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Protein expression is increased by a class III AU-rich element and tethered CUG-BP1

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Abstract

In mammalian somatic cells, the post-transcriptional control of cytokine or proto-oncogene expression is often achieved by factors binding to sequence elements in the 3′ untranslated region (3′UTR). The most studied are the AU-rich elements (ARE) that have been divided into three classes. Here, we show that in mammalian cells, the presence of the class III c-jun ARE in the 3′UTR of a reporter mRNA enhanced reporter protein expression. In contrast, the presence of a class II ARE in the 3′UTR decreased reporter protein expression. CUG-BP1/CELF1 is able to bind c-jun ARE. Protein expression was enhanced similarly to what was observed for c-jun ARE when the reporter mRNA contained a synthetic CUG-BP1/CELF1-binding site, or when this protein was tethered to the 3′UTR of a reporter mRNA. These results reveal an unexpected complexity of ARE-mediated post-transcriptional regulations, and indicate a function for CUG-BP1/CELF1 in class III ARE directed regulations.

Keywords: AU-rich element (ARE); Post-transcriptional control; Embryo deadenylation element-binding protein (EDEN-BP); c-jun; RNA-binding proteins; *Xenopus laevis*; Translation

Post-transcriptional regulations of gene expression are now recognized as mechanisms of widespread importance. This was first demonstrated during metazoan development, in which the period of development centred on fertilization is achieved in the absence of active transcription (reviewed in [1]). In adult cells, post-transcriptional regulations also play a key role in a variety of processes. To give but two examples, intracellular iron homeostasis is achieved by a tight post-transcriptional control on the expression level of a variety of proteins, including ferritin and transferrin receptor [2], and erythropoietic differentiation is characterized by a stabilisation of globin mRNAs and a translational repression of 15-lypoxygenase mRNA [3]. Not surprisingly, a number of human diseases, including inflammatory diseases or certain cancers, are associated with defects in post-transcriptional regulations [4].

Post-transcriptional controls at the mRNA level generally rely on the association of specific proteins with binding sites often situated in the 3′ untranslated regions (3′UTR) of the mRNA. Accordingly, it is these RNA-binding proteins, once bound to the 3′UTR, that determine mRNA degradation and translational fates (reviewed in [5]). Identifying the RNA sequence elements and the associated proteins is therefore a prerequisite toward understanding the mechanisms of post-transcriptional controls. In mammals, a subset of such sequence elements was given the name ARE (AU-rich element) after their nucleotide compositions. AREs are found within the 3′UTRs of a number of unstable mammalian mRNAs, such as those encoding cytokines or proto-oncogenes and have been grouped into three classes depending on the distribution or presence of the AUUUA pentanucleotide (reviewed in [6]). Class I AREs, such as that found in c-fos mRNA, contain several AUUUA motifs interspersed within a less well-defined

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U-rich region, and class II AREs, exemplified by GM-CSF ARE, contain several overlapping AUUUA motifs. Class III AREs, such as c-jun ARE, though still AU-rich, contain no AUUUA [6]. AREs act by promoting mRNA deadenylation (shortening of the poly(A) tail), which causes mRNA degradation [6]. A number of RNA-binding proteins, including HuR, AUF1/hnRNP-D, Tristetraprolin, TIA-1 and the related protein TIAR, and CUG-BP2/CELF2, have been shown to associate with AREs and to be responsible for post-transcriptional controls (reviewed in [7]). As recently documented [7], the role that these ARE-binding proteins (ARE-BP) play in post-transcriptional regulation has mainly been studied with respect to the effect on mRNA stability. However, several of these ARE-BPs have been shown to produce specific effects on the translation or the expression of the protein encoded by the target mRNA. In many cases, the effects on mRNA stability and protein expression are correlated. For instance, binding of Tristetraprolin to GM-CSF and TNF-α mRNAs decreased both the stability of the mRNA [8–10] and the expression of the encoded protein [9,11]. Similarly, binding of HuR to GM-CSF mRNA increased mRNA stability [12] and the amount of GM-CSF protein produced [13]. However, this direct correlation is not always observed. HuR binding to TNF-α or Cyclooxygenase 2 (Cox-2) mRNAs, whilst increasing the stability of these mRNAs [14–16], also decreased their translation [16]. Similar opposing effects have also been reported for CUG-BP2/CELF2 when bound to Cox-2 mRNA [17]. These various reports indicate that mRNA stability and translation may be independently regulated and that a strict correlation is not an absolute rule.

These above results were obtained with class I or II AREs. The work we describe here was aimed to determine the effects of c-jun ARE, a class III ARE, on protein expression, in addition to the already documented mRNA instability. We found that c-jun ARE stimulated protein expression, whereas mRNA steady-state level was decreased, indicating an opposing effect of c-jun ARE on mRNA stability and protein expression. Furthermore, we showed that tethering a class-III ARE-binding protein, CUG-BP1, had the same effect, suggesting a role for this protein in class-III ARE-mediated post-transcriptional control of gene expression.

Materials and methods

Plasmids. A pRL-null (Promega) based plasmid containing, in opposite orientations, the Fluc and the Rluc coding sequences under the control of a bidirectional CMV promoter was furnished by Dr. C. Le Bec (Généthon, Evry). The *Xba*I site immediately downstream of the *Renilla* coding sequence was mutagenized to *Sac*I–*Pvu*II–*Xho*I, these sites are unique in the resulting plasmid. Blunt-ended *Not*I–*Apa*I fragments from the βwt, β-2bs, and β-4bs plasmids (kind gifts of Jens Lykke-Andersen [18]) corresponding to human β-globin 3′UTR with 0, 2 or 4 MS2 sequences were cloned in the *Pvu*II site. This yielded the control bidirectional plasmid (no MS2 sequence) and plasmids with 2 or 4 MS2 repeats. Finally, the 4 MS2 repeats were excised by restriction and replaced by c-jun ARE [19] or annealed oligonucleotides ($(TGTA)_9$ and $(ATTT)_8$) to yield plasmids encoding mRNAs containing the c-jun ARE, $(UGUA)_9$ and $(AUUU)_8$ motifs in the 3′UTR of the Rluc gene.

The MS2-CP plasmid (pCMS2) was described [18]. The plasmid CUG-BP1-MS2-CP was obtained by cloning CUG-BP1/CELF1 coding sequence [20] between the *Bam*HI and *Not*I sites of pCMS2. All the constructions were checked by sequencing.

Cell manipulations and analytical methods. NIH-3T3 and HeLa cells were cultured according to the instructions of the supplier (ECACC). They were transfected with plasmids using either the calcium phosphate procedure or the Lipofectamine 2000 reagent (Invitrogen). Various amounts of plasmid were used in the different experiments as indicated in the figures or the table.

Cell lysis and Luciferase assays were performed using the Promega Dual-luciferase system and a Turner luminometer. Each assay was made using three batches of independently transfected cells. RNAs were extracted with Tri-reagent (Euromedex) and analysed by Northern blotting following standard procedures. Northern probes were obtained by PCR amplification from the bidirectional control plasmid and random priming. Radioactive RNAs were revealed using phosphoimager screens. Quantifications were made with a STORM 860 (Molecular Dynamics) and the ImageQuant software.

Results and discussion

We used plasmid transfections and measurement of a reporter activity (*Renilla* luciferase, Rluc) to analyse the effect of human c-jun ARE sequence element on protein expression. As an internal control for transfection and recovery efficiencies, the transfected plasmids also contained a gene for another reporter (Firefly luciferase, Fluc).

Fig. 1. Effect of class III and class II AREs on protein expression. (A) Structure of the constructs used in plasmid transfection experiments. A CMV bidirectional promoter controls the transcription of both Firefly and *Renilla* luciferases. In the transcribed mRNA, the *Renilla* coding sequence is followed by human β-globin 3′UTR without any supplementary sequence element (0), or one of the following elements: a synthetic class II ARE $(AUUU)_8$; the c-jun class III ARE; a synthetic CUG-BP1/CELF1 binding site $(UGUA)_9$; 2 or 4 MS2 sequences. Measurement of expression from the Firefly luciferase gene allows normalisation between experiments. (B) 6×10^4 NIH-3T3 (left panel) or HeLa (right panel) cells were transfected using the calcium phosphate procedure with 500 ng of the indicated plasmids. Proteins were extracted after 27 h and *Renilla* and Firefly luciferase activities were measured. Results (mean ± SD of three independent transfections) are expressed as the ratio of *Renilla*/Firefly luciferase activities. (C) 5×10^4 HeLa (upper panels) or 7×10^4 NIH-3T3 (lower panels) cells were transfected using the calcium phosphate procedure with 400 or 200 ng, respectively, of the indicated plasmids. Three (HeLa cells) or two (NIH-3T3 cells) series of identical transfections were performed independently with each plamid. After 24 h, RNAs were extracted and submitted to a Northern analysis using first a *Renilla* (above) then a Firefly (below) probe. The radioactive signals were revealed by exposure to phosphoimager screens and quantified by the Storm Image Analysis equipment and ImageQuant software. The brackets indicate the quantified bands. For clarity the order of the lanes was reorganised in the lower panel (NIH-3T3). The relative amounts of each mRNA are given below each lane, the average value obtained for the mRNA extracted from the cells transfected with the control plasmid (no insert) was set to one. The ratio (mean ± SD) of Rluc/Fluc mRNA for the different transfected plasmids is indicated below the panels. UTF corresponds to an extract from untransfected cells.