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Research Article

CUG-BP and 3'UTR sequences influence PARN-mediated deadenylation in mammalian cell extracts

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Abstract

Several mRNAs have been shown to exhibit distinct patterns of poly(A) shortening prior to their decay *in vivo*. In this study, we show that individual transcripts also demonstrate distinct patterns of deadenylation in *in vitro* systems derived from HeLa and Jurkat T cell cytoplasmic extracts. The major patterns observed were slow/synchronous and fast/asynchronous poly(A) tail shortening. For all RNA substrates tested, PARN was shown to be the enzyme responsible for the deadenylation patterns that were observed. Sequences in the 3' untranslated regions influenced the deadenylation pattern. Using a fragment of the 3'UTR of the c-fos mRNA as a model, the interaction of CUG-BP, the human homolog of EDEN-BP - a protein previously implicated in regulated deadenylation in *Xenopus* oocytes - was shown to be associated with changes in PARN-mediated deadenylation patterns. Our results suggest that association of CUG-BP with 3'UTR sequences can modulate the activity of the PARN deadenylase in mammalian cell extracts.

Key words: mRNA decay, deadenylation, mRNA stability, 3'UTR, CUG-BP.

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Introduction

The stability of an mRNA plays an important role in the regulation of gene expression (Wilusz and Wilusz, 2004; Audic and Hartley, 2004; Garcia-Martinez *et al.*, 2004). The major pathways of mRNA decay in eukaryotic cells initiate with the shortening of the 3' poly(A) tail (Meyer *et al.*, 2004; Wilson and Treisman, 1988). Following deadenylation, the body of the transcript is effectively degraded by either decapping/5'-to-3'exonuclease action or by a complex of 3'-to-5' exonucleases (Butler, 2002; Coller and Parker 2004). Elements that regulate the efficiency of mRNA turnover in response to growth, differentiation, environmental stimuli, etc. are often, but not exclusively (*e.g.* Lemm and Ross, 2002; Hughes and Brady, 2005), found in the 3' untranslated region (UTR) of the transcript (Chen *et al.*, 1995). Among them, the best described is the AU-rich element which plays a role in regulating the level of a wide variety of transcripts in multiple eukaryotes (Chen and Shyu, 1995; Guhaniyogi and Brewer, 2001; Raghaven *et al.*, 2004).

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Deadenylation is an attractive mechanism for regulation since it is the first step in the majority of mRNA decay. Numerous candidate deadenylases have been identified to date in mammalian cells. PARN (**P**oly(**A**)-specific 3'-to-5' exo**R**ibo**N**uclease), whose activity is readily demonstrable in extracts (Korner and Wahle, 1997; Korner *et al.* 1998), exhibits an interesting interaction with the 5' cap of transcripts that influences its processivity (Dehlin *et al.*, 2000; Gao *et al.*, 2000; Martinez *et al.*, 2001). In addition, PARN co-purifies with several components of the mRNA decay machinery (Lejeune *et al.*, 2003). At least six homologs of CCR4, a major yeast deadenylase, can be identified in mammalian genomes (Dupressoir *et al.*, 2001). Vertebrate CCR4 isoforms have been shown to co-purify with other mRNA decay components (Collart and Timmers, 2004; Cougot *et al.*, 2004) and one, nocturnin, shows an interesting pattern of gene expression related to circadian rhythms (Baggs and Green, 2003). In addition, Shyu and co-workers recently targeted a human CCR4 isoform with siRNA and demonstrated an effect on the overall decay rate of a reporter mRNA containing a coding region instability element (Yamashita *et al.* 2005). POP2/CAF1 constitutes the third class of candidate deadenylases in human cells. These are found in complex with CCR4 and a mammalian isoform has been shown to possess poly(A) tail shortening activity *in vitro* (Clark *et al.*, 2004; Viswanathan *et al.*, 2004).

Finally, a human homolog of the yeast Pan2/3 deadenylase, which plays a role in poly(A) tail remodeling/length control in the nucleus (Brown and Sachs, 1998), has also recently been described (Dunn *et al.*, 2005; Uchida *et al.*, 2004). Each of these deadenylases is likely to play some role in mRNA metabolism. It will be very important, therefore, to determine the relative roles played by each of these enzymes and assess how the cell regulates their activity.

Relatively little is known about what regulates deadenylase activity in mammalian cells. As mentioned above, PARN interaction with the 5' cap of a transcript stimulates the processivity of poly(A) tail shortening (Dehlin *et al.*, 2000; Gao *et al.*, 2000; Martinez *et al.*, 2001). The availability of the 5' cap, therefore, presents an attractive means of networking mRNA decay and translation. In addition to cap-binding factors, several other proteins have been shown to influence deadenylation (Gherzi *et al.*, 2004; Lai *et al.*, 2003; Tran *et al.*, 2004). Notably, EDEN-BP (Embryo Deadenylation Element-Binding Protein) interacts with a UR-rich motif in the 3'UTR of *Xenopus* mRNAs and triggers their deadenylation, perhaps by PARN, in developing embryos (Bonnet-Corven *et al.*, 2002; Paillard *et al.*, 1998; Takahashi *et al.*, 2000). EDEN-BP, therefore, plays a vital role in the dynamics of gene expression patterns that are observed after oocyte fertilization (Gautier-Courteille *et al.*, 2004). Homologs to EDEN-BP can be identified in numerous eukaryotes including humans, mice, zebrafish, *Drosophila* and *C. elegans*, suggesting that it likely plays a key role in somitic gene expression throughout evolution (Delaunay *et al.*, 2004). The human homolog, CUG-BP (CUG triplet repeat Binding Protein), was first identified as a protein that can interact with a triplet repeat expansion observed in a type of myotonic dystrophy (Timchenko *et al.*, 1996). CUG-BP is localized to both the nucleus and the cytoplasm (Mukhopadhyay *et al.*, 2003; Roberts et at., 1997) and can functionally replace EDEN-BP to promote deadenylation in *Xenopus* embryo extracts (Paillard *et al.*, 2003). Furthermore, the human c-jun mRNA can be rapidly deadenylated by an EDEN-BP specific pathway upon injection into *Xenopus* embryos (Paillard *et al.*, 2002). This conservation of key sequence elements and protein domains, therefore, makes it very likely that CUG-BP regulates poly(A) tail shortening in mammalian cells. Consistent with this prediction, we have recently observed that CUG-BP interacts directly with the PARN deadenylase in human cell extracts and can stimulate poly(A) shortening by this enzyme (Moraes *et al.*, 2006).

We have previously developed an *in vitro* assay using cytoplasmic S100 extracts that faithfully reproduces numerous *in vivo* aspects of mRNA decay (Ford *et al.*, 1999; Gao *et al.*, 2001; Mukherjee *et al.*, 2002). By testing model RNA substrates, deadenylation in HeLa cytoplasmic extracts was shown to be predominantly if not exclusively performed by PARN and could be significantly stimulated by the presence of an AU-rich element derived from the TNFα mRNA (Ford *et al.*, 1999; Gao *et al.* 2000). Whether PARN is the major deadenylase in other cell extracts, how its activity is regulated, or whether it may only act on a subset of mRNA substrates, remain to be determined.

In this study we demonstrate that RNA substrates containing various types of AU-rich elements display at least three distinct patterns of deadenylation in HeLa and Jurkat T cell extracts. Furthermore, regulation of poly(A) tail shortening can be demonstrated in T cell extracts in response to activation, suggesting that the *in vitro* assays replicate several *in vivo* observations with regard to deadenylation. *In vitro* deadenylation assays, therefore, are likely to yield relevant clues to the regulation of poly(A) tail shortening. PARN was shown to be the enzyme responsible for the deadenylation observed *in vitro* in all cases. While AU-rich elements clearly contribute to the deadenylation patterns observed, additional sequences in the 3'UTR were also shown to make a significant contribution. Finally, UV cross-linking analyses with RNA substrates containing variations of the c-Fos 3'UTR clearly implicate CUG-BP as a regulator of PARN activity in mammalian cell extracts. These data suggest a key role for a 3'UTR element and its associated RNA binding protein(s) in regulating deadenylation by the PARN enzyme.

Materials and Methods

Plasmid and DNA template constructions

pGEM-4 plasmids containing 250 bp inserts of the 3'UTR surrounding the ARE elements of representative members of several different subclasses of AU-rich elements were generated by RT-PCR, using RNA prepared from HeLa cells and specific primers for each construction. The primers for cFos-ARE were 5'CCGGAATTCTCTT CAACATCAATGTTCATTGTAATG and 5'CGATCTG CAGT TTATTGACAATGTCTTGGAACAATAAGC; TNFα-ARE were 5'CCGGAATTCTTAGGCCTTCCTC TCTCCAGATGTTTCCAG and 5'CGATCTGCAGACA TGGGAACAGCCTATTGTTCAGCTCC; CYTOR4-ARE were 5'CCGGAATTCGCAGAGAATGAGCTGCAGTC CAG and 5'CCAAGCTTATCTTACAAAAATATCATA CTTGC. The primers for cjun-ARE amplified a 250 bp fragment containing 50 bases upstream and downstream of the ~150 base Class III ARE. The reactions were performed according to the standard conditions described by the supplier (SuperScript™ One-Step RT-PCR with Platinum *Taq*, Invitrogen). To generate pFosARE and pTNFαARE, products from RT-PCR were digested with EcoRI and PstI and cloned into pGEM®-4 (Promega). To generate pCYTOR4ARE, the amplified DNA product was digested with EcoRI and HindIII restriction enzymes and then cloned into pGEM®-4.

DNA fragments used to prepare the ARE deletion constructs cFosΔAREwere generated by a two step PCR re-

action. In the first step, PCR reactions (performed under standard conditions) were done using the three plasmids described above as templates and the following primers: cFosΔARE - sense primer 5'AACAGTTTTCCATGAAA ACGTACCTTGAGGTCTTTTGAC and anti-sense 5'TC AAAAGACCTCAAGGTAGCGTTTTCATGGAAAACT GTT; Final products were cleaved and cloned into pGEM®-4 using the same sites discussed above for the parent constructs.

The pFosARE plasmid described above served as the template to prepare a set of constructs containing defined deletions of 25 base segments. The pFos A construct was constructed using the primer 5'CCGGAATTCCTGATC ATGCATTGTTGAG along with the 3' antisense cFos primer used in the construction of the parent plasmid described above by PCR. The pFos F construct was constructed using the 5' sense primer described above for the parent plasmid along with the primer 5'TCGATCTGCA GAACAATGCTTAAATTTTTCATTC in a PCR reaction. The products were digested with EcoRI and PstI and cloned into pGEM®-4.

The plasmid pFos B was prepared by two rounds of PCR. The first reaction contained the primers 5'AATGTT CATTGTAATGTTAGTTCTGACATTAACAGTTTTC and 5'CGATCTGCAGTTTATTGACAATGTCTTGGAACA ATAAGC. The second reaction was performed using the DNA product from first reaction as a template along with the primers 5'CCGGAATTCTCTTCAACATCAATGTT CATTGTAATG and 5'CGATCTGCAGTTTATTGACA ATGTCTTGGAACAATAAGC. The final product was digested with EcoRI and PstI for cloning into pGem4.

Deletion clones pFosC and pFosE were created by PCR utilizing overlapping primers that lacked specific sequences in a strategy similar to the ΔARE described above. Reaction 1 contained the primer 5'CCGGAATTCTCT TCAACATCAATGTTCATTGTAATG and a construct-specific antisense primer (pFosC - 5'ATTAAAAACAC AATAAAAATTCAGACCACCTCAACAATGC or pFosE 5'TCGATCTGCAGAACAATGCTTAAATTTTTCATT C). Reaction 2 contained the primer 5'CGATCTGCAG TTTATTGACAATGTCTTGGAACAATAAGC and a construct-specific sense primer (pFosC: 5'ATTGTTGA GGTGGTCTGAATTTTTATTGTGTTTTTAATT or pFosE: 5'ATGTCTTGGAACAATAAGCACTTTCCACATGTC AAAAGAC). PCR products were combined and amplified using the common sense and antisense primers listed above. Reaction products were digested with EcoRI and PstI and cloned into pGEM®-4.

Deletion construct pFos D was prepared by a similar two step PCR reaction as described for the two previous constructs. The unique primers were 5'GATCGTTTAAA AAAATAAAATAAAAATATAAATATCTGAG for Reaction 1 and 5'TGAATTTGAATGAAAAATTTAAGCA TTG for Reaction 2. The DNA product from reaction 1 was digested with Dra I and EcoRI. And the product from reaction 2 was performed was digested with PstI. The restricted DNA fragments were combined and amplified in a standard PCR reaction using 5'CCGGAATTCTCTTCAACATCA ATGTTCATTGTAATG and 5'CGATCTGCAGTTTAT TGACAATGTCTTGGAACAATAAGC. The product from this reaction digested with EcoRI and PstI and cloned into pGEM®-4.

In order to create DNA templates that would yield an RNA with a 60 base poly(A) tail after *in vitro* transcription, plasmids were linearized using HindIII and sequences encoding a 60 base poly(A) tail were added to DNA using a ligation-PCR approach previously described (Ford *et al.* 1999).

All clones were sequenced prior to being used for *in vitro* transcription.

In vitro transcription and RNA purification

RNAs were transcribed *in vitro* using SP6 RNA polymerase and purified on denaturing acrylamide gels. Transcripts were capped co-transcriptionally by the addition of 5 mM ^{7me} GpppG in the transcription reaction.

Preparation of cell extracts

Cytoplasmic extracts were prepared from HeLa and Jurkat T cells as described previously (Ford and Wilusz, 1999). HeLa cells were grown in spinner culture in 5% horse serum. T cells were grown in 175 cm^2 flasks in RPMI 1640 and induced using 15 ng/mL TPA and 1 μg/mL A23187 or treated with control vehicle DMSO solution.

In vitro mRNA decay/ deadenylation assay

Polyadenylated RNAs radiolabeled internally at U-residues were incubated in HeLa S100 cytoplasmic extracts or Jurkat T cell extracts at 30 °C as described previously (Ford and Wilusz, 1999). Reaction products were analyzed on 4% polyacrylamide gels containing 7 M urea.

Immunodepletions

Hela and Jurkat T cell cytoplasmic extracts were immunodepleted using the same conditions previously described (Gao *et al.*, 2000) using either control rabbit serum or an anti-PARN polyclonal antibody kindly provided by Dr. Michael Wormington. Briefly, immunodepletions were performed using 3 μL of antisera and 1 μL of RNAsin. Immune complexes were precipitated using Protein A sepharose and depleted extracts were used in the *in vitro* mRNA decay system described above. Western blotting experiments were performed in order to verify PARN immunodepletion of > 95% (data not shown).

UV cross linking/ Immunopreciptation/ Competition assays

UV cross-linking analyses were performed as described (Ford *et al.*, 1999). Cross-linking assays were done in the presence of 25 mM EDTA to inhibit RNA turnover

and allow for accurate comparisons between samples. After digestion with RNase A, cross-linked proteins were analyzed on 10% acrylamide gels containing SDS.

For immunoprecipitation analysis following UV cross-linking, 400 µL of Net2 buffer (50 mM Tris [pH 7.6], 75 mM NaCl, 0.05% Nonidet P-40) was added to samples and mixtures were centrifuged for 2 min to remove precipated/aggregated proteins. Pre-cleared samples were incubated at 4 °C with antibodies under gently shaking conditions for 3 h. Antigen-antibody complexes were collected on protein A-positive *Staphylococcus aureus* cells and washed five times with Net2 buffer. The immunoprecipitated cross-linked proteins were separated on a 10% acrylamide gel containing SDS. Polyclonal antisera specific for CUG-BP were the kind gift of Dr. Maurice Swanson.

Results and Discussion

Different mRNAs have been shown to have distinct deadenylation kinetics *in vivo* (Chen *et al.*, 1995; Xu *et al.*, 1997). The Class II ARE-containing GM-CSF and TNF-α mRNAs, for example, demonstrate asynchronous cytoplasmic deadenylation which is consistent with processive activity of the poly(A) shortening enzyme. Class I (c-fos) and Class III (c-jun), on the other hand, show synchronous poly(A) shortening, consistent with distributive deadenylation kinetics. Other transcripts bear poly(A) tails that are very stable and refractory to deadenylation. We wished to assess whether we could reproduce deadenylation patterns in cytoplasmic extracts that were consistent with these *in vivo* observations.

Capped and polyadenylated substrates containing a 250 base fragment of the 3'UTR surrounding the ARE of members of the seven proposed classes of ARE elements (Garneau *et al.*, 2007) were incubated in HeLa cytoplasmic S100 extracts in our *in vitro* mRNA decay system. As seen in Figure 1A and B, three patterns of deadenylation were observed. The cFos-A60 RNA substrate was deadenylated in a slow and synchronous manner. A similar slow and synchronous pattern was observed with a c-jun-A60 RNA substrate (Figure 1A, bottom right panel). This is very consistent with the kinetics of poly(A) shortening of Class I (c-fos) and Class III (c-jun) ARE-containing transcripts *in vivo* (Chen *et al.*, 1995; Xu *et al.*, 1997) and suggestive of a distributive mechanism of deadenylation. Just as was observed *in vivo* (Chen *et al.*, 1995; Xu *et al.*, 1997), the Class IIA ARE containing TNFα-A60 RNA substrate was deadenylated in a fast and asynchronous manner *in vitro*. This is suggestive of a processive mechanism of poly(A) shortening on this transcript. Finally, RNA substrates such as the Class IIE ARE containing CYTOR4-A60 RNA in Figure 1 exhibited a pattern of no or very little deadenylation *in vitro* (CYTOR-4 is cytokine receptor related protein-4 (Genbank accession no. AF046059)). We conclude that deadenylation patterns in HeLa cytoplasmic extracts are RNA substrate-dependent and generally fall into three categories - fast/asynchronous, slow/synchronous, and no poly(A) shortening. Furthermore, these appear to faithfully reproduce patterns that have been observed *in vivo*.

Independent deadenylase enzymes could contribute to the differential deadenylation patterns discussed above. As mentioned above numerous candidate deadenylase enzymes have been identified in mammalian cells including, for example, PARN, CCR4, Caf1/POP2 and Pan2/3 (Fritz *et al.*, 2003). We have previously shown that PARN was the major deadenylase in HeLa cytoplasmic extracts for RNA substrates containing the TNFα ARE and a polylinker-derived transcript (Gao *et al.*, 2000), but had not tested other RNAs. We wished to determine, therefore, whether PARN was the major deadenylase for other RNA substrates or if there were, perhaps, transcript-specific deadenylases that were active *in vitro*. The role of PARN in the poly(A) shortening of RNA substrates that display slow/synchronous or fast/asynchronous patterns of deadenylation was investigated in HeLa cytoplasmic extracts using an immunodepletion approach. As seen in Figure 1C, while immunodepletion of HeLa extracts with control sera had no or minimal effects, depletion of ~95% of PARN protein (as determined by western blotting (Gao *et al.*, 2000; data not shown) blocked most of the poly(A) shortening observed on either the cFos-A60 or TNFα-A60 RNA substrates. Addition of recombinant PARN protein to extracts immunodepleted with PARN antisera effectively restored deadenylation. Similar observations were made using Jurkat T cell extracts (Figure 1D). The deadenylation of all RNA substrates that we have tested to date is essentially blocked in cytoplasmic extracts that have been immunodepleted of PARN protein or have PARN activity sequestered by the addition of cap analog to the reactions (Gao *et al.* 2000). We conclude, therefore, that at least in cytoplasmic extracts of HeLa and Jurkat T cells, if not most cells, PARN is the major enzyme responsible for poly(A) shortening. The activity of PARN, therefore, must be regulated to account for the differences in deadenylation patterns that were observed *in vitro*.

We next wished to test whether deadenylation patterns observed with HeLa cytoplasmic extracts could be reproduced in extracts from other cell types. Furthermore, we wished to assess whether *in vitro* deadenylation patterns could be altered to reflect regulatory changes that occur *in vivo*. Jurkat cells, an immortalized T cell line, can be stimulated by phorbol esters to produce IL2 and are widely used to study the molecular responses associated with T cell activation. Jurkat T cells were grown under normal uninduced conditions or activated by the addition of TPA (12-O-tetradecanoylphorbol-13-acetate)/A23187 (a calcium ionophore) and cytoplasmic S100 extracts were prepared. As seen in Figure 2, the patterns of deadenylation of cFos-A60, TNFα-A60 and CYTOR-4-A60 RNA substrates in extracts

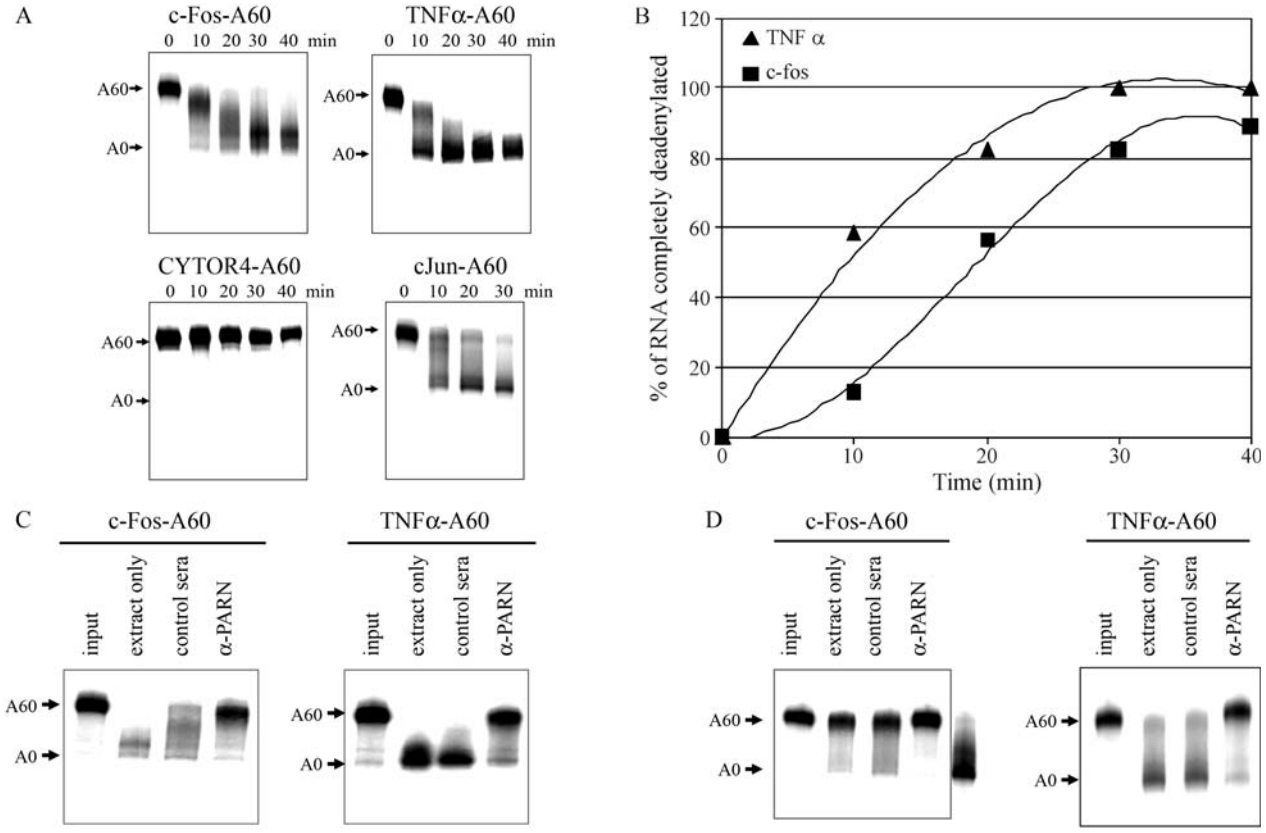

Figure 1 - Three main patterns of deadenylation can be observed on RNA substrates that contain AU-rich elements in HeLa extracts. Panel 1A. pGEM-4 plasmids containing 250 bp inserts of the 3'UTR surrounding the ARE elements of representative members of several different subclasses of AU-rich elements were generated by RT-PCR. Capped and polyadenylated RNAs containing 250 base segments of the 3'UTR of cFos (cFos-A60), TNFα (TNFα-A60), CYTOR-4 (CYTOR4-A60) or cJun (cJun-A60) mRNA were incubated with HeLa cytoplasmic S100 extracts in an *in vitro* mRNA deadenylation/decay system for the times indicated. Reaction products were analyzed on 4% acrylamide gels containing 7 M urea and visualized by phosphorimaging. The positions of the adenylated (A60) and unadenylated (A0) RNAs are indicated to the left of each frame. Panel 1B. Graphical representation of the data obtained for cFos and TNFα in panel A. Panel C. Capped and polyadenylated RNAs containing 250 base segments of the 3'UTR of cFos (cFos-A60) or TNFα (TNFα-A60) mRNA were incubated in HeLa cytoplasmic extracts S100 extracts in an *in vitro* mRNA deadenylation/decay system for 0 time (input lanes) or 40 min (extract only lanes). Some extracts were treated with control rabbit serum (control serum lanes) or immunodepleted using PARN-specific antisera (α-PARN lanes) prior to use in the assay as previously described (Gao *et al.*, 2000). Reaction products were analyzed on 4% acrylamide gels containing 7 M urea and visualized by phosphorimaging. The positions of the adenylated (A60) and unadenylated (A0) RNAs are indicated to the left of each frame. Panel D. Same as panel C, but Jurkat cytoplasmic extracts were used instead of HeLa cytoplasmic extracts.

from untreated T cells were very similar to that observed with HeLa cells. cFos-A60 was deadenylated in a slow synchronous pattern, TNFα-A60 was deadenylated in a fast, asynchronous manner, and the poly(A) tail of the CYTOR-4-A60 RNA substrate was essentially stable. In extracts from TPA-induced T cells, the ARE-containing RNA substrates became extremely stable, and the pattern of any poly(A) shortening that was observed was very slow/synchronous. Deadenylation of a control, polylinker-derived transcript was not altered in extracts from induced Jurkat cells (data not shown). This is consistent with the stabilization of ARE-containing mRNAs that occurs in T cells upon TPA/A23187 treatment (Raghaven *et al.*, 2004). We conclude that deadenylation patterns observed in HeLa cells can be generalized to T cell extracts and perhaps other cell types as well. Furthermore, the regulation of deadenylation that can be observed *in vivo* upon T cell induction can be reproduced in cytoplasmic extracts. Therefore the study of deadenylation mechanisms *in vitro* should provide some insight into *in vivo* phenomena.

Since a fast/asynchronous pattern of deadenylation has been observed on polylinker-derived RNA substrates (Ford *et al.*, 1999; Gao *et al.*, 2000), other aspects of the c-fos 3'UTR must be contributing to the observed slow/synchronous pattern of deadenylation on this RNA substrate. Our next goal was to localize the regions responsible for the observed pattern of poly(A) shortening by PARN on this substrate.

In order to localize additional elements in the cFos RNA substrate that influence its deadenylation pattern, a series of deletion constructs were made that are diagrammed in Figure 3A. As seen in Figure 3, panels B and C, a

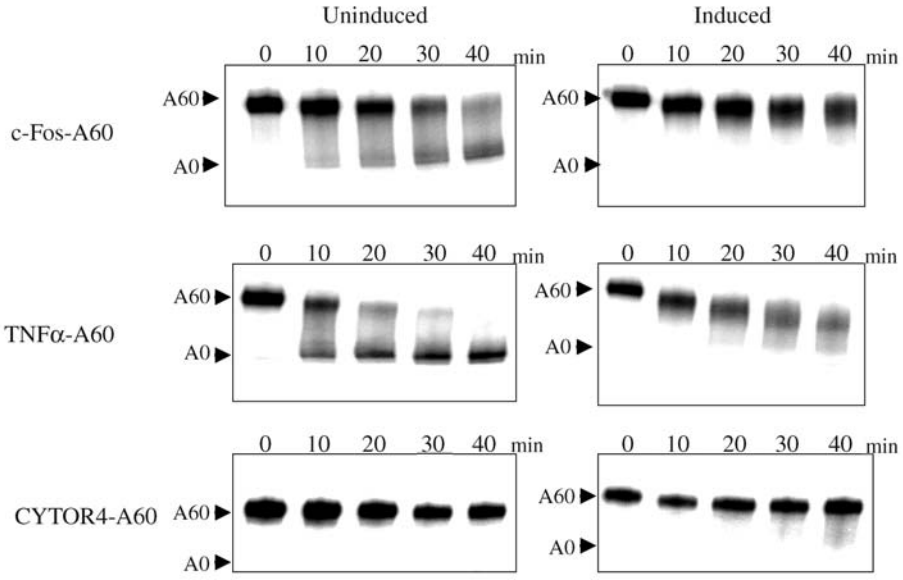

Figure 2 - Regulated patterns of deadenylation on AU-rich element-containing RNAs can also be observed in cytoplasmic extracts from Jurkat cells. Capped and polyadenylated RNAs containing 250 base segments of the 3'UTR of cFos (cFos-A60), TNFα (TNFα-A60) or CYTOR-4 (CYTOR4-A60) mRNA were incubated with Jurkat cytoplasmic S100 extracts that were made from cells treated with DMSO (uninduced panel) or with TPA and A23187 (induced panel) in an *in vitro* mRNA deadenylation/decay system (Ford *et al.* 1999) for the times indicated. Reaction products were analyzed on 4% acrylamide gels containing 7 M urea and visualized by phosphorimaging. The positions of the adenylated (A60) and unadenylated (A0) RNAs are indicated to the left of each frame.

switch from slow/synchronous to fast/asynchronous deadenylation was clearly observed with deletion variants cFos ΔB, cFos ΔC and cFos ΔE. These represent deletions of blocks of sequence both before and after the ARE element. We conclude that either a large and diffuse 3'UTR element, or multiple regions that interact in some fashion, perhaps through the formation of a secondary structure, significantly influences the deadenylation pattern of the cFos RNA.

We next wished to identify RNA binding proteins that may influence the deadenylation pattern of cFos mRNA. UV cross-linking analyses were performed on the wild type cFos-A60 RNA as well as the six variants outlined in Figure 3A. When radiolabeled cFos-A60 RNA was incubated in HeLa cytoplasmic S100 extracts, multiple cross-linked protein species were observed (Figure 4B, lane cFos). Interestingly, when the data for each of the cFos A-F variant RNAs was analyzed, a protein of ~50kDa was consistently observed to cross-link more effectively to the ΔB, ΔC and ΔE cFos variant transcripts (Figure 4B, compare lanes A-F). The cFos ΔB, ΔC and ΔE variants were precisely the same set of variants that caused the switch in a deadenylation pattern from slow/synchronous to fast/asynchronous (Figure 3B). The cross-linking of this 50 kDa protein, therefore, correlates perfectly with the switch in the pattern of PARN-mediated deadenylation associated with these three variants.

We have recently demonstrated that CUG-BP, a human homolog of the EDEN-BP protein previously implicated in regulated deadenylation in *Xenopus* embryos (Bonnet-Corven *et al.*, 2002; Paillaird *et al.*, 1998), can interact with PARN and stimulate deadenylation efficiency in HeLa cell extracts (Moraes *et al.*, 2006). Consistent with this observation, antibodies to CUG-BP precipitated the cross-linked 50 kDa protein (Figure 4C). Control sera or unrelated antibodies failed to precipitate the 50 kDa protein (data not shown). As seen in Figure 4C, the amount of cross-linked CUG-BP that was immunoprecipitated from the ΔB, ΔC and ΔE variants was also relatively higher than the other three variants. The ~two fold effect observed in these data was highly reproducible. We conclude, therefore, that an increase in CUG-BP cross-linking with our set of variant cFos-A60 RNA substrates directly correlated with a change in the deadenylation pattern from slow/synchronous to fast/asynchronous. The cFos-A60 3'UTR region contains several UGUR sequences which have been previously implicated in CUB-BP binding (Figure 4A). We hypothesize that the cFos ΔB, ΔC and ΔE deletion variants may juxtapose UGUR sites in the RNA substrate and create a more efficient CUG-BP binding region that results in more effective UV cross-linking and faster deadenylation kinetics. This model is consistent with the recent observation by Cosson *et al.* (2006) that oligomerization of the CUG-BP homolog EDEN-BP is required for efficient RNA binding and deadenylation in *Xenopus*.

In summary, the data presented above establish four interesting points concerning the process of deadenylation. First, various patterns of deadenylation can be effectively reproduced in cytoplasmic extracts, providing a valuable technical resource to gain potential mechanistic insights into the process. Second, PARN is the major deadenylase in

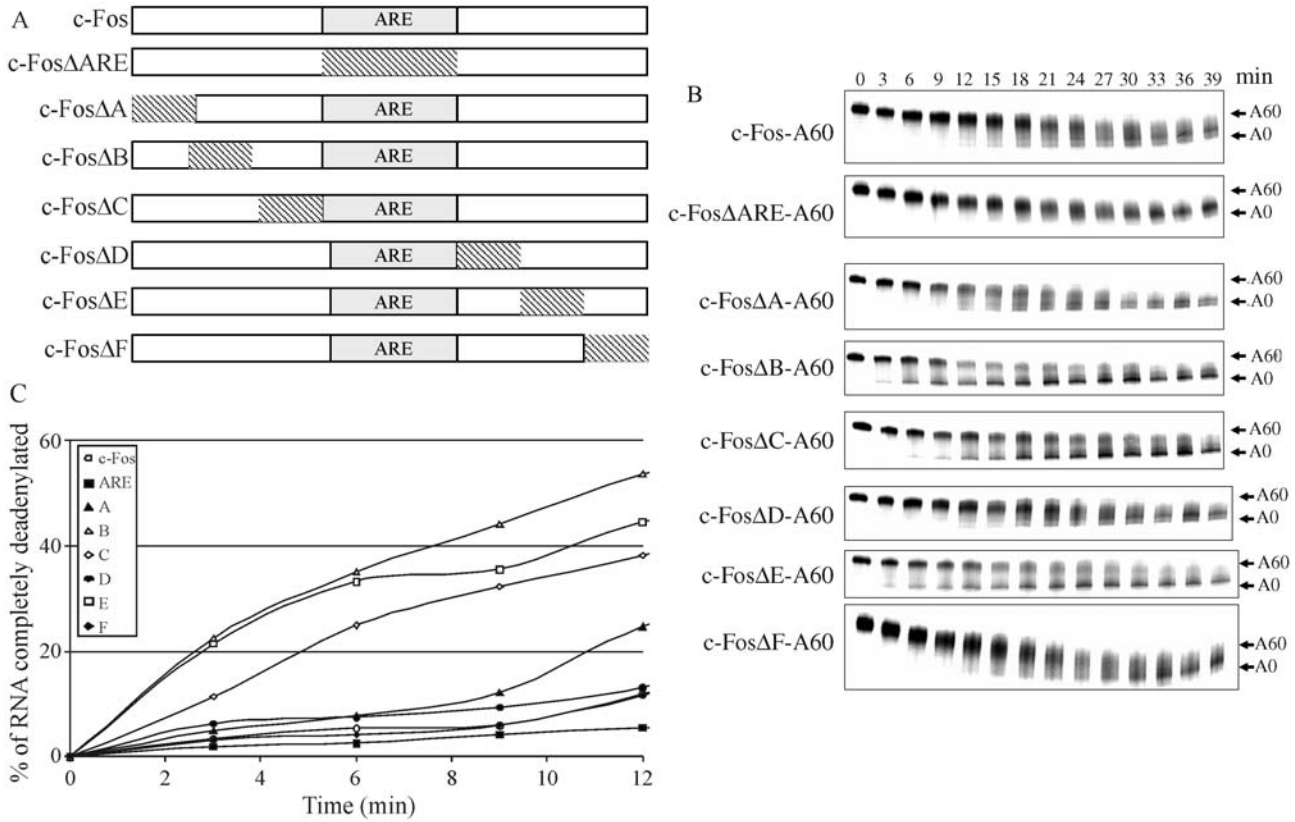

Figure 3 - Deletion of specific regions outside the AU-rich element significantly alters the deadenylation pattern of the cFos-A60 RNA substrate. Panel A. Diagrammatic representation of the RNAs used in the assays. Deletion constructs were made by a PCR approach using specific primers to generate the indicated variants. Panel B. Capped and polyadenylated RNAs containing the deletions outlined in panel A were incubated in HeLa extracts in the *in vitro* deadenylation/decay system for the times indicated. Reaction products were analyzed on 4% acrylamide gels containing 7 M urea and visualized by phosphorimaging. The positions of the adenylated (A60) and unadenylated (A0) RNAs are indicated to the left. Arrows on the left highlight the three variants that demonstrated a fast/asynchronous pattern of deadenylation. Panel C. Graphical representation of the data presented in Panel B.

all tested cytoplasmic extracts and can act on a variety of mRNA substrates - as well as be influenced by *cis*-acting elements present on the RNA substrate. Third, 3'UTR sequences, including but not limited to AU-rich elements, can regulate PARN deadenylation patterns. Finally, CUG-BP, a close homolog of a known developmental regulator of deadenylation in *Xenopus* embryos, was identified as a candidate regulator of PARN. Collectively, these observations suggest that PARN may play an important role in regulated deadenylation through functional interactions with 3'UTR elements and binding factors.

Deletion of the ARE in the cFos-A60 substrate in Figure 3B demonstrated that this element was not required for the slow/synchronous pattern of deadenylation observed for this RNA substrate. The ARE, however, may play a role in changing the deadenylation pattern of the transcript to fast/asynchronous that can occur under certain conditions. The deletion of the ARE in the TNFα-A60 RNA substrate resulted in a reproducible change in the pattern of deadenylation from fast/asynchronous to a slower/synchronous one (data not shown). The Class IIA ARE in the TNFα RNA substrate, therefore, does significantly contribute to the deadenylation pattern that is observed *in vitro*. Surprisingly, deletion of the Class IIE ARE in the CITOR4-A60 RNA substrate failed to activate deadenylation *in vitro*. In conclusion, while an ARE can clearly contribute to the fast/asynchronous deadenylation pattern as in the case of the TNFα RNA substrate, other sequences in the 3'UTR of the mRNA appear to play a significant role as well in determining the slow/synchronous pattern as well as the repression of poly(A) shortening. Therefore the ARE is only one of several contributing factors to the overall pattern of deadenylation that is observed with individual transcripts.

The model that emerges from these observations suggests that the combination of protein factors or RNA structures that assembles on the 3'UTR of an mRNA dictate how the transcript will associate with the relevant deadenylase. This assembled complex then determines the kinetics of the deadenylation process. The 3'UTR complex, along with perhaps the rate of poly(A) tail shortening, may allow the mRNA to effectively be targeted for storage and subsequent re-adenylation / translation rather than rapidly degraded by an exonucleolytic pathway. The fact that these exonucleolytic pathways also are regulated by an array of protein factors (Garneau *et al.*, 2007) suggests that the deadenylation process may serve as a gatekeeper for sev-

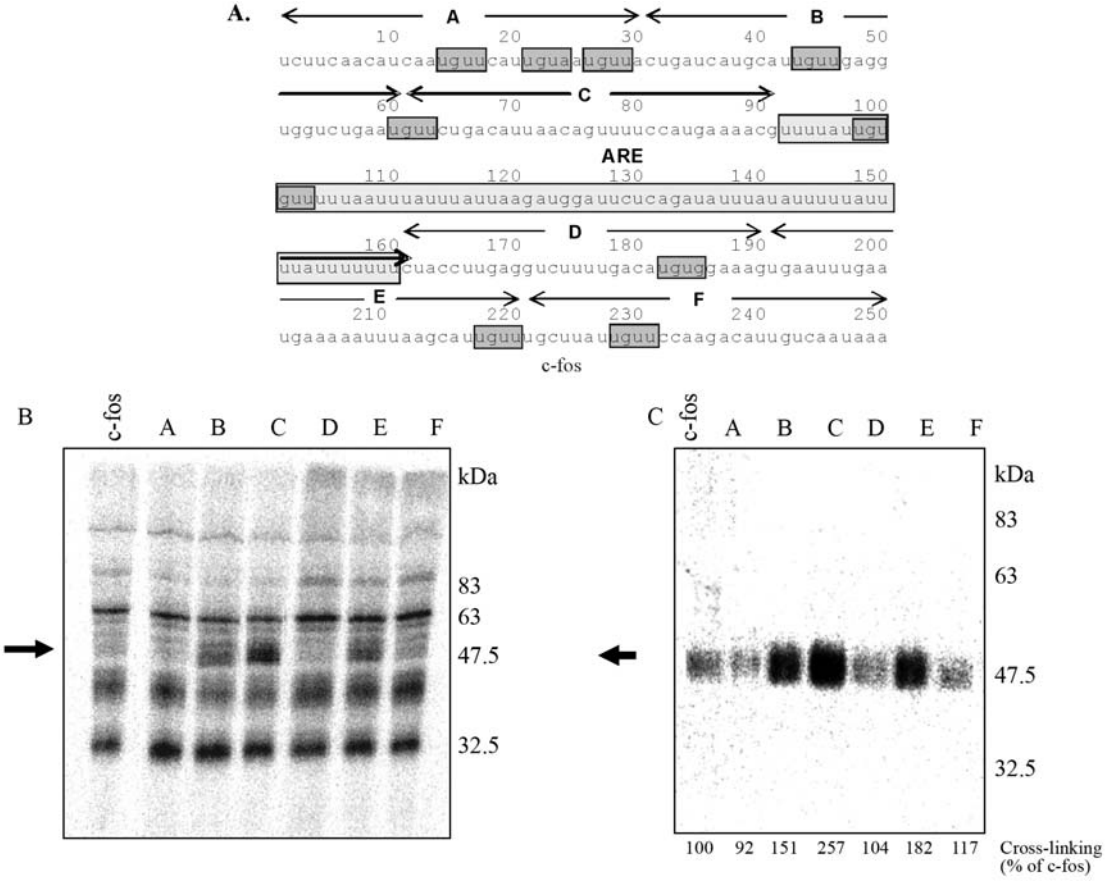

Figure 4 - The interaction of CUG-BP with the cFos RNA substrate correlates with a shift to a fast/asynchronous deadenylation pattern. Panel A. Sequence of the 250 base region of the cFos 3'UTR used in these assays. Potential UGUR and ARE CUG-BP binding sites are boxed and the regions deleted in the regions deleted in the A-F variants are indicated by arrows. Panel B. cFos-A60 RNA or the indicated deletion variants that are outlined in Figure 3A were radiolabeled at U residues and incubated with HeLa cytoplasmic extracts in the *in vitro* mRNA decay system in the presence of EDTA. Reaction mixtures were irradiated with UV light to cross-link closely associated proteins, treated with RNase A and cross-linked proteins were analyzed on a 10% SDS gel and visualized by phosphorimaging. The arrow at the left indicates a ~50 kDa protein whose cross-linking intensity correlates with a switch in deadenylation to a fast/asynchronous pattern. Panel C. Same as panel B, except that reaction mixtures were immunoprecipitated with anti-CUG-BP sera prior to SDS-PAGE analysis.

eral possible fates of the transcript rather than simply acting as the first step in its degradation. Thus, it will be important to fully understand the relationship of deadenylation and 3'UTR complex formation in order to gain a better perspective on this aspect of the post-transcriptional control of gene expression. It is interesting to note that CUGBP2 has in fact recently been implicated in translational silencing of the Cox-2 mRNA in cells following radiation treatment (Mukhopadhyay *et al.*, 2003).

While the data presented in this study clearly support a role for PARN in deadenylation of mammalian mRNAs, they do not exclude a role of one or more of the other candidate deadenylase enzymes that have been identified. Although PARN is highly active and can form complexes with other decay factors, it appears to be non-essential in both fungi and *C. elegans* (Tucker *et al.*, 2001). CCR4, which has been shown to be a major deadenylase in yeast (Tucker *et al.*, 2001), has also more recently been shown to be the major deadenylase in *Drosophila* (Temme *et al.*, 2004). Both yeast and *Drosophila* do, however, lack a good PARN homolog. One interesting explanation for the exquisite stability of the CITOR4-A60 RNA substrate in our study could be that it contains 3'UTR elements that specifically target the transcript for poly(A) tail shortening by an enzyme that is not present or active in the S100 cytoplasmic extracts. In addition to the data that we report here for PARN, CCR4, Caf1 and Pan2/3 activities have been shown to be influenced by 3'UTR structure and/or sequences (Lowell *et al.*, 1992; Chen *et al.*, 2002; Viswanathan *et al.*, 2003), and CCR4 may be targeted by a coding region instability determinant as well (Chang *et al.*, 2004). Therefore internal sequences in the body of the mRNA appear to play a large role in determining the efficiency of shortening of the 3' poly(A) tail once an mRNA has been targeted for turnover.

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