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CUG binding protein 1 binds to a specific region within the human albumin 3′ untranslated region

Siavash Khaziapoul [a], Mark J. Pearson [a], Ian F. Pryme [b,c], Beate Stern [b,c], John E. Hesketh [a,*]

[a] Institute for Cell and Molecular Biosciences, The Medical School, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK
[b] UniTargetingResearch AS, Thormøhlensgate 51, N-5006 Bergen, Norway
[c] Department of Biomedicine, University of Bergen, Jonas Lies vei 91, N-5009 Bergen, Norway

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ABSTRACT

3′ Untranslated regions (3′UTRs) of messenger RNAs have important roles in post-transcriptional regulation of gene expression and this is partly achieved through binding of specific proteins to sequences or structures within these regions. Previously, replacement of a native luciferase 3′UTR with the human albumin 3′UTR has been found to lead to a 10-fold increase in luciferase reporter activity. In this work we investigated protein binding to the human albumin 3′UTR. Electrophoretic mobility shift and UV cross-linking assays indicate that a ~50 kDa protein from Chinese Hamster Ovary (CHO) cells binds to the albumin 3′UTR, and affinity experiments followed by proteomics identified this protein as CUG binding protein 1 (CUG-BP1, also known as CELF1). Deletion analysis of the albumin 3′UTR showed that nucleotides 1–50 and nucleotides 101–150 are not required for binding but that removal of nucleotides 51–100 caused a loss in binding. The results suggest that CUG-BP1 binds to nucleotides 51–100 of the human albumin 3′UTR. In human cells CUG-BP1 binding may thus play a role in regulation of albumin expression and, additionally, it may have a function in post-transcriptional control in CHO cells.

1. Introduction

In mammals the 3′ untranslated regions (3′UTRs) of messenger RNAs (mRNAs) are longer, have greater tertiary structure and have a wider functionality than the 5′UTRs [1,2]. It is now known that 3′UTRs are important in the regulation of gene expression through effects on mRNA stability, localisation and translation [3–6]. Usually a motif within a discrete part of the 3′UTR, not the whole region, is a sufficient signal to bring about such a regulatory effect and this motif can either be a sequence or a specific RNA structure (e.g. [3]). 3′UTR signals achieve their biological effects through binding to *trans*-acting factors or miRNAs [3–6].

Recently, in proof of principle experiments to test whether it was possible through rational selection and exchange of 3′UTR sequences in a reporter luciferase vector system to significantly enhance recombinant protein titre, we found that the human albumin 3′UTR increased reporter activity 10-fold [7]. Since nothing is known about the binding of proteins to the albumin 3′UTR, the aim of the present work was to firstly investigate whether proteins in fact do bind and secondly, if so, to determine their nature. Using electrophoretic mobility shift assays, UV cross-linking and RNA pull-down experiments we found the albumin 3′UTR to bind CUG binding protein 1 (CUG-BP1), a known RNA-binding protein that is a member of the CELF/Bruno-like family of RNA-binding proteins [8].

2. Materials and methods

2.1. Cell culture

The CHO AA8 tet-off cell line (Clontech) was used throughout. Cells were cultured in Dulbecco's modified Eagle's medium complete growth medium (DMEM + GlutaMAX; Gibco) supplemented with 10% foetal calf serum (FCS; Gibco), 1% penicillin/streptomycin (10,000 U penicillin sodium, 10,000 μg/ml streptomycin sulphate in 0.85% saline; Gibco), and gentamycin (300 μg/ml; Gibco). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. In vitro transcription, electrophoretic mobility shift assays and UV cross-linking

RNA transcripts were synthesised with the MEGAshortscript™ Kit (Ambion) by performing *in vitro* transcription and subsequently quantified by spectrophotometry. Templates for *in vitro* transcription were generated by PCR using primers (listed in Table 1) corresponding either to the 5′ and 3′ ends of the full-length human

* Corresponding author. Fax: +44 191 222 7424.
E-mail address: j.e.hesketh@ncl.ac.uk (J.E. Hesketh).

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Table 1
Primers used for *in vitro* transcription. Regions underlined in italics indicate the minimal T7 promoter.

Oligo	Sequences (5′–3′)
Full3UTR.for	*TAATACGACTCACTATAGG*CATCTACATTTAAAAGC
Del(1–50).for	*TAATACGACTCACTATAGG*ATGAAGATCAAAAGC
Del(1–100).for	*TAATACGACTCACTATAGG*CAACACCCTGTCTAAAAAAC
Del(1–150).for	*TAATACGACTCACTATAGG*CTCTGTGCTTCAATTAATA
Del(1–50 and 101–150).for	*TAATACGACTCACTATAGG*ATGAAGATCAAAAGCTTATTC
GloFor141_T7	*TAATACGACTCACTATAGG*GCAGGCTGCTGGTTGTCTAC

albumin 3′UTR or its respective deletions. A T7 minimal promoter sequence was included in the forward primers. ^{32}P-labelled and non-labelled RNA transcripts were produced using 0.8 μg of purified PCR products (Qiagen). Reaction mixtures were prepared in an RNase-free microcentrifuge tube at room temperature according to the manufacturer's instructions and incubated for 3 h. To remove the template, 1 μl of turbo DNase (Ambion) was added and mixed well. The reaction was incubated at 37 °C for 15 min at room temperature. Electrophoretic mobility shift assays (EMSA) were performed with 1–3 μg of S-100 CHO cell protein extract (hereafter termed CHO cell protein extract) and 12 fmol of ^{32}P-labelled RNA transcript in binding buffer (40 mM NaCl, 4 mM $MgCl_2$, 1 M dithiothreitol, 30 mM Tris–HCl, pH 7.6 with ½ tablet EDTA-free complete protease inhibitor) in a total volume of 8 μl at room temperature for 15 min. For competition assays, labelled and unlabelled transcripts were added simultaneously. After incubation, 40 U of RNase T1 was added and the samples were incubated for a further 5 min at room temperature. The native polyacrylamide gel was pre-run in 0.5× TBE buffer at 120 V. Next, 2 μl of 20% (w/v) Ficoll was added to samples and electrophoresis was carried out in 0.5× TBE buffer for 2 h at 120 V. The gel was then dried and exposed to Kodak Biomax XAR film. UV cross-linking experiments were performed with 50 fmol of ^{32}P-labelled transcript and 2.5–3 μg of S-100 CHO cell protein extract in 40 mM NaCl, 5 mM $MgCl_2$, 30 mM Tris–HCl, pH 7.6 (lysis buffer). The total volume was made up to 13 μl with lysis buffer and incubated at room temperature for 15 min. In competition experiments labelled transcripts and excess unlabelled transcripts were added simultaneously. The reaction mixture was cross-linked for 12 min in SpectroLinker XL 1000 V Cross-Linker on ice and digested with 10 μg of RNase A at 37 °C for 60 min. The samples were analysed by SDS–PAGE.

2.3. Protein isolation using streptavidin-coated paramagnetic particles

RNA-binding proteins were isolated using biotinylated albumin 3′UTR linked to paramagnetic particles [9]. The biotinylated albumin sequence was produced in an *in vitro* transcription reaction using the albumin 3′UTR sequence generated by PCR (see Section 2.2) with the exception of adding 2 μl of Biotin-UTP16 (Roche). The synthesised biotinylated transcripts were subjected to phenol/chloroform extraction and quantified by spectrophotometry. Twenty micrograms of biotinylated transcript were heated at 70 °C for 5 min, then at 40 °C for 20 min and then allowed to cool down at room temperature. The MagneSphere Streptavidin-Coated Paramagnetic Particles (SA-PMP; Promega) were incubated with 100 μl of 0.5× SSC buffer, 10 μg of BSA and 10 μg of yeast tRNA for 60 min at room temperature with shaking. The SA-PMP were washed twice with 300 μl of 0.5× SSC buffer (containing 4.38 mg/ml of NaCl, 2.205 mg/ml of sodium citrate, pH 7.0) and incubated with 20 μg of biotinylated transcript in 300 μl of 0.5× SSC buffer for 10 min at room temperature. Particles were washed with 0.3 ml of 0.5× SSC buffer and incubated with 1 mg of CHO cell protein extract in 500 μl of 40 mM lysis buffer with an additional 25 μg of yeast tRNA, 10 μg of BSA and 800 U/ml of RNasin at 4 °C for 60 min with shaking. Particles were pelleted magnetically by placing the tubes onto MagnaRack™ (Life Technologies). The supernatant fluid was removed and particles washed 5 times with 1 ml of 40 mM lysis buffer. Particles were then re-suspended in 25 μl of 40 mM lysis buffer. Ten microlitres of this re-suspension was mixed with 2× dissociation buffer (90 mM Tris–HCl, pH 6.8, 20% [w/v] glycerol, 2% [w/v] SDS, 2% [w/v] β-mercaptoethanol, 0.02% [w/v] Bromophenol Blue). After denaturation by heating for 5 min at 95 °C the resuspended protein–particles mixture was loaded onto a 10% SDS–PAGE gel. After electrophoresis, the gel was visualised by Novex Colloidal Blue (Life Technologies).

2.4. Proteomic analysis and Western blotting

For protein identification bands in Colloidal Blue stained gels were excised for in-gel trypsin digestion followed by Liquid Chromatography Mass Spectrometry analysis (performed by NEPAF, Newcastle-upon-Tyne). In addition, for Western blotting proteins were transferred to a PVDF membrane (Roche) by a semi-dry transfer method and incubated with a polyclonal antibody to CUG-BP1 (Abcam, 1:1000 dilution) following the manufacturer's instructions. After washing and incubation with anti-rabbit IgG antibody linked to horseradish peroxidase (Sigma – Aldrich, 5000 dilution) bands were visualised using an ECL detection system (GE Healthcare) and Kodak Biomax XAR film (Sigma – Aldrich).

2.5. siRNA transfection

The Chinese hamster CUG-BP1 gene sequence is not known and therefore a consensus sequence was constructed from the CUG-BP1 mRNA sequence in *Rattus norvegicus* (Accession number: NM_001025421) and sequence alignment with isoforms of CUG-BP1 from *Mus musculus* (Accession numbers: NM_198683.1 & NM_017368.2), *Homo sapiens* (Accession numbers: NM_006560, & NM_198700 & NM_001025596) and *Canis familiaris* (Accession numbers: XM_533186 & NM_855451). A specific CUG-BP1 siRNA (sense AAACCUUGGCAGACACGACAUUCCC, antisense GGGAAUGUCGUGUCUGCCAAGGUUU) was then designed using the consensus sequence and Blok-iT™ RNAi designer software (Invitrogen). Cells (5×10^5) were seeded in a 6 well plate 24 h before transfection in order to reach 30–50% confluency. On the day of transfection, the growth medium was replaced with fresh medium containing only 5% FCS. Eighty pmol of CUG-BP1 specific siRNA or negative control siRNA (Life Technologies) in a volume of 4 μl water were added to 246 μl of OptiMEM medium for each well and incubated for 10 min at room temperature. Separately, for each well, 5 μl of Lipofectamine™ 2000 and 245 μl of OptiMEM were mixed and incubated for 10 min at room temperature. The siRNA and lipofectamine OptiMEM solutions were mixed and incubated for further 25 min before being gently added to the cells in the 6 well plate. The medium was replenished the next day and cells were collected 1–4 days after siRNA transfection.

3. Results and discussion

3.1. Protein binding to the human albumin 3′UTR

Since the albumin 3′UTR was found to modulate the level of luciferase reporter transcripts and reporter activity in the culture medium [7] we used RNA–protein binding assays to explore the potential *trans*-acting factors. Incubation of labelled full-length albumin 3′UTR transcripts with CHO cell protein extract led to retardation of the transcripts when analysed in electrophoretic mobility shift assays (EMSA) (Fig. 1), thus providing evidence of RNA–protein complex formation. Investigation of the region of the 3′UTR which was involved in the interaction between transcripts and cell lysate protein was carried out using competitive EMSA with unlabelled 3′UTR sequences in which a range of different short regions were deleted. As shown in Fig. 1 transcripts in which nucleotides 1–50 were deleted from the albumin 3′UTR were able to compete with the full length transcripts in EMSA, suggesting that nucleotides 51–186 are sufficient for protein binding; transcripts in which nucleotides 101–150 were deleted from the albumin 3′UTR were also able to compete with the full length transcripts in EMSA, consistent with nucleotides 51–100 being critical for binding. UV cross-linking assays using CHO cell protein extract and labelled albumin 3′UTR transcripts indicated binding of more than four proteins to the albumin 3′UTR including one major component with a molecular weight of ~50 kDa (Fig. 2). Competitive UV cross-linking assays showed that short regions of the 3′UTR with nucleotides 1–50 deleted competed strongly for binding of the ~50 kDa protein in CHO cell protein extracts. Transcripts with nucleotides 101–150 deleted also competed for binding. Thus, the EMSA and UV cross-linking data indicate that a ~50 kDa protein in CHO cell protein extracts binds to the albumin 3′UTR and that neither loss of nucleotides 1–50 nor loss of nucleotides 101–150 had a major effect on binding. Interestingly, deletion of both nucleotides 1–50 and 101–150 together abrogated binding, and this may be due to a change in RNA structure following removal of such a large proportion of the 3′UTR.

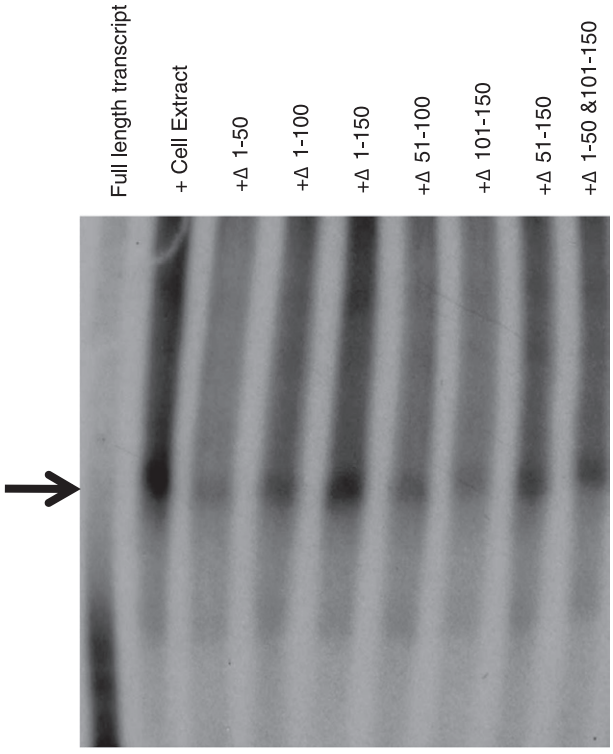

Fig. 1. Electrophoretic mobility shift assays showing RNA–protein complex formation between proteins in CHO cell protein extracts and human albumin 3′UTR sequences. ^{32}P-labelled full length human albumin 3′UTR (186 nt) and non-labelled transcripts with parts of the 3′UTR deleted were prepared by *in vitro* transcription, incubated with CHO cell extracts and subjected to polyacrylamide gel electrophoresis as described in Section 2. In the presence of cell extracts (Lane 2) the labelled transcripts were retarded in the gel, indicating formation of a RNA–protein complex (arrow). Competition assays were carried out using the albumin 3′UTR deletion constructs as competitors. Addition of unlabelled transcripts with nucleotides 1–50 (lane 3) or nucleotides 101–150 (Lane 7) deleted led to reduced complex formation indicating competition for binding. Other deletions did not compete for binding. Lane 1 represents the radiolabelled full length albumin 3′UTR transcript; lane 2 represents radiolabelled transcripts with the addition of CHO cell extract. Lanes 3–9 represent the radiolabelled transcripts with the addition of cell protein extracts and transcripts with the following nucleotide regions deleted: 1–50, 1–100, 1–150, 51–100, 101–150, 51–150, 1–50 and 101–150.

3.2. CUG binding protein 1 binds to the human albumin 3′UTR

On the basis of these data, nucleotides 51–186 of the albumin 3′UTR were used as bait in pull-down assays to isolate the proteins interacting with the albumin 3′UTR. In control assays, protein was incubated with rabbit β-globin 3′UTR sequences. As shown in Fig. 3, SDS–PAGE of protein bound to the transcripts revealed that several proteins interact with the control β-globin sequences but that three additional proteins were observed to bind to the albumin 3′UTR but not to control transcripts (boxed). These proteins were observed migrating in the region of the gel corresponding to a molecular weight of 40–60 kDa and this part of the gel was cut out and subjected to proteomic analysis. Mass spectrometry analysis identified three proteins in the sample that were bound to SA-PMP linked to nucleotides 51–186 of the albumin 3′UTR but which were not present in the negative control. These proteins were identified as Far Upstream Element (FUSE) binding protein 3, Regulator of Differentiation 1 (Rod 1) and CUG binding protein 1 (CUG-BP1). There were three reasons for considering that recovery of CUG-BP1 in these experiments reflected its specific binding to

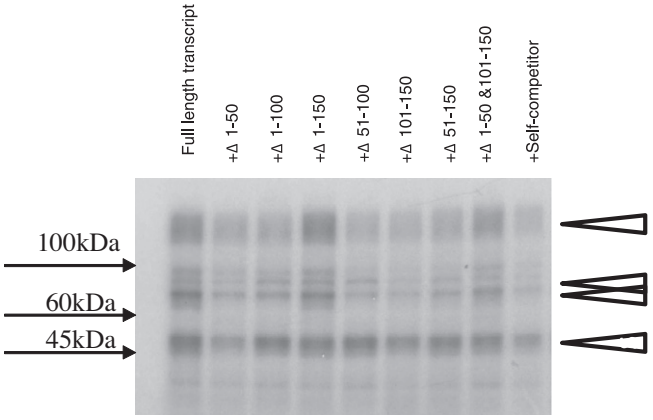

Fig. 2. Protein binding to the human albumin 3′UTR assessed by competitive UV cross-linking competition assay. ^{32}P-labelled full length human albumin 3′UTR (186 nt) and non-labelled transcripts with parts of the 3′UTR deleted were prepared by *in vitro* transcription, incubated with CHO cell protein extracts and then subjected to UV cross-linking, treatment with RNase T1 and RNase A and finally SDS–PAGE was performed. Positions of molecular weight markers are indicated by arrows. Unlabelled transcripts corresponding to sections of the albumin 3′UTR were generated from deletion constructs and used as specific competitors. Lane 1 represents results with radiolabelled full length albumin 3′UTR transcripts with the addition of protein extract, RNaseT1 and RNase A. Lanes 2–9 indicate data obtained with radiolabelled transcripts, cell extract and unlabelled transcripts derived from the following deletion constructs: 1–50, 1–100, 1–150, 51–100, 101–150, 51–150, 1–50 and 101–150 – or unlabelled full length albumin 3′UTR transcripts added as self competitor. In the presence of cell extracts (Lane 1) four major labelled protein bands (open arrowheads) were detected which were lost in the presence of unlabelled competitor full albumin 3′UTR (lane 9). Addition of unlabelled transcripts with nucleotides 1–50 (lane 2) or 101–150 (Lane 6) deleted from the albumin 3′UTR showed reduced complex formation indicating competition for binding. Other deletions did not compete for binding.

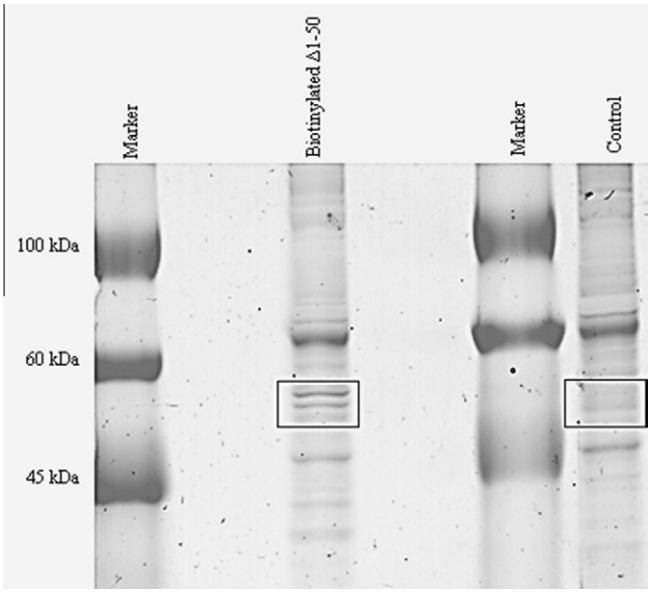

Fig. 3. Isolation of proteins attached to albumin 3′UTR by RNA-affinity pull down protocol. CHO cell protein extract was incubated with magnetic particles linked to biotinylated transcripts corresponding to either nucleotides 51–186 of the human albumin 3′UTR or rabbit β-globin 3′UTR sequences as a control, and after washing, the proteins attached to the particles were dissociated from the particles, denatured and subjected to 10% SDS–PAGE gel electrophoresis. Positions of molecular weight markers are indicated. The gel was stained with colloidal blue and the regions indicated by boxes were excised and analysed by mass spectrometry.

the albumin 3′UTR. First, CUG-BP1 is a ~50 kDa member of the CELF/Bruno-like family of RNA-binding proteins [8,10]. Second, the albumin 3′UTR contains a U-rich sequence between nucleotides 75 and 85 which is similar to an 11-mer nucleotide sequence termed the GU-rich element (GRE) in the 3′UTRs of many short-lived transcripts; and third, there is considerable evidence for the binding of CUG-BP1 to CUG and UG repeats. Therefore, we focused further experiments on obtaining additional evidence for interaction of CUG-BP1 with the albumin 3′UTR.

Our approach was to use siRNA to knock-down CUG-BP1 expression and assess complex formation between the albumin 3′UTR and CUG-BP1. EMSA reactions were carried out with albumin 3′UTR transcripts and protein extracts from either CHO cells in which CUG-BP1 expression had been knocked down by treatment with a specific siRNA or control CHO cells treated with a negative control siRNA. Western blotting analysis showed the antibody to detect multiple bands but that 2 bands of ~50 kDa showed markedly lower expression 3 days after transfection with the specific CUG-BP1, indicating that siRNA treatment reduced CUG-BP1 expression (Fig. 4A). EMSA showed that treatment with the specific CUG-BP1 siRNA abolished the ability of proteins in cell extracts to form a complex with the albumin 3′UTR (Fig. 4B) so providing further evidence that CUG-BP1 binds to the albumin 3′UTR.

In conclusion, the present work strongly indicates that the CUG-BP1, a known RNA binding protein, binds to the albumin 3′UTR. CUG-BP1 is a member of the highly conserved CELF/Bruno-like family of RNA-binding proteins [8,11] and therefore, although at present the functional significance of the binding of CUG-BP1 to the albumin 3′UTR is unclear, we speculate that it plays a role in post-transcriptional control of albumin expression. CUG-BP1 has been reported to act as modulator of alternative splicing [12], translation [13] and deadenylation [14,15] in a variety of cell lines. There is evidence that CUG-BP1 specifically binds to UG motifs and dinucleotide UG repeats [16] and the CELF family of RNA-binding proteins has also been shown to interact with GU-rich elements and regulate mRNA degradation, translation and deadenylation [8,10,13]. Computational analysis suggested that a conserved 11-mer GU-rich element (GRE) UGUUUGUUUGU is present in the 3′UTR regions of many short-lived mRNA transcripts and that CUG-BP1 specifically interacts with GRE and modulates GRE-dependent decay of target mRNA transcripts [10].

Since the albumin 3′UTR contains a UGUUUUCUUUU sequence within the region of nucleotides 51–100, we hypothesise that

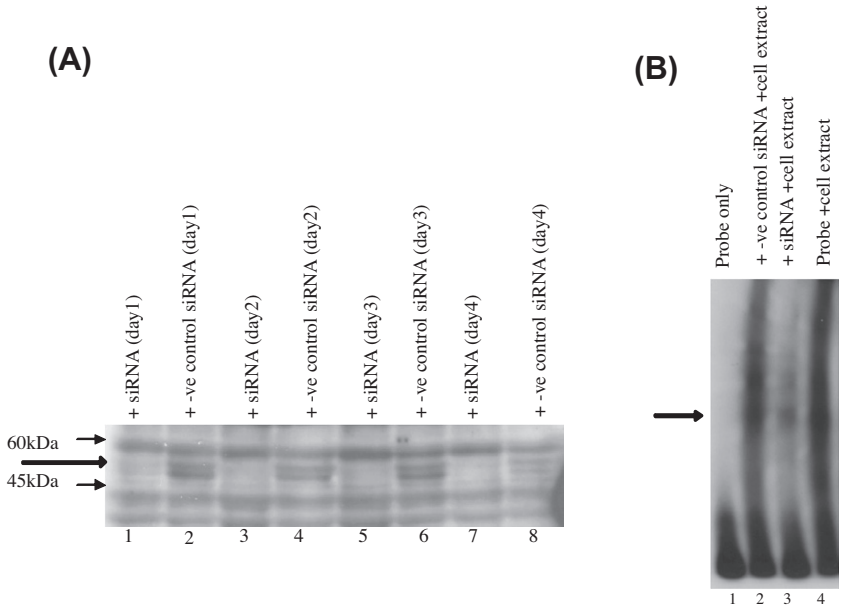

Fig. 4. Effect of CUG-BP1 silencing on RNA–protein complex formation. (A) Western Blot showing extent of CUG-BP1 knockdown. CHO cells were transfected with siRNA for CUG-BP1 or a negative control siRNA and cell proteins extracted after 2–4 days. Small arrows indicate migration of molecular weight markers. The anti-CUG-BP1 antibody recognised two bands of ~50 kDa in CHO cell protein extracts from cells treated with negative control siRNA (long, broad arrow) but these bands were essentially absent from cells treated with the siRNA for CUG-BP1. B. EMSA with CHO cell protein extract from cells treated with either siRNA specific to CUG-BP1 or negative control. Complex formation between full albumin 3′UTR transcripts and CHO cell protein extract (indicated by arrow) was evident with either cell protein extract obtained from non-treated cells (Lane 4) or cells treated with negative control siRNA (lane 2) but was greatly reduced with cell protein extract from the cell line treated with specific CUG-BP1 siRNA (lane 3). Lane 1 shows the transcript alone.

CUG-BP1 binds to this element but at present it is not known whether the binding modulates mRNA translation or stability. Previously, deletion analysis of the albumin 3′UTR has shown that loss of nucleotides 1–50, which removed an AU-rich complex stem loop region, caused significant reductions in both luciferase protein expression and luciferase mRNA levels [7]. Thus binding of the ~50 kDa protein to the nucleotides 51–100 of the human albumin 3′UTR does not appear to play a role in the high reporter protein expression observed in CHO cells. The difference in effects of deleting nucleotides 1–50 and 51–100 on protein binding and reporter expression suggest that the albumin 3′UTR contains at least two regulatory motifs. Further work is required to define the potential role of CUG-BP1 binding to mRNA in post-transcriptional control of expression of albumin and other target proteins, including the role of any possible binding to native CHO transcripts.

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