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The ARE-associated factor AUF1 binds poly(A) *in vitro* in competition with PABP

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Running title: AUF1 binds the poly(A) *in vitro*.

Synopsis

The AU-rich element (ARE) is a post-transcriptional element controlling both mRNA turnover and translation initiation by primarily inducing poly(A) tail shortening. The mechanisms by which the ARE-associated proteins induce deadenylation are still obscure. One possibility amongst others would be that an ARE/ARE-binding protein complex intervenes on the PABP/poly(A) tail association and facilitates poly(A) tail accessibility to deadenylases. Here we show by several experimental approaches that AUF1/hnRNP D, an mRNA-destabilizing ARE-binding protein, can bind poly(A) sequence *in vitro*. First, endogenous AUF1 proteins from HeLa cells specifically bound poly(A), independently of PABP. Second, using polyadenylated RNA probes, we showed that (i) the four recombinant AUF1 isoforms bind poly(A) as efficiently as PABP, (ii) the AUF1 binding to poly(A) does not change when the polyadenylated probe contains the GM-CSF ARE, suggesting that *in vitro*, the AUF1/poly(A) association was independent of the ARE sequence *per se*. *In vitro*, the binding of AUF1 isoforms to poly(A) displayed oligomeric and cooperative properties and AUF1 efficiently displaced PABP from the poly(A). Finally, the AUF1 molar concentration in HeLa cytoplasm was only twice lower than that of PABP, whereas in the nucleus, its molar concentration was similar to that of PABP. These *in vitro* results suggest that *in vivo*, AUF1 could compete with PABP for the binding to poly(A). Altogether our data may suggest a role for AUF1 in controlling PABP/poly(A) tail association.

Keywords: post-transcriptional regulation, hnRNP D/AUF1, PABP, AU-rich elements, poly(A) tail.

Introduction

The Adenosine Uridine-rich element (ARE) is a powerful *cis*-regulatory determinant found in the 3'-untranslated region of many unstable mRNAs [1] that controls both mRNA turnover and translation [2-5]. In mammalian cells, the ARE induces mRNA decay by triggering deadenylation as a first and necessary step; deadenylation is then immediately followed by decapping and exonucleic digestions [4, 6]. We recently reported that the ARE intervenes on the binding of poly(A)-binding protein (PABP) to poly(A) [7]. The ARE-mediated PABP release may unprotect the poly(A) tail, opening access to deadenylases and allowing rapid deadenylation. Direct consequences of this deadenylation may be a break of the protective interactions mediated by the mRNA extremities leading to a decircularization of the transcript, translational inhibition and mRNA body degradation [4, 8]. Therefore the PABP/poly(A) tail association may be the primary target of the ARE-mediated mechanisms.

Many ARE-binding proteins (ARE-BPs) have been described [2, 4, 5]. They participate in ARE functions by favouring or impeding either decay or translation. Controversial data have been reported pertaining to the role of the protein AUF1/hnRNP D in ARE functions [9]. Whereas AUF1 triggers rapid decay of many ARE-containing mRNAs in various cellular environments [2, 4, 5, 10], its ectopic expression stabilises mRNAs containing either various AREs or the major coding region determinant of instability (mCRD) present in the *c-fos* mRNA [11]. The high AUF1 expression levels obtained in the latter report are far from physiologic and could account for this disparity [12]. Therefore AUF1 is mostly considered as a destabilizing factor and this is supported by its ability to interact with the exosome machinery responsible for the 3' to 5' RNA body degradation [13]. However investigations on the role of AUF1 in ARE functions have been obscured by the fact that (i) four AUF1 isoforms of 37, 40, 42 and 45kDa, generated by alternative splicing, are present simultaneously [10], (ii) these may have opposite roles in ARE-mediated decay [5, 14], (iii) hundreds of mRNAs are bound by AUF1 amongst which some do not contain obvious ARE [15, 16], (iv) a non-ARE mRNA destabilizing complex contains AUF1 [17].

One model to explain the ARE-mediated deadenylation process postulates that the ARE complex, comprising the ARE and its associated ARE-BPs, induces the removing of the PABP from the poly(A) tail, exposing the naked poly(A) tail to the degradation by either the deadenylase PARN or the exosome [4, 18]. In this model, the PABP/poly(A) tail dissociation is mediated by the ARE/ARE-BP complex [4]. Our recent results support such a model [7]. However it is noteworthy that (i) PABP can bind to the ARE [19, 20], (ii) both AUF1 and

PABP participate in ARE [21] and non-ARE [17] destabilizing complexes. Thus, alternatively, the ARE-BPs could control the activation status of the deadenylases or the decapping enzymes and/or their access to their RNA targets. Supporting this hypothesis, a direct interaction between the ARE-associated destabilizing factor KSRP and the deadenylase PARN has been reported [22]. Moreover, the ARE-binding protein TTP was shown to recruit and activate mRNA decay enzymes by two internal domains [23]. Thus, the molecular mechanism of the ARE-mediated deadenylation is far from being clear and the role of AUF1 in this step remains fully obscure.

Here, we show by several experimental approaches that the endogenous and recombinant AUF1 proteins can bind poly(A) in an oligomeric fashion and that AUF1 can efficiently displace PABP from the poly(A) by molecular competition, thus supporting the model described in [4].

Experimental

Plasmids and small interfering RNA

The pBAD/His-AUF1 plasmids were kindly provided by Gary Brewer, Piscataway, USA. The Flag-AUF1 plasmids were kindly provided by Robert J. Schneider, New York, USA. GC-UTRp(A+), AU-UTRp(A+), pGEM-GC and pGEM-AT vectors were kindly provided by Dr Joan A. Steitz, New Haven, USA. The pTrcHisB-PABP vector was kindly provided by Richard E. Lloyd, Houston, USA. A cocktail of four small interfering RNAs (siRNA) directed against PABP was purchased from Dharmacon. The control scramble siRNA was purchased from Eurogentec. The siRNAs were transfected in HeLa cells with the Transmessenger Reagent (Qiagen).

Cell culture and cytoplasmic extract preparations

HeLa cells were cultured in DMEM, 10% Fetal Bovine Serum. Cytoplasmic lysates were prepared from HeLa cells as described [7].

Agarose poly(A)-binding assay

Poly(A)- or poly(C)-agarose beads (Sigma) were washed 3 times in binding buffer (BB: 25mM Hepes [pH 7.9], 150mM KCl, 2mM EDTA [pH 8.0], 1mM DTT, 5% glycerol, 0.5% NP40), incubated with 400µg whole HeLa cytoplasmic proteins for 4hrs at +4°C under rotation and finally washed 4 times in BB. The bead pellet was resuspended in Laemmli buffer, boiled for 3 min and resolved on a 10% SDS-polyacrylamide gel. The amount of beads was estimated to get 7.5µg of polynucleotide in each reaction. When indicated, Micrococcal nuclease (0.7U/µl) was added to the protein extracts in presence of CaCl₂ (1 mM final) and incubated 15min at RT. The reaction was stopped by adding EGTA (4mM final) prior to selection on agarose beads.

Antibodies and western blot analyses

Western blot analyses were carried out as described [7, 17]. Antibodies against hnRNP K, GST (HRP-labelled) and U1 snRNP70k were from Santa Cruz. Antibody against AUF1 (5B9) was from Euromedex/Upstate. Antibodies against Actin and alpha-Tubulin were from Sigma. The mouse monoclonal 10E10 antibody against PABP was kindly provided by Dr Gideon Dreyfuss (Philadelphia, USA). In western blotting experiments, the protein amount was determined by a 5 min incubation of the membrane with the Supersignal West Dura

Extended Duration Substrate (Pierce) and subsequent measure of chemiluminescence using a VersaDoc Imaging system (Biorad). In the poly(A)-agarose experiments, 'one hundred percent' corresponds to the amount of either PABP or AUF1 selected on the poly(A)-agarose column when using the control extracts.

Recombinant proteins

His₆-AUF1 proteins: The pBAD/HisB plasmids (Invitrogen) containing the whole coding region of either 37, 40, 42 or 45 kDa AUF1 isoform were each introduced into TOP10 bacteria (Invitrogen). AUF1 expression was induced by adding 0.0002% arabinose in culture media for 5hrs [24]. After induction, each histidine-tagged protein was purified from bacteria pellet using the Talon purification kit (BD Biosciences, Clontech) in non-denaturing conditions. The purification was completed by High Performance Gel Filtration chromatography using AKTA purifier system (Amersham Biosciences). An aliquot of each isoform (200 µl) was submitted to chromatography on a Superdex 75 HR 10/30 column equilibrated with the TGN buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl and 10 % glycerol) at a flow rate of 0.2 ml/min. Proteins monitored by absorbance at 280 nm, were recovered in 250 µl fractions. Selected fractions were electrophoresed and the protein profile assessed by Silver staining. Fractions of interest were pooled and concentrated using an Amicon Ultra-4 centrifugal filter device (Millipore, Molsheim, France). Concentrates were aliquoted and stored at -80°C in TGN buffer. Purified recombinant AUF1 concentration was determined by colorimetry using the BCA protein assay kit (Pierce) and using Coomassie blue-stained SDS-polyacrylamide gels.

His₆-PABP: The pTrcHisB plasmid (Invitrogen) containing the whole coding region of the PABP [25] was introduced into TOP10 bacteria and the PABP expression was induced by adding 1 mM IPTG in culture media for 5hrs. After induction, the his₆-PABP was purified from bacteria pellet using the Talon purification kit (BD Biosciences, Clontech) as above. Protein purity was checked on a Coomassie blue-stained SDS-10% polyacrylamide gel. The protein was then concentrated and stored as described above.

GST-PAIP2: The GST-PAIP2 protein was kindly provided by Dr Nahum Sonenberg (Montréal, Canada).

Preparation of RNA probes

In vitro transcription reactions were performed using T7 RNA polymerase (Promega) and either the Sac I-linearized AU-UTRp(A)⁺ and GC-UTRp(A)⁺ plasmids (polyadenylated

RNA probes) or Xba I-linearized AU-UTRpGEM and GC-UTRpGEM (non-adenylated RNA probes) plasmids as templates [26]. Labelled RNA transcripts were produced by including [α - 32 P] ATP (3000 Ci/mmol, Perkin Elmer) in the reaction mixtures. Non-adenylated RNA probes were purified on polyacrylamide-urea gel before use.

Analysis of ribonucleoprotein interactions with purified proteins

The binding reactions were performed in binding buffer A (10 mM Hepes [pH 7.6], 40 mM KCl, 5% glycerol, 0.5% NP40, 3 mM MgCl₂, 2 mM DTT, 0.3 mg/ml Heparin, 0.2 mg/ml tRNA and 150 nM bovine serum albumin (BSA)) as described previously [7]. The volume of each reaction mixture was 10 μ l and incubations were done at room temperature. Each purified protein (concentrations as indicated) was incubated for 15 min with 1 nM of the indicated 32 P-labeled RNA probe. When using the polyadenylated RNA probes, mixtures were further treated with RNase T1 (5U/tube) for 20 min prior to loading onto a non-denaturing low-ionic-strength 6%-polyacrylamide gel. Finally, the gel was dried, scanned using an Instant Imager (Packard) and exposed to a film.

Protein affinity for the non-adenylated probes was determined by measuring the amount of the unbound RNA probes (counts). The amount of unbound RNA versus log his₆-protein concentration was plotted using GraphPad Prism 3.0 computer software. The apparent K_d was determined as the EC₅₀, i.e. the protein concentration at which 50% of RNA was bound.

Protein affinity for the poly(A) tail was determined by measuring the amount of poly(A)-specific RNP complexes formed with the polyadenylated probes (counts). In all conditions, a concentration of 360nM in his₆-protein was sufficient to reach the maximal binding on polyadenylated RNA probes. The amount of his₆-protein/RNA complex versus log his₆-protein concentration was plotted using GraphPad Prism 3.0 computer software. The apparent K_d was determined as the EC₅₀ which is the protein concentration corresponding to the half-maximal binding of the his₆-protein to the poly(A) part of the RNA probes.

Results

Endogenous AUF1 proteins specifically bind to poly(A) in vitro

We recently showed that the GM-CSF ARE/ARE-BP complex intervenes in the PABP/poly(A) association [7]. This led us to hypothesize that some ARE-BPs may be involved in the PABP removal from the poly(A) tail. We hypothesized that this removal could be mediated by protein competition for the poly(A). Following the report of Moraes KC and co-authors showing that AUF1 had structural and binding similarities with PABP [27], we investigated whether AUF1 could specifically associate with the poly(A) *in vitro*. To do so, we performed affinity chromatography using a poly(A)-agarose resin and HeLa cytoplasmic extracts followed by western blot analyses. As control, similar affinity chromatography using a poly(C)-agarose resin was performed in parallel and the specific binding of either hnRNP K [28] to the poly(C) (Figures 1A and B, lower panel) or PABP to the poly(A) (Figures 1A) was verified. These results validated specific selection of our protein of interest when using the chosen resins. Blotting with the polyclonal anti-AUF1 antibody clearly showed that three bands, corresponding to the 37, 40 + 42, and 45 kDa AUF1 isoforms [12, 14, 29] respectively, were strictly selected on the poly(A)-agarose (Figures 1A, middle panel). The western blot data did not enable us to determine whether the middle band contained the 40 kDa, the 42 kDa or both isoforms raising the possibility that one of them was not selected in our experimental conditions. However all recombinant AUF1 isoforms were able to bind poly(A) *in vitro* (see below) and each ectopically-expressed Flag-tagged AUF1 isoform was specifically selected on the poly(A)-agarose column, whereas HuR, another ARE-BP [2, 4, 16], was not (not shown). Treatment of the HeLa cytoplasmic extract with the Micrococcal nuclease prior to selection on agarose beads increased the amount of PABP molecules selected on poly(A)-agarose resin (Figure 1A, upper panel, compare lanes 5 and 7), likely due to an increase in the amount of free PABP following mRNA digestion. The Micrococcal nuclease treatment did not decrease the amount of AUF1 isoforms bound to poly(A) (Figure 1A, middle panel, compare lanes 5 and 7) indicating that the AUF1 isoforms were not selected on poly(A)-agarose resin by mRNA tethering. To support the binding specificity on the poly(A)-agarose, we added increasing amounts of free poly(A) during our affinity chromatography selections. Whereas excess of poly(A) did not alter the binding of the hnRNP K to the poly(C) resin (Figure 1B, lower panel), it strongly competed with the binding of both PABP and AUF1 isoforms on the poly(A) resin (Figure 1B). Finally, we checked whether

AUF1 specific binding to poly(A)-agarose could be consecutive to a co-purification with the PABP since given RNP complexes were shown to contain both AUF1 and PABP [17, 21]. To do so, PABP expression in HeLa cells was knocked-down by transfection with a cocktail of four specific small interfering RNAs (siRNAs) directed against the human PABPC1 mRNA (Acc N°: NM_002568). Similar experiments were performed with a non-specific scramble siRNA as control. In each case, PABP expression was followed by western blotting during the next three days (not shown). The amount of either PABP or AUF1 in the cytoplasmic extracts (Figure 1C, CE) was calculated and normalized by comparison with the amount of Actin used as protein-loading control. Semi-quantitative experiments (Figure 1C, lanes 1 and 2) showed that PABP expression was reduced to 43 % in cells transfected with the cocktail of anti-PABP siRNAs in comparison with the cells transfected with the control siRNA (Figure 1C, compare lanes 1 and 2). In the same conditions, the amount of AUF1 did not change (Figure 1C, compare lanes 1 and 2). Poly(A)-affinity chromatography was then performed using 400µg cytoplasmic extracts prepared from HeLa cells grown for three days after siRNA transfections (CE, Figure 1C). The amount of PABP, selected on the poly(A)-agarose resin, was clearly reduced to 42 % in cytoplasmic extracts from cells transfected with the anti-PABP siRNAs as compared to control cell extracts (Figure 1C, compare lanes 3 and 4). In the same conditions, the amount of the AUF1 proteins selected on the resin did not change (Figure 1C, compare lanes 3 and 4). Altogether these results demonstrated that the binding of the different cytoplasmic AUF1 isoforms to the poly(A) is specific and independent of the amount of PABP in the cytoplasm.

Recombinant AUF1 proteins specifically bind to poly(A) in vitro

To further study the AUF1/poly(A) tail association, we performed gel retardation assays with several RNA probes and increasing amounts of purified histidine-tagged proteins. The *in vitro*-transcribed RNA probes used here were as previously described [7, 26]. We first evaluated the ability of the purified p37^{AUF1} to bind to the non-adenylated RNA probes. As shown (Figure 2), p37^{AUF1} specifically bound to the AU-UTR(A-), the GM-CSF-ARE containing probe, in an oligomeric fashion while its concentration increased (Figure 2, upper left panel, $K_d=80\pm11$ nM), but not to its non-ARE mutated counterpart onto which no cytoplasmic proteins bound, the GC-UTR(A-) probe (Figure 2, upper left panel, $K_d>1500$ nM). Because p37^{AUF1} binding to AU-UTR(A-) resulted in the formation of multiple protein/RNA complexes, the K_d determined in these experiments is only indicative and shows the specific binding of p37^{AUF1} to the ARE. It should be noted that this result is consistent

with previous data showing a sequential association of AUF1 dimers with the ARE [29, 30]. When performing control experiments with a purified histidine-tagged PABP, we observed a clear and specific binding of PABP to the ARE (Figure 2, lower left panel, $K_d=148\pm4$ nM), with a twice-lower affinity than $p37^{AUF1}$, supporting previously reported data [19, 20]. The PABP binding to the GM-CSF ARE-containing AU-UTR(A-), seemed less oligomeric in comparison to $p37^{AUF1}$. Here again, since two protein/RNA complexes formed when the PABP amount increased, the K_d values is only indicative and shows a specific binding of PABP to the ARE and not to the GC-UTR(A-) probe. In our hands, affinities of these two proteins for the GM-CSF ARE were higher than those described in previous reports [20, 29, 30], which may be explained by the use of various types of AREs or different GMSA conditions.

We then investigated the ability of $p37^{AUF1}$ to bind to the polyadenylated RNA probes AU-UTR(A+) and GC-UTR(A+) constructed from AU-UTR(A-) and GC-UTR(A-) respectively, to which a 100 nt-long polyA tail had been added. Note that as GC-UTR(A-) does not bind any protein, GC-UTR(A+) can be considered as a polyA sequence. When loaded on a non-denaturing low-ionic-strength 6%-polyacrylamide gel, the undigested polyadenylated RNA probes were smearing, whereas they showed mostly a single band when loaded on a 6 % urea-containing polyacrylamide gel (data not shown). As this smear was not observed with the deadenylated GC-UTR(A-) or AU-UTR(A-) probe (see Figure 2, left panels), it is likely due to the poly(A) sequence. In accordance with this observation, similar results were obtained in non-denaturing low-ionic-strength polyacrylamide gel by other authors when using a 100nt-long RNA probe containing a 85nt-long poly(A) sequence [31]. Finally, in order to distinguish between the ARE- and the poly(A)-specific RNP complexes, we treated the binding mixtures with RNase T1 prior to loading onto gel. As shown in Figure 2, upper right panel, poly(A)-specific RNP complexes were easily detectable with 45 nM of $p37^{AUF1}$ and 1 nM of either GC-UTR(A+) or AU-UTR(A+) probe (lanes 6 and 15 respectively). Here again the binding pattern of $p37^{AUF1}$ displayed an oligomeric pattern with a ladder of RNP complexes (arrow heads). Moreover the intensity of the higher oligomeric complexes dose-dependently increased while AUF1 concentration increased. Because the binding samples were treated with RNase T1, the apparent affinity of $p37^{AUF1}$ for the poly(A) ($K_d=35\pm5$ nM) was evaluated through the amount of RNP complexes formed with the poly(A) part of each polyadenylated probe and cannot be compared to the affinity obtained with the ARE where the K_d s were calculated with the remaining free RNA probe (see Materials and Methods section for details). Affinity of $p37^{AUF1}$ for the poly(A) tail was similar in the

presence (GC-UTR(A+)) or the absence (AU-UTR(A+)) of the GM-CSF ARE (Figure 2, upper right panel). Control GMSA performed with PABP and the GC-UTR(A+) probe showed as expected, that the PABP associated *in vitro* with its natural ligand (Figure 2, lower right panel, $K_d=47\pm11$ nM) with an affinity comparable to that of p37^{AUF1}. Here again, the presence of the ARE did not notably change the PABP-binding affinity. Interestingly, the binding pattern of PABP was different from that obtained with p37^{AUF1}. Indeed, as noted before, p37^{AUF1} binding to the poly(A) part of GC-UTR(A+) was oligomeric (Figure 2, upper right panel). In the case of PABP, a similar degree of oligomerization was obtained with the poly(A) at a concentration about 4-times higher than with p37^{AUF1} (Figure 2, lower right panel). It is noteworthy that an increase of 0.92 log units in p37^{AUF1} concentration was required to increase the fraction of p37^{AUF1}/poly(A) complex from 10% to 90% indicating a possible cooperativity in the binding of p37^{AUF1} to poly(A) [32]. To further support the specificity of the AUF1/poly(A) tail association, we performed competitive experiments using 1 nM of GC-UTR(A+) probe, 45 nM p37^{AUF1} and an increasing amount of a cold 23-mer oligo(A). As shown in Figure 3, a 12.5-fold excess of cold 23-mer oligo(A) very efficiently abolished the binding of p37^{AUF1} to the poly(A) demonstrating the specificity of the interaction between p37^{AUF1} and the poly(A). It is noteworthy that when the cold competitor was in a 2.5-fold excess, the binding of p37^{AUF1} to GC-UTR(A+) remained mainly unchanged. However when the competitor was in 12.5-fold excess, the binding of p37^{AUF1} to GC-UTR(A+) was suddenly lost. These results are consistent with the results of the Figure 2 and support the idea that *in vitro*, both association and dissociation of p37^{AUF1} with or from the 100nt-long poly(A) are cooperative. The ability of a cold 23-mer oligo(A) to efficiently compete with the binding of p37^{AUF1} to a 100nt-long poly(A) also suggests that the binding site of AUF1 for poly(A) spans 23 Adenine bases or less. Since both GC-UTR(A+) and AU-UTR(A+) RNA probes are 100nt-long, these probes may potentially contain at least four AUF1-binding sites. This hypothesis is supported by the fact that the oligomeric pattern of AUF1 binding to the poly(A) sequence of either GC-UTR(A+) or AU-UTR(A+) showed five potential protein/RNA complexes (arrow heads, Figure 2, upper right panel). Finally we compared the *in vitro* binding affinity of the four different AUF1 isoforms for the poly(A). Our results (Figure 4) showed that the four isoforms bound to the poly(A) in an oligomeric and cooperative way as efficiently as shown for p37^{AUF1} and with a ladder of RNP complexes corresponding to five potential binding sites on the 100nt-long poly(A) (arrow heads). Here again the ARE did not notably change the binding affinity of the four AUF1 isoforms for the poly(A) tail (Figure 4).

Recombinant AUF1 proteins efficiently compete with PABP for the binding to poly(A)

The observation of AUF1 binding to poly(A) led us to investigate whether this could play a role in the ARE-mediated removal of PABP from the poly(A) tail [7]. Since p37^{AUF1} and PABP had a similar affinity for the poly(A) (Figure 2), we hypothesized that the interference with the PABP/poly(A) tail association by the ARE/ARE-BP complex [7] and the destabilizing effect of AUF1 in mRNA decay could be due to a competition between AUF1 and PABP for the poly(A). We first assessed that the formation of the PABP/poly(A) complexes in our GMSA conditions could be efficiently abrogated *in vitro* by a specific inhibitor. This was done by performing competition binding assays using 1 nM GC-UTR(A+) probe, 50 nM PABP and an increasing amount of purified PAIP2, a PABP-interacting protein known to inhibit the binding of PABP to poly(A) [33]. As expected, PAIP2 specifically inhibited the binding of PABP to poly(A) whereas it was unable to dislodge p37^{AUF1} from the poly(A) (data not shown). Secondly we checked whether or not AUF1 was able to interact with PABP in our GMSA conditions. PAIP2 was used as a control for our interaction assays. Two hundred nM of GST-PAIP2 (PABP has two PAIP2 binding sites [33]) or 100 nM of p37^{AUF1} were resolved on a native polyacrylamide gel in the presence or absence of 100 nM of recombinant PABP (and in the presence of 150 nM BSA). As shown in Figure 5, when the mixture of PABP and PAIP2 was resolved on the native gel, a retardation of the migration of both proteins was observed (lane 6, arrow heads). Unexpectedly PABP was also partially detected by the 5B9 polyclonal anti-AUF1 antibody (Figure 5, upper panel, lane 2). This cross-immunodetection allowed us to observe the retardation of PABP migration in the presence of PAIP2 (Figure 5, upper panel, lane 6, asterisk). Taken together these results clearly demonstrated that PAIP2 and PABP interacted with each other in our GMSA conditions. Surprisingly the detection of PABP by immunoblotting was strongly decreased in the presence of PAIP2 (Figure 5, middle panels, compare lanes 2 and 4 with lane 6). It should be reminded that the electrophoresis and protein transfer were made in non-denaturing conditions. Therefore, this observation may suggest that the antigenic epitope recognized by the 10E10 anti-PABP antibody was masked by the interaction of PAIP2 with PABP and is localized in one of the two PAIP2 binding sites for PABP. In the presence of 100 nM of p37^{AUF1}, no retardation in the migration of either PABP (Figure 5, middle panels, compare lanes 2 and 4) or PAIP2 (Figure 5, bottom panel, compare lanes 3 and 5) was observed demonstrating that in our GMSA conditions, p37^{AUF1} did not interact with either PABP or PAIP2. Similar results were obtained with 100 nM of the three other AUF1 isoforms (not

shown). We then investigated whether the different AUF1 isoforms could compete with PABP for the poly(A) *in vitro*. Competition assays were first performed using 1 nM GC-UTR(A⁺) probe, 50 nM PABP and an increasing amount of each recombinant AUF1 isoforms. That concentration of PABP was chosen as it is close to the K_d of PABP for the poly(A) and because at this concentration, the PABP/poly(A) complexes displayed a low degree of oligomerization (Figure 2, lower right panel). Results in Figure 6 showed that increasing concentrations of AUF1 dose-dependently reduced the formation of the PABP/poly(A) complexes while there was a progressive increase in the formation of the AUF1/poly(A) complexes. About 50% of reduction in the formation of the PABP/poly(A) complexes were obtained using a 4-fold molar excess of either p37^{AUF1} or p40^{AUF1} (Figure 6, see graph). A similar percentage of reduction in the formation of the PABP/poly(A) complexes was obtained using an 8-fold molar excess of p42^{AUF1} or a 12-fold molar excess of p45^{AUF1}. These results demonstrated that the ability of the different AUF1 isoforms to displace the PABP from the poly(A) was not equivalent and that p37^{AUF1} and p40^{AUF1} were the most efficient competitors, whereas p45^{AUF1} was the least efficient. As appearance of a new complex containing PABP, AUF1 and the polyadenylated probe was not observed in our assays, we concluded that in our conditions, AUF1 and PABP were unable to interact with each other in the presence of the poly(A). Altogether these results allowed us to conclude that AUF1 isoforms (in this order p37^{AUF1} = p40^{AUF1} > p42^{AUF1} > p45^{AUF1}) can efficiently displace the PABP from the poly(A) *in vitro*. In converse experiments, the reduction of the AUF1/poly(A) complexes could not be easily observed because of the co-migration of the PABP/poly(A) complexes when the PABP molar concentration increased. However we observed a dose-dependent reduction in the formation of a part of the p37^{AUF1}/poly(A) and p40^{AUF1}/poly(A) complexes when PABP molar concentrations increased (not shown). But because of this co-migration, we were unable to estimate the reduction of the p37^{AUF1}/poly(A) or p40^{AUF1}/poly(A) complexes when the concentration of PABP increased. From these results, we can conclude that, at least for p37^{AUF1} and p40^{AUF1}, AUF1 and PABP can alternatively displace each other from the poly(A).

As PABP, AUF1 is an abundant protein in HeLa cytoplasm and nucleus

On the basis of these results, we wondered whether the respective amounts of AUF1 and PABP in the cytoplasm and nuclear compartments may allow such competition to occur *in vivo*. To address this question, AUF1 and PABP were quantified in HeLa cytoplasmic and nuclear extracts using a semi-quantitative western blotting procedure. We first assessed the

purity of three different cytoplasmic and nuclear HeLa extracts by western blotting. Alpha-Tubulin and U1 small nuclear ribonucleoprotein 70k protein (U1 snRNP70k), which are respectively specific of the cytoplasm and the nucleus, were only detected in their specific cellular compartment (Figure 6). We thus proceeded to the determination of AUF1 and PABP amounts in these extracts. A standard curve was created for each protein by plotting the amounts of recombinant protein against the chemiluminescence signal. Several quantities of extract were tested in order to obtain a chemiluminescence signal in the linear range of the standard curve. One representative experiment is shown in Figure 6B with the cytoplasmic extracts and Figure 6D with the nuclear extracts. By this strategy, we found that the amounts of AUF1 and PABP in the cytoplasm were respectively of 32.3 ± 26.9 and 61.8 ± 31.4 fmol per μg of cytoplasmic proteins (Figure 6C, this difference was statistically significant by using a *t* test, $p < 0.03$). In the nucleus, the amounts of AUF1 and PABP were respectively of 62.53 ± 14.60 and 56.06 ± 8.56 fmol per μg of nuclear proteins (Figure 6E). Because the PABP/AUF1 molar ratio is only of 1.91 in HeLa cell cytoplasm and of 0.90 in the nucleus, these results show that AUF1 is abundant in both HeLa cytoplasm and nucleus, and support the idea that AUF1 and PABP proteins could compete *in vivo* for the poly(A).

Discussion

Since we previously showed that the GM-CSF ARE/ARE-BP complex intervenes on the binding of PABP to poly(A) and knowing that HuR and TIAR do not bind to the poly(A) part of our polyadenylated RNA probes [7], we investigated the hypothesis that other ARE-BPs could bind to poly(A) and intervene on the PABP/poly(A) association. We focused our investigations on AUF1 since its RNA recognition domain-2 (RRM-2) had been shown to share structural and binding similarities with that of PABP [27]. Results from our affinity chromatography experiments confirmed that like PABP, AUF1 can specifically associate with the poly(A) sequence (Figure 1). Previous reports have shown by co-immunoprecipitation that AUF1 and PABP can belong to the same multimolecular RNP complex [17, 21]. Here we showed that the AUF1/poly(A) association was independent of the PABP amount in the cytoplasmic extract (Figure 1C). This suggests that *in vivo* AUF1 and PABP do not directly or stably interact with each other. This is in accordance with the fact that results of the investigations done with the aim to identify the proteic partners of either AUF1 or PABP [34-36], failed to show a direct interaction between these two proteins. An exception to this concerns the recent report by Lu and co-authors [37] showing that AUF1, eIF4G and PABP could directly interact with each other. However the display of these interactions required the use of a chemical cross-linking agent suggesting their weakness or instability. An interesting point however is the observation that the weak interaction observed between AUF1 and PABP is abrogated by the binding of AUF1 to the ARE [37], a property that may help AUF1 to act later as a competitor for the poly(A). In our interaction assays (Figure 5), whereas PABP directly and firmly interacted with PAIP2, we were unable to show any interaction of AUF1 with either PABP or PAIP2. It is therefore likely that AUF1 and PABP participate in the same regulatory processes since they belong to the same multimolecular RNP complex [17, 21] and bind to and compete for the poly(A) (this report). However our results do not support the idea that these two proteins directly or firmly interact with each other.

Whereas association of AUF1 with the ARE is widely documented [2, 4], its association with the poly(A) had never been investigated. We therefore further investigated the AUF1/poly(A) association with recombinant histidine-purified AUF1 proteins. Our *in vitro* results showed that the four AUF1 isoforms bound to the poly(A) sequence with an affinity comparable to that of PABP. Complementary experiments showed that this binding did not depend on the GM-CSF ARE sequence *per se*. The binding pattern of the four AUF1 proteins to our 100nt-long poly(A) sequence displayed a clear oligomeric pattern (Figures 2

and 4) respecting some property of a cooperative binding [32]. Similar results were obtained when AUF1 dissociated from our poly(A) (Figure 3). AUF1, which is dimeric in solution, can bind to AREs in the form of tetramers or hexamers [10, 29, 30]. It is assumed that such a large structure might serve to the packaging of the RNA or facilitate its interaction with other regulatory proteins [10]. It is therefore likely that AUF1 binds to poly(A) with similar or even higher degrees of oligomerization than previously reported. This is supported by the binding pattern of the AUF1 proteins to the poly(A) showing a ladder likely to correspond to a successive association of AUF1 dimers. When using the PABP as positive control for the binding to our polyadenylated RNA probes, an oligomeric pattern was also observed with the poly(A) sequence. However higher concentrations of PABP were required in order to display an oligomerization amount comparable to that of p37^{AUF1}. Therefore, not only AUF1 specifically bound *in vitro* to the poly(A) tail with an affinity close to that obtained with the PABP, but it could also generate, at a given concentration, higher ranks of oligomerization with the poly(A) than PABP. This feature could help AUF1 to efficiently prevent the PABP/poly(A) tail association and/or provoke the PABP/poly(A) dissociation. The ability of AUF1 to remove the PABP from the poly(A) was demonstrated in our competitive experiments (Figure 6) where an increasing amount of AUF1 dose-dependently displaced the PABP from the poly(A) by generating poly(A)/AUF1 complexes of higher molecular weight. In our hands, the four AUF1 isoforms efficiently compete with PABP for the poly(A) in this order p37^{AUF1} = p40^{AUF1} > p42^{AUF1} > p45^{AUF1}. Because *in vitro* the different AUF1 isoforms specifically bind to poly(A) in an oligomeric fashion with a binding affinity close to that of PABP and compete with PABP, AUF1 could be considered as an efficient PABP competitor *in vivo*.

Some published data indirectly suggested an interaction of AUF1 with non-ARE parts of the messenger RNA. First the different AUF1 isoforms were shown to reduce the half-life of a non-ARE reporter mRNA and p37^{AUF1} was shown to directly interact with this reporter mRNA [12]. Second a hepatocyte-mRNA population significantly reduced the interaction between AUF1 and the GM-CSF ARE in the presence of tRNAs used as non-specific competitors [15]. The identification of ribonucleoprotein-RNA interactions *in vivo* showed that AUF1 bound to the 5' and 3' non-translated regions of the *ankylosis* mRNA into cortical neurons [38]. Cellular factors, amongst which AUF1 were shown to associate with the non translated region of the Hepatitis C virus RNA (+) genome [39]. Finally, two different strategies led to the identification of hundreds of AUF1-interacting mRNAs, of which some do not contain obvious AREs [15, 16].

Concerning the interaction of AUF1 with the poly(A), here again, published data supported such an interaction. By comparative molecular modelling, RRM2 of AUF1 was shown to have structural similarities with the RRM2 of PABP, and to be able to elaborate either base-specific or non-base-specific interactions with the adenine residues through hydrogen bond or stacking interaction respectively [27]. In the same report, GMSA experiments with a purified his₆-tagged p37^{AUF1} showed that AUF1 had comparable affinities for various RNA probes constituted by either the wild type TNF- α ARE, several mutants of this ARE or a 25nt-long poly(A) [27]. Another report corresponding to the original discovery of AUF1 [31] showed that a 7S complex from the human erythroleukemia K562 post-ribosomal S130 supernatant containing AUF1 and displaying an mRNA decay activity could bind to the *c-myc* ARE. The binding of this complex to the *c-myc* ARE was displaced by either a poly(U) or a poly(A) competitor. However AUF1 binding was not detected when using a radio-labelled poly(A) probe [31].

Immunofluorescence experiments have shown that PABP is mainly cytoplasmic [40], whereas AUF1 is largely but not exclusively localized in the nucleus [12]. Because it is presumed that PABP is more abundant than AUF1 in the cytoplasm, it is difficult to understand how AUF1 could compete *in vivo* with PABP in this compartment. To precisely assess this point, we determined by western blotting the amount of each protein in HeLa cytoplasm and nucleus. Our results showed that, like PABP, AUF1 is an abundant protein in HeLa cells, being roughly twice less abundant than PABP in the cytoplasm and similarly abundant in the nucleus. Because AUF1 can bind the poly(A) and is an abundant protein, this raises the idea that AUF1 may compete with PABP *in vivo*.

It is therefore possible that in the nucleus, AUF1, with the help of other factors and/or depending on its phosphorylation status, may associate with both the ARE and poly(A) tail of the same mRNA before its export to the cytoplasm. Once in the cytoplasm, since PABP is (two times) more concentrated than AUF1 in this compartment, it may compete with AUF1 for the poly(A) and maybe also for the ARE (since PABP binds to the ARE [19, 20], this study) to allow mRNA translation. Therefore, the balance between the association of either AUF1 or PABP with the poly(A) and/or the ARE may control the fate of the ARE-containing mRNAs by triggering either their decay or their translation. Such a model is compatible with the interesting observations that (i) AUF1 activity in mRNA decay depends on its nuclear import [41], (ii) AUF1 is associated with cytosolic fraction [16] whereas PABP is found in polysome-bound fraction, (iii) AUF1-induced mRNA decay is linked to the exosome [13] and the ubiquitin-proteasome pathway [21]

In conclusion, from the *in vitro* data presented in this study, we propose that the destabilizing ARE-BP AUF1 could play a major role in the ARE-mediated dissociation or non-association of PABP with the poly(A) tail. The direct consequences of this non-association would be an activation of the poly(A)-shortening, decapping and of mRNA body degradation [4, 5, 18]. *In vivo* studies are currently under way to investigate the physiological relevance of the AUF1 binding to the poly(A) in the ARE-mediated processes.

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Abbreviations

A, adenine; ARE, AU-rich element ; ARE-BP, AU-rich element-binding protein ; AUF1, AU-rich element RNA-binding protein 1 ; BSA, Bovine serum albumin; C, cytidine; eIF, eukaryotic initiation factor; GM-CSF, granulocyte macrophage-colony stimulating factor ; GMSA, gel mobility shift assay ; hnRNP, heterogeneous nuclear ribonucleoprotein; KSRP, K homology RNA-binding protein ; mCRD, *c-fos* major coding region determinant of instability ; mRNA, messenger RNA ; NSAP-1, NS1-associating protein-1 ; PABP, poly(A)-binding protein ; PAIP-1, PABP-interacting protein-1 ; PARN, poly(A) ribonuclease ; RNA, ribonucleic acid ; RNP, ribonucleoprotein; RRM, RNA recognition motif ; siRNA: small interfering RNA; snRNP, small nuclear RNP; TNF- α , tumor necrosis factor-alpha; tRNA, transfer RNA; U, uridine; UNR, Upstream of *N-ras*.

Figure legends

Fig.1. Endogenous AUF1 proteins bind to poly(A).

Four hundred micrograms of HeLa cytoplasmic proteins were incubated with either poly(A)- or poly(C)-agarose beads as described in Materials and Methods section. Proteins from HeLa cytoplasmic lysates (CE; Panel A, lane 1, 10µg total proteins; Panel C, lanes 1 and 2, 20µg total proteins) or selected on either poly(C)- (Panel A, lanes 2 to 4; Panel B, lanes 1 to 3) or poly(A)-agarose resin (Panel A, lanes 5 to 7; Panel B, lanes 4 to 6; Panel C, lanes 3 and 4) were resolved on a 10% polyacrylamide gel and analysed by western blotting using either an anti-PABP (Panels A, B and C, upper panels), anti-AUF1 (Panels A, B and C, middle panels), anti-hnRNP K (Panels A and B, lower panels) or anti-Actin (Panel C, lower panel) antibody. Panel A: Prior to selection on the columns, cell extracts were either untreated (lanes 2 and 5) or incubated in Nuclease buffer without (lanes 3 and 6) or with (lanes 4 and 7) Micrococcal Nuclease. Panel B: Prior to selection on the columns, cell extracts were added with either no (lanes 1 and 4), 10-fold (lanes 2 and 5) or 100-fold (lanes 3 and 6) excess amount of free poly(A). Panel C: HeLa cytoplasmic extracts, prepared from cells transfected with either a control siRNA (lanes 1 and 3) or an anti-PABP siRNA cocktail (lanes 2 and 4), were resolved on gel before (lanes 1 and 2) or after (lanes 3 and 4) selection on poly(A)-agarose resin. The amount of either PABP or AUF1 (means of two independent experiments) was calculated as described in Materials and Methods section. The name and size of the identified proteins are as shown on the left. Molecular weight markers are as shown on the right.

Fig.2. Recombinant p37^{AUF1} and PABP bind to the GM-CSF ARE and poly(A) *in vitro*.

The AU-UTR(A-) probe contains the GM-CSF ARE. The GC-UTR(A-) probe is the non-ARE mutated counterpart of the AU-UTR(A-) probe onto which no cytoplasmic proteins bound [7]. The GC-UTR(A+) probe contains the non-ARE GC-UTR(A-) probe sequence followed by a 100 nt-long poly(A) sequence. As the GC-UTR part does not bind any proteins, the GC-UTR(A+) probe can be considered as a poly(A) sequence. The AU-UTR(A+) probe contains both the ARE sequence from the AU-UTR(A-) probe and a 100 nt-long poly(A) sequence. All gel retardations described in this report were done in the presence of 150 nM BSA as an irrelevant protein and tRNA as non-specific RNA competitor. Purified his₆-p37^{AUF1} (top panels) or his₆-PABP (bottom panels) (concentrations as indicated) was mixed with 1 nM of either ³²P-labeled GC-UTR(A-), AU-UTR(A-), GC-UTR(A+) or AU-UTR(A+)

RNA probe as indicated at the top of the panels. When using GC-UTR(A+) or AU-UTR(A+) RNA probe, the reaction mixtures were treated with RNase T1 prior to loading onto gel. The unbound RNA and the ARE- or poly(A)-specific RNP complexes are shown in brackets. Arrow heads show the ladder of the p37^{AUF1}/poly(A) complexes. K_d values were calculated from three independent experiments as described in Materials and Methods section. A representative curve corresponding to the binding of each his₆-protein to the poly(A) sequence of the two polyadenylated RNA probes according to the increasing protein concentration is shown below the corresponding gel. The asterisk shows our inability to distinguish, in the spanned area, between the upper-migrating ARE-specific complexes and the lower-migrating poly(A)-specific complexes.

Fig.3. The binding of p37^{AUF1} to the poly(A) is specific.

Purified his₆-p37^{AUF1} (45 nM) was mixed with 1 nM of ³²P-labeled GC-UTR(A+) and an increasing amount of cold oligo(A)₂₃ (concentrations as indicated). Mixtures were treated by RNase T1 prior to loading onto gel.

Fig.4. *In vitro* the four AUF1 isoforms bind similarly to poly(A).

Purified his₆-p37^{AUF1} (upper left panel), his₆-p40^{AUF1} (upper right panel), his₆-p42^{AUF1} (lower left panel) or his₆-p45^{AUF1} (lower right panel) (concentrations as indicated) was mixed with 1 nM of either ³²P-labeled GC-UTR(A+) or AU-UTR(A+) as indicated above the panels. The reaction mixtures were treated with RNase T1 prior to loading onto gel. Arrow heads show the ladder of the AUF1 proteins/poly(A) complexes. K_d values were calculated from the presented experiments.

Fig.5. AUF1 does not interact with PABP in our GMSA conditions, whereas PABP and PAIP2 do.

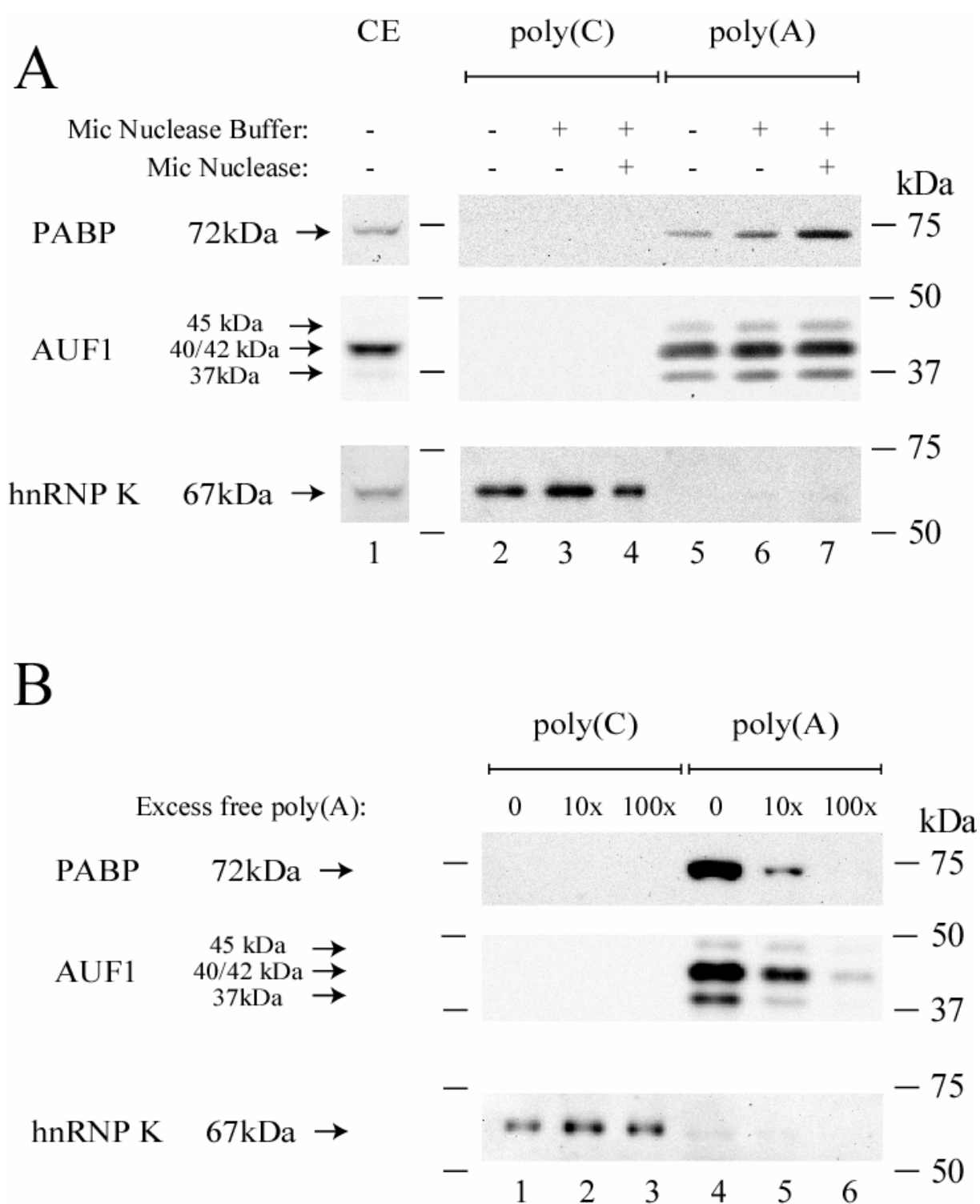
Purified his₆-p37^{AUF1} (100 nM), his₆-PABP (100 nM) or GST-PAIP2 (200 nM) were incubated 15min at room temperature in Buffer A alone or together as indicated. The reaction mixtures were then resolved on a non-denaturing low-ionic-strength 6% polyacrylamide gel. After transfer in TBE 0.5X buffer, the proteins were detected by western blotting using the 5B9 anti-AUF1, 10E10 anti-PABP and anti-GST HRP-labelled antibodies respectively. Arrow heads show the retardation of the migration of either PABP or PAIP2. The asterisk shows the retardation of the migration of PABP revealed by the cross-reactivity using the 5B9 polyclonal anti-AUF1 antibody.

Fig.6. AUF1 proteins compete with PABP for the binding to poly(A).

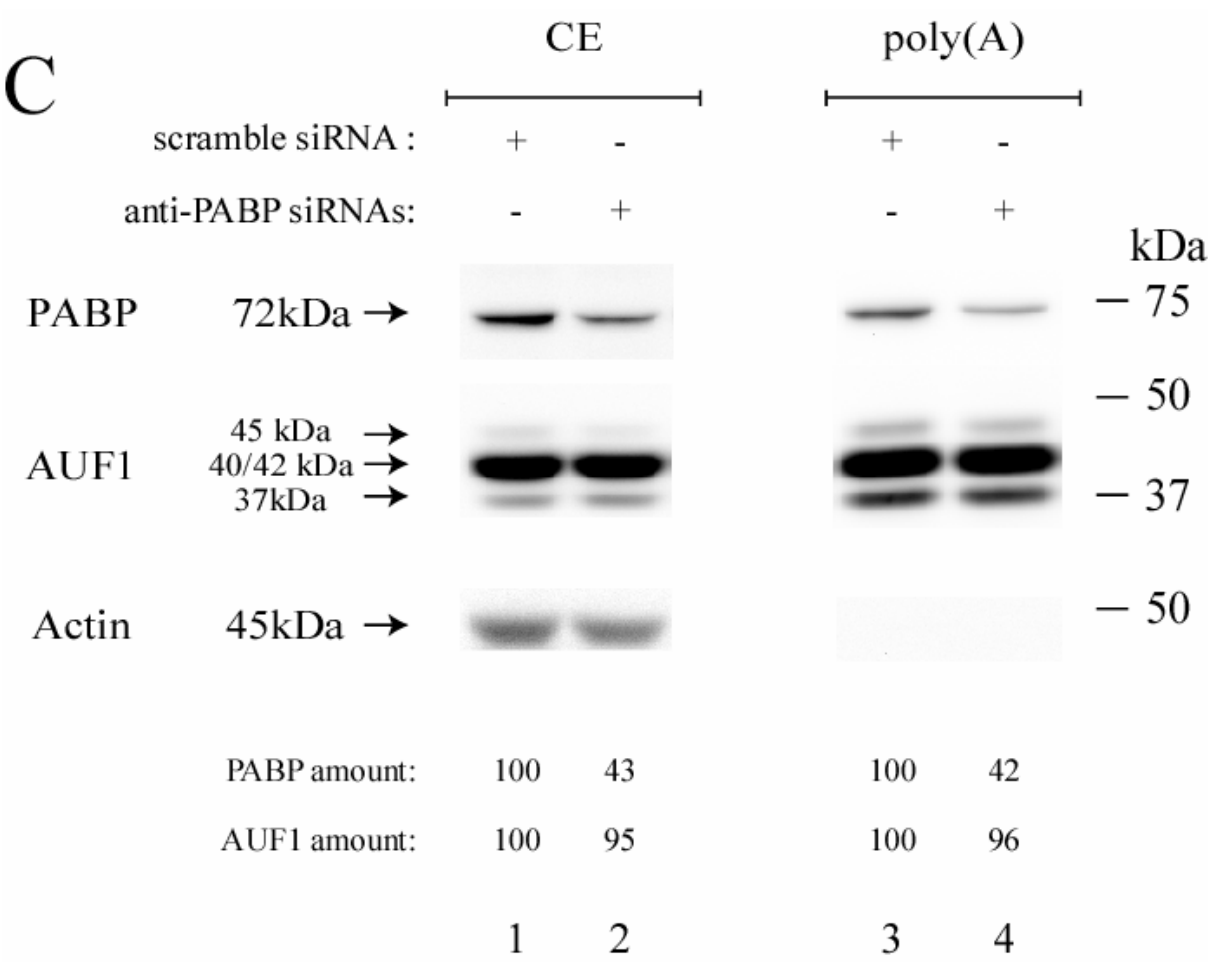
Purified his₆-p37^{AUF1} (upper left panel), his₆-p40^{AUF1} (upper right panel), his₆-p42^{AUF1} (middle left panel) or his₆-p45^{AUF1} (middle right panel) (concentrations as indicated) was mixed with 1 nM of ³²P-labeled GC-UTR(A+) and 50 nM of his₆-PABP (except in lanes 9). The reaction mixtures were treated with RNase T1 prior to loading onto gel. The percentages of the remaining poly(A)/PABP complexes (shown in dashed brackets) were plotted against the concentration of each recombinant AUF1 protein. One hundred percent corresponds to the amount of poly(A)/PABP complex formed from the mixture of 1 nM of ³²P-labeled GC-UTR(A+) and 50 nM of his₆-PABP (lane 2) in the absence of recombinant AUF1 proteins. The results are summarized in the graph at the bottom of the panel. Representative of three independent experiments.

Fig.7. AUF1 is an abundant protein in HeLa cytoplasm and nucleus.

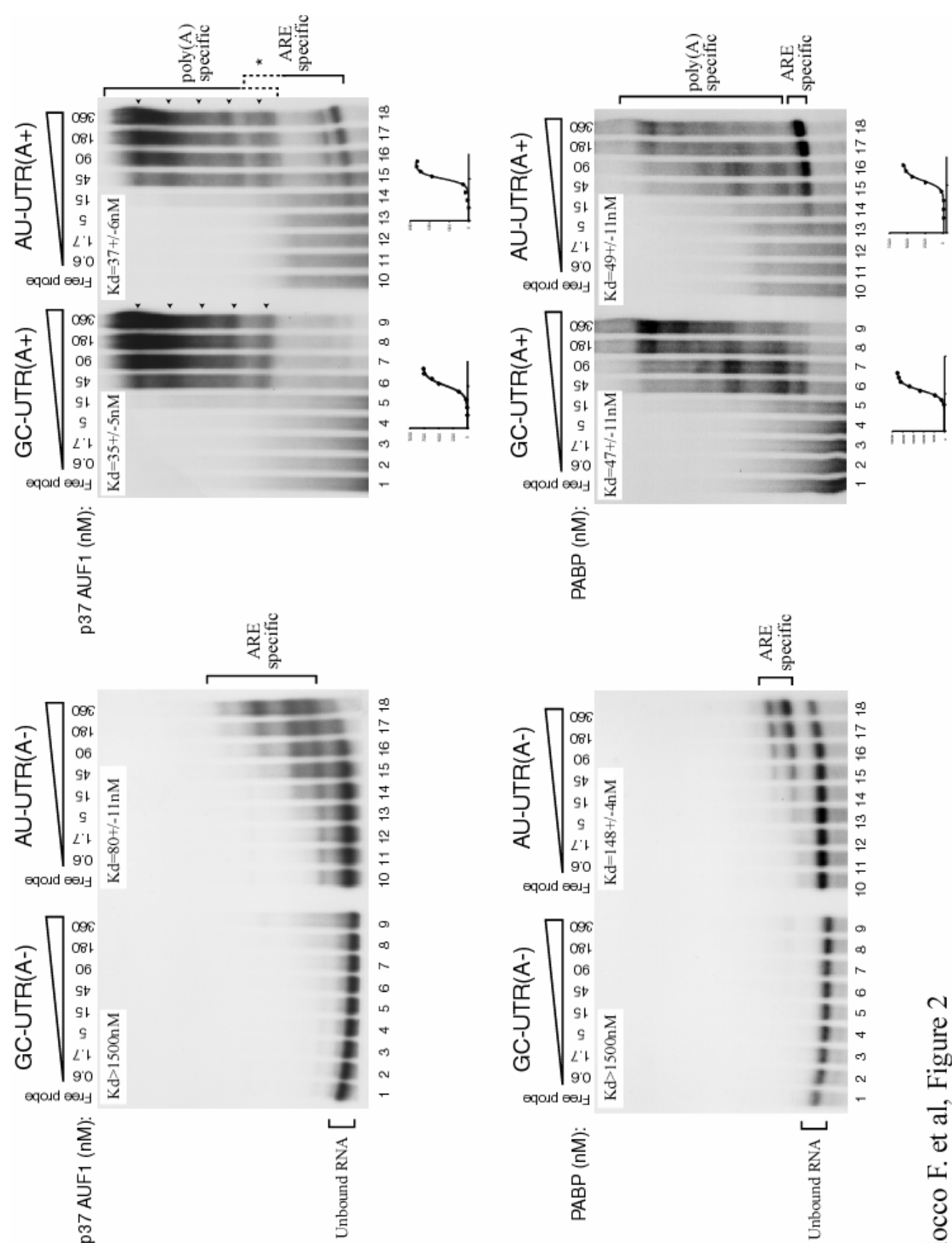
Panel A: Twenty µg of cytoplasmic extracts (CE) and 5 µg of nuclear extracts (NE) from three distinct preparations were resolved on a 10% polyacrylamide gel and analysed by western blotting using either an anti- α -Tubulin or anti-U1 snRNP70k antibody as indicated. Panel B: Various amounts of HeLa cytoplasmic proteins (CE, lanes 1 to 4, indicated in µg), his₆-PABP (lanes 5 to 9, top panel, indicated in ng) or his₆-p40^{AUF1} (lanes 5 to 9, bottom panel, indicated in ng) were resolved on an 8% polyacrylamide gel and analysed by western blotting using either an anti-PABP (upper panel) or anti-AUF1 (bottom panel) antibody. Panel C: The table summarizes the results from independent experiments (number as indicated) using the three distinct HeLa cytoplasmic extracts shown in Panel A. Results are expressed in either ng or fmol of corresponding protein per µg of HeLa cytoplasmic extract \pm standard deviation. Panel D: Various amounts of HeLa nuclear proteins (NE, lanes 1 to 3, indicated in µg) and cytoplasmic proteins (CE, lane 4, indicated in µg), his₆-PABP (lanes 5 to 9, top panel, indicated in ng) or his₆-p40^{AUF1} (lanes 5 to 9, bottom panel, indicated in ng) were resolved on an 8% polyacrylamide gel and analysed by western blotting using either an anti-PABP (upper panel) or anti-AUF1 (bottom panel) antibody. Panel E: The table summarizes the results from independent experiments (number as indicated) using the three distinct HeLa nuclear extracts shown in Panel A. Results are expressed in either ng or fmol of corresponding protein per µg of HeLa cytoplasmic extract \pm standard deviation.



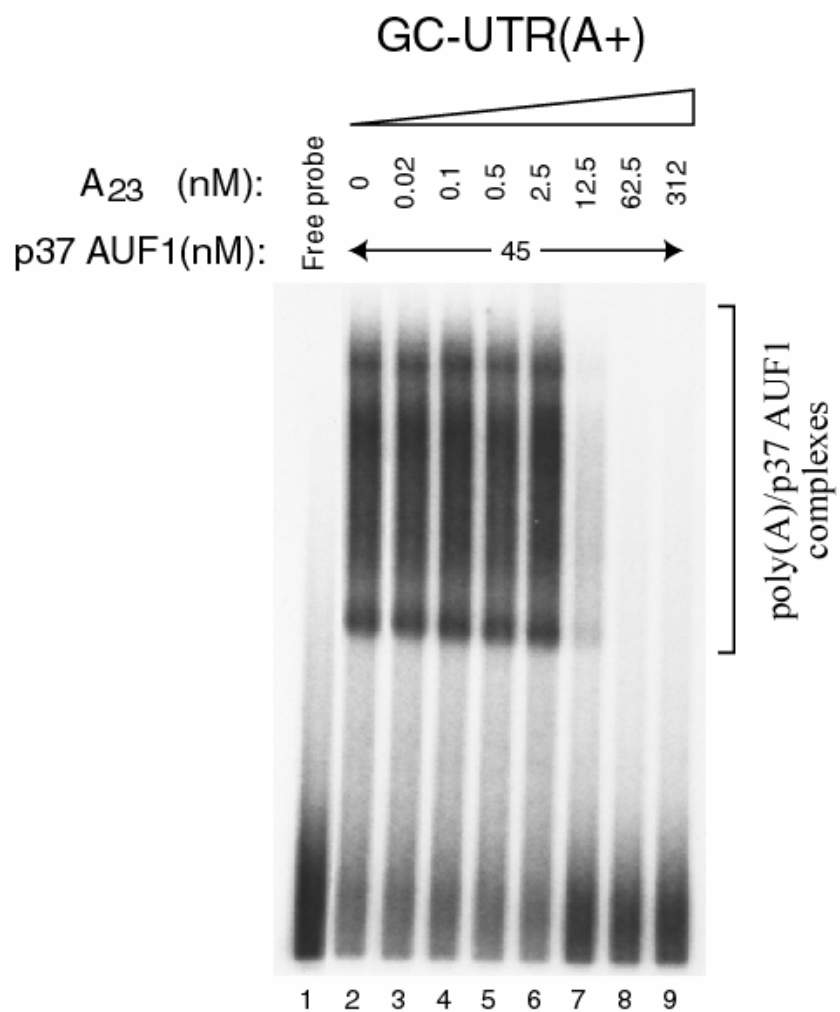
Sagliocco F. et al, Figure 1



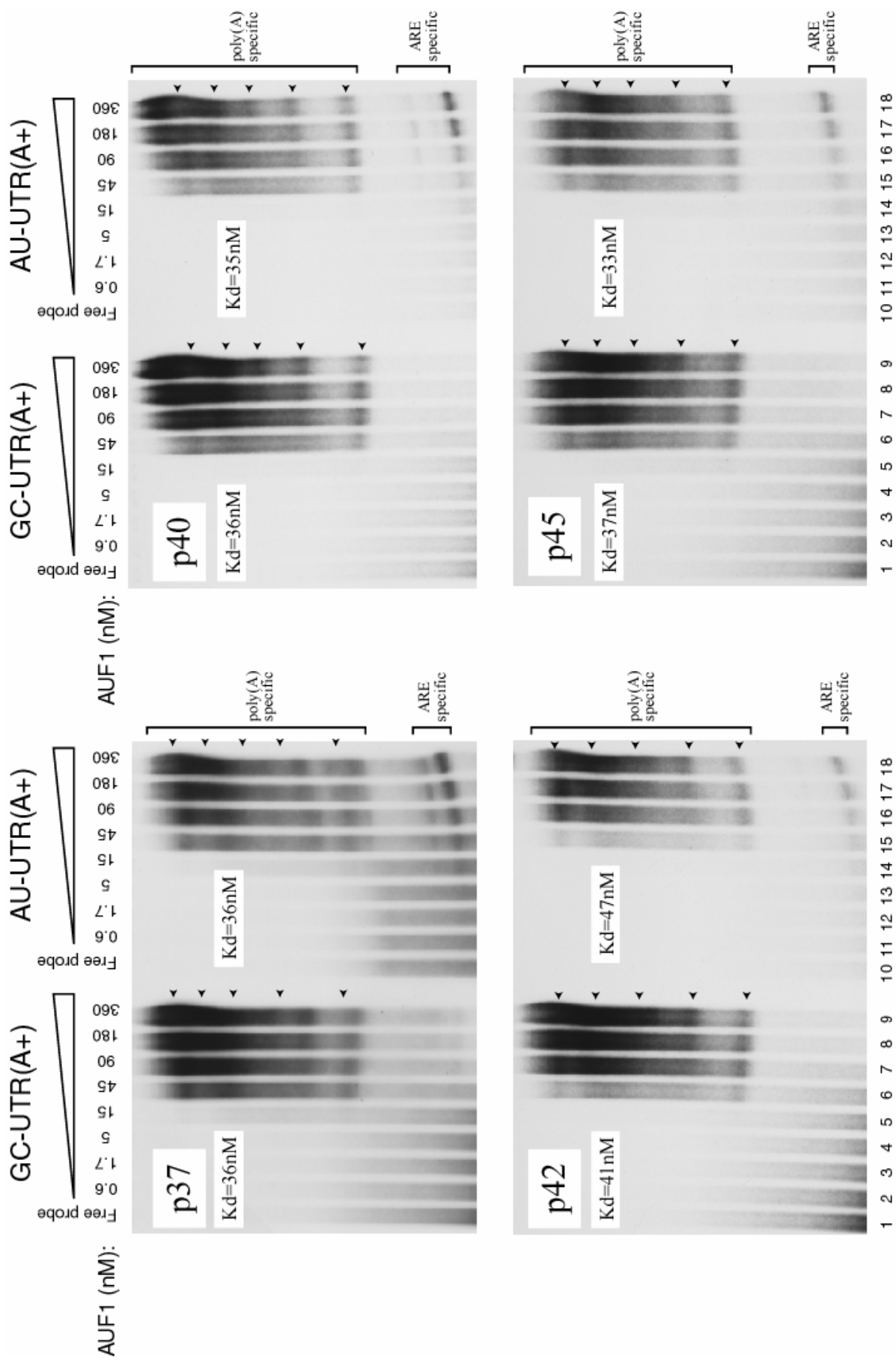
Sagliocco F. et al, Figure 1



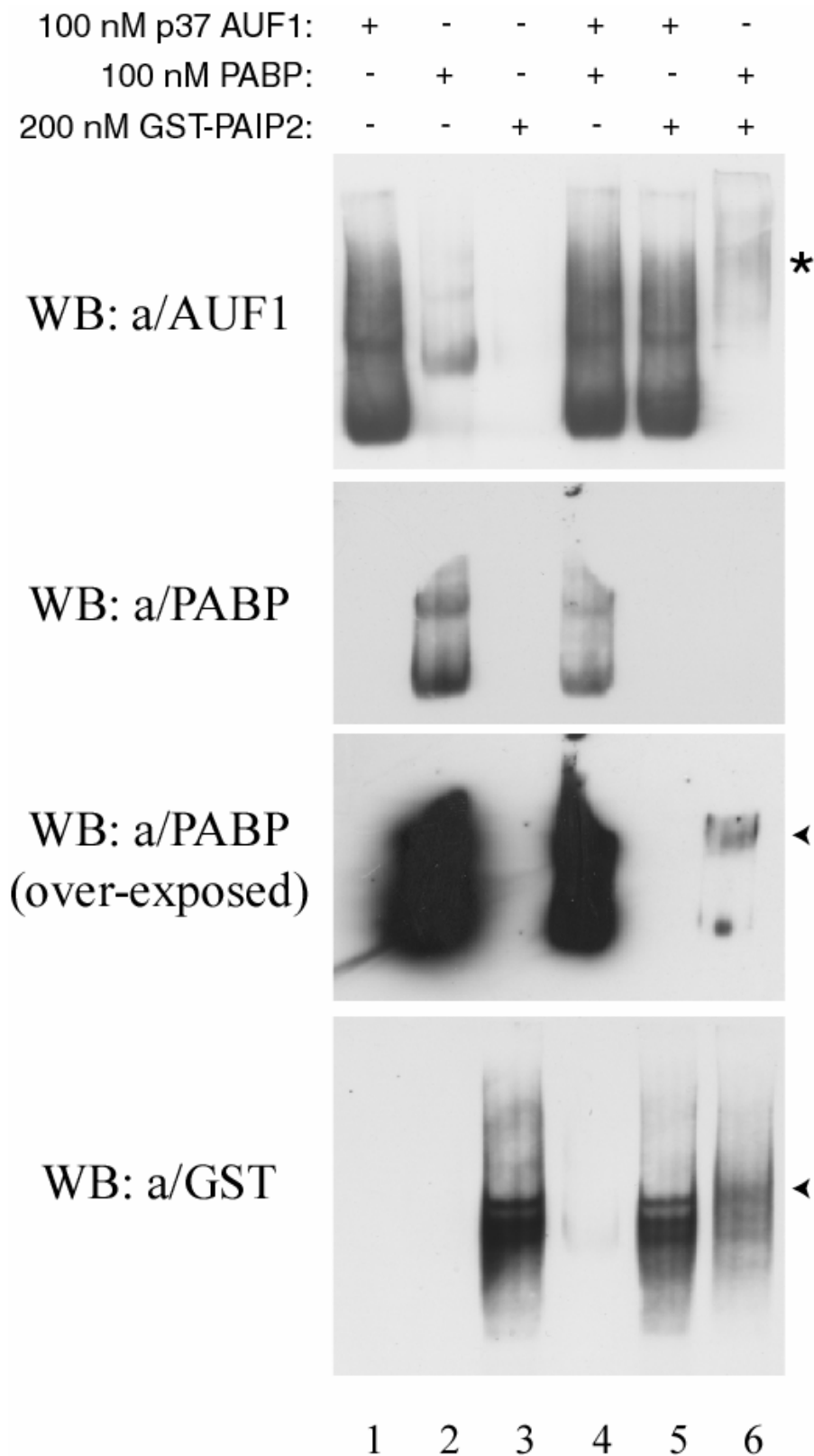
Sagliocco F. et al, Figure 2



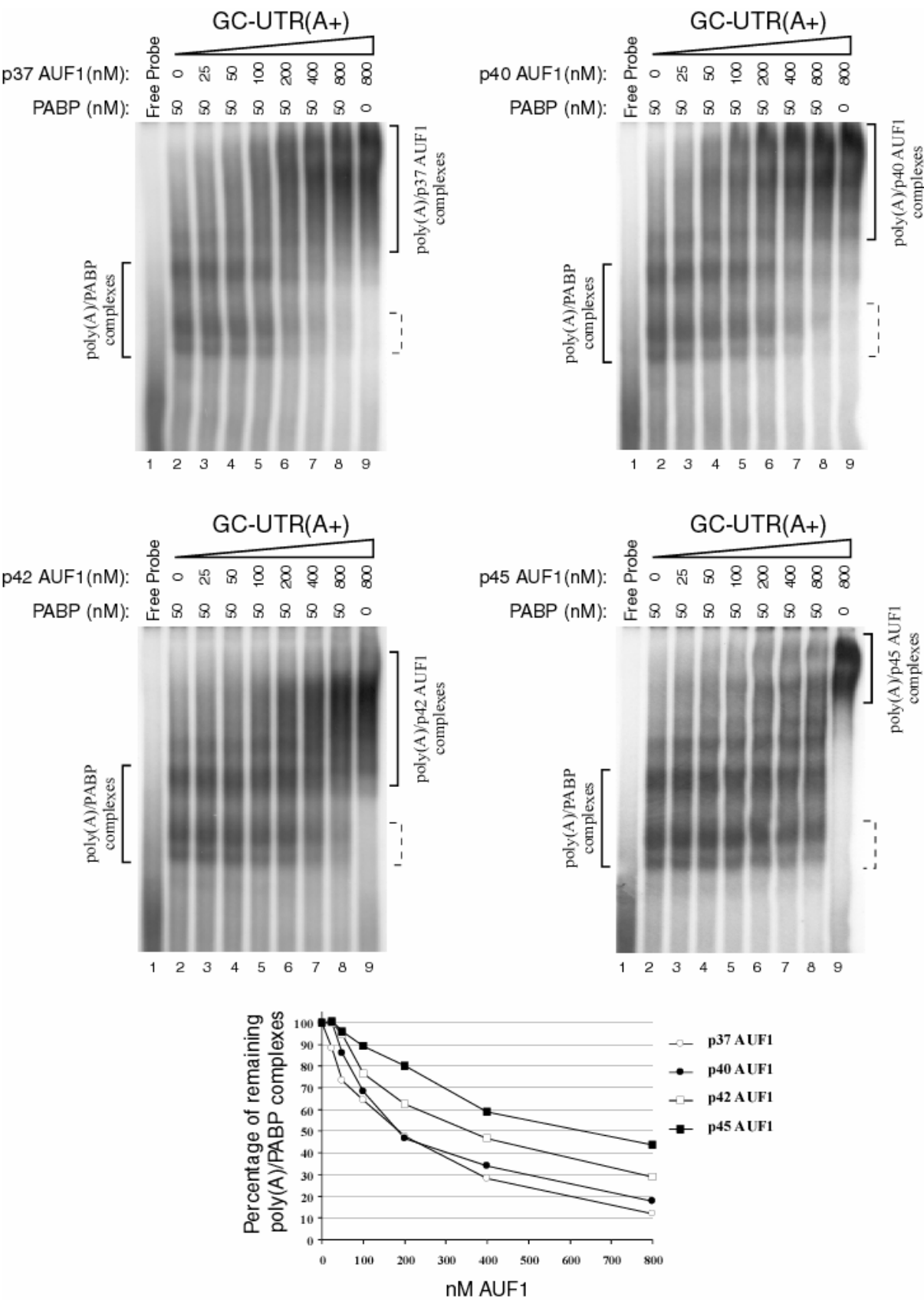
Sagliocco F. et al, Figure 3



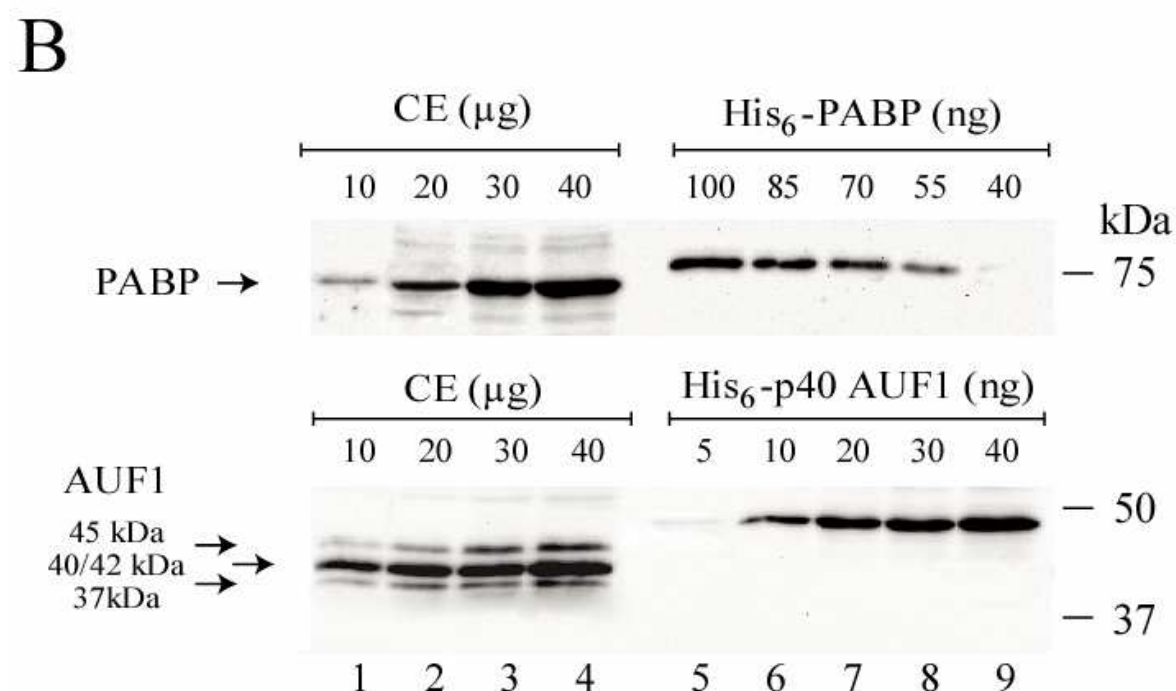
Sagliocco F. et al, Figure 4



Sagliocco F. et al, Figure 5



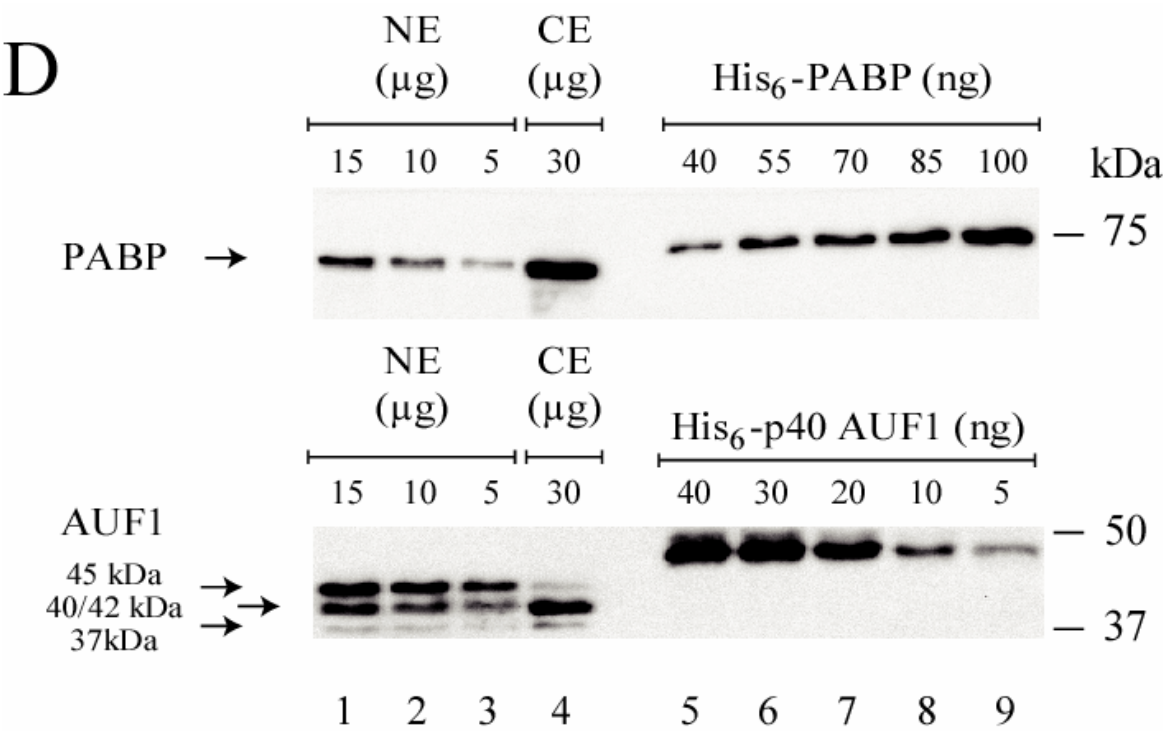
Sagliocco F. et al, Figure 6



C

| Cytoplasm | AUF1 (n=15) | | | | PABP (n=10) | |
|------------------|-------------|-------------|-------------|-------------|-------------|--------------------------|
| | p37 | p40/42 | p45 | all | | |
| ng/μg CE +/-SD | 0.24+/-0.24 | 0.94+/-0.78 | 0.13+/-0.13 | 1.31+/-1.11 | 4.45+/-2.26 | Molar ratio PABP/AUF1 |
| fmol/μg CE +/-SD | 6.4+/-6.4 | 22.9+/-19.1 | 3.0+/-2.8 | 32.3+/-26.9 | 61.8+/-31.4 | 1.91 |

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E

| Nuclear | AUF1 (n=6) | | | | PABP (n=6) | |
|------------------|------------|-------------|--------------|--------------|--------------|-----------------------|
| | p37 | p40/42 | p45 | all | | |
| ng/μg NE +/-SD | 0.49+/-0.1 | 0.98+/-0.33 | 1.14+/-0.22 | 2.61+/-0.60 | 4.04+/-0.61 | Molar ratio PABP/AUF1 |
| fmol/μg NE +/-SD | 13.2+/-2.9 | 23.98+/-8.1 | 25.35+/-4.94 | 62.53+/-14.6 | 56.06+/-8.56 | 0.90 |

Sagliocco F. et al, Figure 7