involved in the regulation of translation of CUG (or CCG) repeat containing mRNAs. Although CUGBP1 was originally identified as a protein that binds to CUG repeats in RNA (23,24), results from this paper show that

CCG repeats can also be bound by bacterially expressed CUGBP1 and by endogenous cytoplasmic CUGBP1. This suggests that CUGBP1 may have a more broad set of RNA targets. Here we characterized the role of CUGBP1 in translation of C/EBPβ mRNA, since CUGBP1 binding sites were identified in the 5′ regulatory region of C/EBPβ mRNA. Several other mRNAs contain CUG/CCG repeats within coding regions or in the 3′ UTR (such as DMPK) and also may be regulated by CUGBP1 at the levels of translation or mRNA stabilization. Future experiments will show whether other CUG/CCG containing mRNAs are regulated by CUGBP1. The identification of a novel function for CUGBP1, regulation of translation, suggests that alterations of CUGBP1 in myotonic dystrophy may cause abnormal translation of CUG/CCG containing mRNAs.

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