ELAV proteins stabilize deadenylated intermediates in a novel in vitro mRNA deadenylation/degradation system

Lance P. Ford,[1] Janice Watson,[2] Jack D. Keene,[2] and Jeffrey Wilusz[1,3]

[1]Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey (UMDNJ), New Jersey Medical School, Newark, New Jersey 07103 USA; [2]Department of Microbiology, Duke University Medical Center, Durham, North Carolina 27710 USA

We have developed an in vitro mRNA stability system using HeLa cell cytoplasmic S100 extracts and exogenous polyadenylated RNA substrates that reproduces regulated aspects of mRNA decay. The addition of cold poly(A) competitor RNA activated both a sequence-specific deadenylase activity in the extracts as well as a potent, ATP-dependent ribonucleolytic activity. The rates of both deadenylation and degradation were up-regulated by the presence of a variety of AU-rich elements in the body of substrate RNAs. Competition analyses demonstrated that *trans*-acting factors were required for RNA destabilization by AU-rich elements. The ~30-kD ELAV protein HuR specifically bound to RNAs containing an AU-rich element derived from the TNF-α mRNA in the in vitro system. Interaction of HuR with AU-rich elements, however, was not associated with RNA destabilization. Interestingly, recombinant ELAV proteins specifically stabilized deadenylated intermediates generated from the turnover of AU-rich element-containing substrate RNAs. These data suggest that mammalian ELAV proteins play a role in regulating mRNA stability by influencing the access of degradative enzymes to RNA substrates.

[*Key Words*: Deadenylation; mRNA degradation; poly(A); ELAV proteins; mRNA stability]

Received August 7, 1998; revised version accepted November 23, 1998.

The relative stability of mRNA is an important regulator of gene expression. The half-life of a specific mRNA can play a role in determining both its steady-state level of expression, as well as the rate at which its gene product is induced (for review, see Ross 1995; Caponigro and Parker 1996). Furthermore, mutations that affect the stability of mRNAs encoding regulatory factors can promote oncogenic transformation and immune dysregulation (Aghib et al. 1990; Schiavi et al. 1992). In general, many short-lived proteins, including those derived from cytokines and proto-oncogenes, are encoded by short-lived mRNAs. Several mRNAs that encode stable proteins, such as α-globin, have also been shown to have extraordinarily long half-lives (Holcik and Liebhaber 1997). In addition, surveillance mechanisms that identify and reduce the half-lives of aberrant mRNAs, which contain nonsense codon mutations, have been described (Maquat 1995; Jacobson and Peltz 1996). Therefore, regulation of the half-life of mRNAs can have dramatic consequences on cellular responses and functional outcomes during growth and development.

Through the application of genetics, the mechanisms and factors involved in the turnover of mRNA in *Saccharomyces cerevisiae* are beginning to be identified. Multiple pathways of mRNA turnover are present in yeast, allowing for numerous levels of regulation and fine-tuning of gene expression. One general pathway of mRNA decay involves poly(A) tail shortening followed by decapping and 5′ → 3′ exonucleolytic decay (Muhlrad et al. 1994). A second general pathway involves deadenylation followed by 3′ → 5′ turnover of the body of the mRNA (Anderson and Parker 1998). Endonucleolytic cleavage of some mRNAs has also been demonstrated (Presutti et al. 1995). Finally, another alternative decay pathway that bypasses deadenylation is involved in the translation-dependent degradation of nonsense codon-containing mRNAs (Weng et al. 1997). Several degradation enzymes and regulatory proteins that play a role in mRNA stability in yeast have been identified (Caponigro and Parker 1996; Weng et al. 1997). Functionally significant interactions between the cap structure and the 3′ poly(A) tail of yeast mRNAs have also been described (Tarun and Sachs 1997). Whether these observations are generally applicable to mammalian cells, however, remains to be established.

In vivo observations are beginning to allow some generalizations concerning major pathways of mRNA turnover in mammalian cells. A poly(A) tail of ~200 bases is added to most mRNAs during processing in the nucleus

This paper is dedicated to the memory of John C. Wilusz.

[3]Corresponding author.
E-MAIL wilusz@umdnj.edu; FAX (973) 972-3644.

(Colgan and Manley 1997). The poly(A) tail serves at least two known functions in mRNA stability. First, in association with poly(A)-binding proteins (Bernstein et al. 1989; Ford et al. 1997), it protects the mRNA from 3′ → 5′ exonucleases. Second, the poly(A) tail serves as an initiation site for the turnover of the mRNA. The poly(A) tail can be shortened progressively throughout the lifetime of a mRNA in the cytoplasm. Controlling the rate of deadenylation appears to be an important regulatory point in mRNA stability (Wilson and Treisman 1988; Xu et al. 1997). Once the poly(A) tail is shortened to ~30–65 bases, the body of the mRNA appears to be degraded in a rapid fashion in vivo without the accumulation of discernible intermediates (Chen et al. 1995; Xu et al. 1997). Little is known, however, concerning the enzymes and regulatory components involved in mammalian mRNA turnover.

In addition to the poly(A) tail, several *cis*-acting elements have been shown to play a role in mRNA stability. The 5′ terminal cap structure protects the transcript from exonucleases (Furuichi et al. 1977). Several destabilizing elements (Caput et al. 1986; Shyu et al. 1989; Bonnieu et al. 1990; Peng et al. 1996), as well as stabilizing elements (Stefonovic et al. 1997), located in the body of the mRNA have also been identified. One well-characterized element that regulates mRNA stability is an AU-rich sequence (ARE) found in the 3′ untranslated region of many short-lived mRNAs (Shaw and Kamen 1986). These AREs primarily consist of AUUUA repeats or a related nonameric sequence (Lagnado et al. 1994; Zubiaga et al. 1995; Xu et al. 1997) and have been divided into three classes on the basis of sequence characteristics and degradation kinetics (Xu et al. 1997). In general, AREs have been shown to increase the rate of deadenylation and RNA turnover in a translation-independent fashion (Chen et al. 1995; Fan et al. 1997). The underlying mechanism behind ARE function, however, remains to be determined.

Numerous proteins have been described that can bind in vitro to AU-rich elements (e.g., Malter 1989; Bohjanen et al. 1991; Brewer 1991; Vakalopoulou et al. 1991; Hamilton et al. 1993; Levine et al. 1993; Katz et al. 1994; Nakagawa et al. 1995; Ma et al. 1996), but the exact role of each factor in the process of mRNA turnover remains to be defined. The ELAV family of ARE-binding proteins is conserved evolutionarily and expressed differentially in tissues throughout the development of vertebrates (for review, see Antic and Keene 1997). Although ELAV proteins have been found in both the cytoplasm and the nucleus (Gao and Keene 1996), the most ubiquitously expressed form, HuR, can shuttle between the nucleus and the cytoplasm (Atasoy et al. 1998; Fan and Steitz 1998; Peng et al. 1998). ELAV proteins play an important role in growth and development, as the *Drosophila* homolog is genetically essential for development and maintenance of the nervous system (Campos et al. 1985; Robinow and White 1988). In addition, mammalian ELAV proteins are induced during differentiation and are distributed in RNP granules along dendrites (Gao and Keene 1996). Several lines of evidence suggest that ELAV proteins control aspects of post-transcriptional gene expression (Gao and Keene 1996; Koushika et al. 1996; Ma et al. 1997; Myer et al. 1997; Antic and Keene 1998). Overexpression of ELAV family members, for example, has been shown to affect accumulation of selected mRNAs (Jain et al. 1997; Fan and Steitz 1998; Levy et al. 1998; Peng et al. 1998). The precise role of ELAV proteins and other ARE-binding factors, however, remains to be established.

Mechanistic questions in mammalian cells are usually best approached using biochemical systems because of the inherent difficulties with mammalian cells as a genetic system. It has been difficult, however, to establish a versatile in vitro system to study mRNA stability and turnover. On the basis of in vivo observations and practical considerations, an optimal in vitro system to study the process of mRNA stability should have the following properties: First, the system should be efficient and highly reproducible. Second, minimal amounts (preferably zero) of RNA degradation in the system should be attributable to random degradation by nonspecific contaminating ribonucleases. Third, deadenylation should occur before general degradation of the mRNA body is observed. Fourth, degradation of the mRNA body should occur in an apparently highly processive fashion without detectable intermediates. Fifth, regulation of the rate of overall deadenylation and degradation should be observed in a sequence-specific manner. Finally, the system should work on exogenous RNAs to allow ease of experimental manipulation.

In this study we report the establishment of an in vitro mRNA stability system using cytoplasmic S100 extracts that fulfills all of the criteria listed above and possesses all of the properties known to be involved in ARE-mediated mRNA turnover. We have used this system to demonstrate a role for the AU-rich element-binding proteins of the ELAV family in mRNA stability. These findings suggest that ELAV proteins can affect a default pathway of ARE-mediated degradation by either protecting the mRNA from nuclease attack or by displacing factors that otherwise mark these short-lived transcripts for degradation. This in vitro system should allow the identification of cellular factors involved in mRNA turnover and help elucidate mechanisms involved in the post-transcriptional regulation of gene expression.

Results

Development of an in vitro system that deadenylates and degrades RNA substrates

The development of an in vitro system to study mRNA turnover requires the generation of a convenient source of poly(A)$^+$ RNA substrate and an active cellular extract. To obtain substrate RNAs that were both polyadenylated and easy to identify using standard acrylamide gel technology, we used a versatile ligation PCR approach that can attach a template encoding a 60-base poly(A) tail to the 3′ end of DNA fragments that contain a *Hin*dIII site (Ford et al. 1997; see Materials and Methods). The

sequence of all short, poly(A)$^+$ RNA substrates used in this study is shown in Table 1. In initial studies to develop an in vitro RNA stability system, we attached a 60-base poly(A) tail to a 54-base polylinker-derived sequence (Gem-A60). The small size of this polyadenylated transcript made it easy to analyze intermediates in the pathway of RNA turnover on acrylamide gels. Cellular extracts were prepared following a standard cytoplasmic S100 protocol (Dignam et al. 1983) using hypotonically lysed HeLa spinner cells with minor variations as described in the Materials and Methods. S100 extracts were chosen for their ease of preparation, as well as the fact that they have been shown previously to have some in vitro ribonuclease activity (e.g., Levy et al. 1998). The relevance of this ribonuclease activity to mRNA deadenylation/degradation observed in vivo, however, remains to be established.

Gem-A60 RNA was incubated in S100 extracts in the presence of ATP. As seen in Figure 1A (left), very little turnover of the Gem-A60 RNA was noted after 60 min of incubation. This reproducible slow rate of turnover prompted us to hypothesize that an inhibitor of the deadenylation/degradation process might be present in S100 extracts. This hypothesis was based on several observations. First, our previous work with nuclear extracts determined that poly(A)-binding proteins (PABPs) were strong inhibitors of a 3′ → 5′ exonuclease activity (Ford et al. 1997). Second, the activity of a partially purified mammalian deadenylase preparation was inhibited by high amounts of PABP (Korner and Wahle 1997). Third, overexpression of PABP in *Xenopus* oocytes inhibits maturation-specific deadenylation (Wormington et al. 1996). To test whether excess amounts of PABPs were responsible for inhibiting the deadenylation of Gem-A60 RNA in S100 extracts, we added increasing amounts of cold poly(A) competitor RNA to reaction mixtures to sequester PABPs. As shown in Figure 1A (right), the addition of poly(A) competitor activated a degradation activity in the S100 extracts. The Gem-A60 RNA was shortened to a species slightly larger than the size of a deadenylated marker (Gem-A0) and ~30% of the input RNA was degraded. Titration experiments performed in coordination with UV cross-linking studies demonstrated that the amount of poly(A) competitor RNA required to activate the S100 extract corresponded precisely with the ability of the competitor to inhibit binding of proteins to the poly(A) tail of the substrate RNA (data not shown). Furthermore, the nucleolytic activities activated by the addition of cold poly(A) RNA as competitor to the S100 extracts were still observable at concentrations of poly(A) >500 ng (data not shown). These data suggest that the activated nucleases are highly refractory to competition by poly(A).

The progressive shortening of the Gem-A60 RNA substrate observed with incubation in S100 extract supplemented with poly(A) competitor RNA was determined to be attributable to a 3′ → 5′, poly(A) tail-specific exonuclease based on the following observations: First, RNA substrates ^{32}P-labeled exclusively at their 5′ cap structures were progressively shortened in the system in a similar fashion as uniformly labeled transcripts (Fig. 1, cf. A and B). These data suggest that the shortening of the input RNA occurred in a 3′ → 5′ direction. This conclusion was confirmed by separately analyzing the 5′ and 3′ portions of RNA products from the in vitro system by RNase H digestion before gel electrophoresis. As shown in Figure 1C, the 3′ portion of the substrate RNA [which consists primarily of the 60-base poly(A) tail] was clearly being degraded before any turnover of the 5′ portion of the transcript was detected. After 9 min of incubation, 72% of the 3′ fragment containing the poly(A) tail is degraded, whereas only 19% of the 5′ fragment has been turned over. Finally, to ascertain whether this 3′ → 5′ exonuclease activity was indeed a poly(A)-specific deadenylase, we added 15 bases of nonadenylate sequence onto the 3′ end of the Gem-A60 RNA (Gem-A60-15). As seen in Figure 1D, although the Gem-A60 transcript [which contains a 3′ poly(A) tail] is an excellent substrate for the 3′ exonuclease activity, the Gem-A60-15 RNA, which has its poly(A) tract internalized 15 bases, was not.

From these data we conclude that the addition of poly(A) competitor RNA to an S100 extract activates a deadenylase that is active on exogenous, poly(A)$^+$ substrate RNAs. The in vitro system reproduces several aspects of mRNA stability observed in vivo. The surprising observation that the deadenylase itself is not apparently inhibited by cold poly(A) suggests that the native enzyme may not have high affinity for its substrate. This suggestion is also supported by the absence of a lag in the kinetics of deadenylation in the system as well as the observation that we cannot block deadenylation with very high amounts of cold poly(A). The deadenylase activity may contain additional RNA-binding activities that anchor it to mRNAs, perhaps as part of a multicomponent complex.

Table 1. *The sequence of small capped RNAs used as substrates in the in vitro system*

Gem-A60	GAAUACACGGAAUUCGAGCUCGGUACCCGGGGAUCCUCUAGAGUCGACCUGCAGGCAUGCAAGCUA(60)
Gem-A60-15	GAAUACACGGAAUUCGAGCUCGGUACCCGGGGAUCCUCUAGAGUCGACCUGCAGGCAUGCAAGCUA(60)UAUUGAGGUGCUCGAGGU
ARE-A60	GAAUACACAUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUAAGCUA(60)
MT-ARE-A60	GAAUACACGUUAGUAUUCAUUUGUUUACUAUUGAUUUCUUUAAGCUA(60)
FOS-A60	GAAUACACAAAUUUUAUUGUGUUUUUAAUUUAUUUAUUAAGAUGGAUUCUCUCUAUAAAUAUAAAAAUAAAAUAAAAAAAAGCUA(60)
CX-A60	GAAUACACCCCAACGGGCCCUCCUCCCCUCCUUGCACCAUCAUCGCAUCACGAGCUA(60)

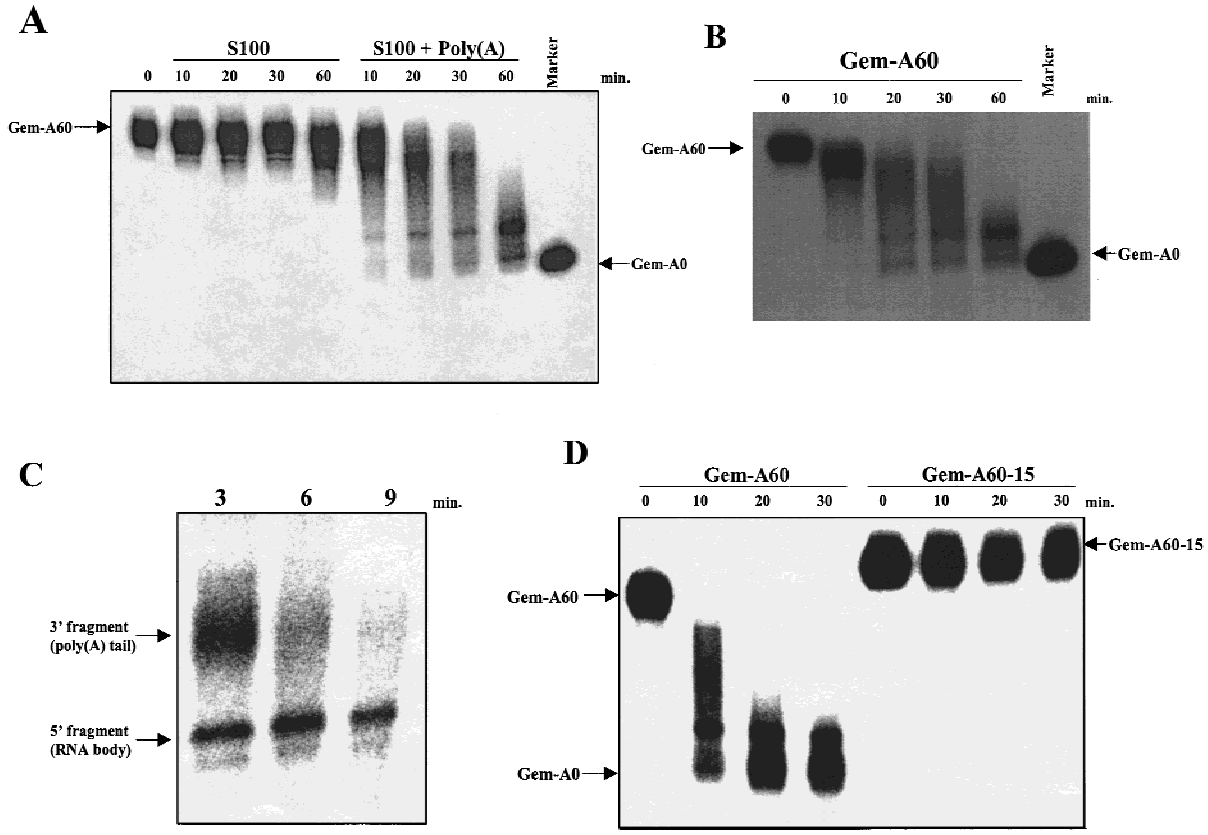

Figure 1. The addition of poly(A) to cytoplasmic S100 extracts activates specific deadenylase and degradation activities. (*A*) Poly(A) competitor RNA activates nucleolytic activities in the extract. A capped, radiolabeled 54-base RNA containing a 60-base poly(A) tail (Gem-A60) was incubated at 30°C with S100 extract in the absence (lanes marked S100) or presence [lanes marked S100 + Poly(A)] of 500 ng of cold poly(A) RNA as described in Materials and Methods for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The position of a deadenylated, 54-base transcript (Gem-A0) is indicated at *right*. (*B*) The shortening of input transcripts is attributable to a 3′ → 5′ exonuclease. Gem-A60 RNA, labeled exclusively at the 5′ cap, was incubated in the in vitro mRNA stability system for the times indicated. Reaction products were analyzed on a 5% acrylamide gel containing 7 M urea. The position of a deadenylated, 54-base transcript (Gem-A0) is indicated on the right. (*C*) An alternative approach also demonstrates that the shortening of input transcripts is attributable to a 3′ → 5′ exonuclease. ARE-A60 RNA, radiolabeled at A residues, was incubated in the in vitro stability system for the times indicated. Reaction products were hybridized to a DNA oligonucleotide and cleaved into 5′ and 3′ fragments using RNase H. Fragments were analyzed on a 5% acrylamide gel containing 7 M urea. (*D*) The 3′ → 5′ exonuclease activity is a specific deadenylase. Gem-A60 RNA or a variant that contains 18 extra nucleotides after the poly(A) tract (Gem-A60-15) were incubated in the in vitro stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The position of a deadenylated, 54-base transcript (Gem-A0) is indicated at *left*. 31 ± 11% of the input Gem-A60 RNA was deadenylated/degraded in 30 min.

RNA turnover in the in vitro system is regulated by AU-rich instability elements

We addressed whether the RNA turnover activities exhibited by the S100 extract system could be influenced by sequences in the body of the transcript in a specific manner. The relative stability of small polyadenylated RNAs containing either a 54-base polylinker sequence (Gem-A60), a 34-base AU-rich element (ARE) from tumor-necrosis factor α (TNF-α) mRNA (ARE-A60), or a 72-base ARE from the c-*fos* mRNA (Fos-A60) was determined in the in vitro stability system. As shown in Figure 2, A and B, the turnover of both of the ARE-containing RNAs was increased dramatically compared to the Gem-A60 control transcript. To assess directly whether regulation by AREs was occurring in a sequence-specific fashion, we extensively mutated the TNF-α–ARE as described in Materials and Methods. Similar mutations in AU-rich instability elements were shown previously to greatly increase mRNA half-life in vivo (Myer et al. 1997). As seen in Figure 2C, mutations in the ARE reduced the rate and extent of deadenylation/degradation more than threefold in the in vitro system. We conclude, therefore, that RNA turnover in the in vitro system can be regulated by AU-rich instability elements in a sequence-specific fashion.

All of the RNA substrates we have examined above contain a body of ~50–70 bases attached to a poly(A) tail. The next question we asked was whether we could detect regulated turnover using larger polyadenylated RNA substrates. As shown in Figure 2D, a polyadenylated 250-base RNA derived from the 3′ UTR of the SV40 late

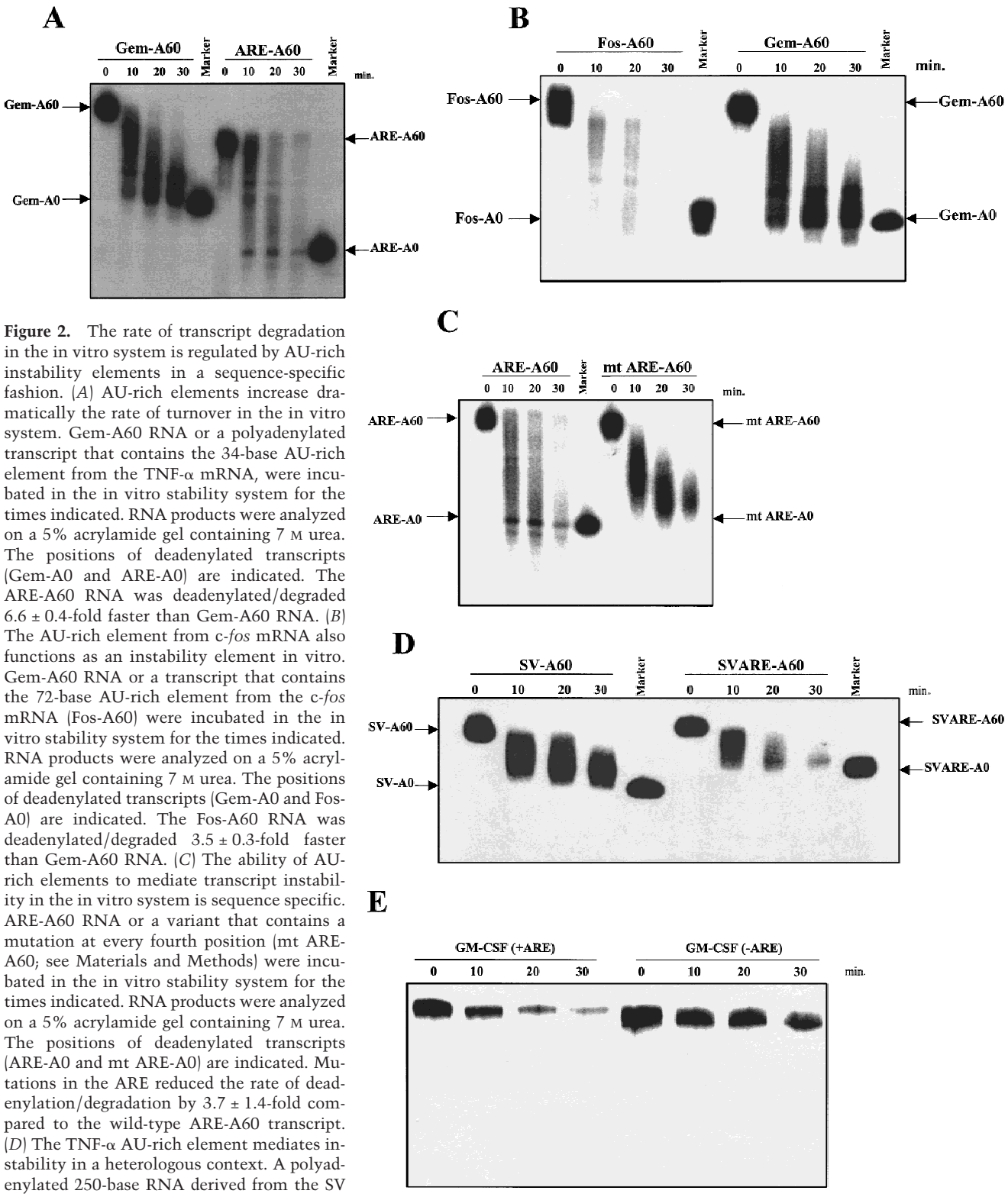

Figure 2. The rate of transcript degradation in the in vitro system is regulated by AU-rich instability elements in a sequence-specific fashion. (*A*) AU-rich elements increase dramatically the rate of turnover in the in vitro system. Gem-A60 RNA or a polyadenylated transcript that contains the 34-base AU-rich element from the TNF-α mRNA, were incubated in the in vitro stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The positions of deadenylated transcripts (Gem-A0 and ARE-A0) are indicated. The ARE-A60 RNA was deadenylated/degraded 6.6 ± 0.4-fold faster than Gem-A60 RNA. (*B*) The AU-rich element from c-*fos* mRNA also functions as an instability element in vitro. Gem-A60 RNA or a transcript that contains the 72-base AU-rich element from the c-*fos* mRNA (Fos-A60) were incubated in the in vitro stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The positions of deadenylated transcripts (Gem-A0 and Fos-A0) are indicated. The Fos-A60 RNA was deadenylated/degraded 3.5 ± 0.3-fold faster than Gem-A60 RNA. (*C*) The ability of AU-rich elements to mediate transcript instability in the in vitro system is sequence specific. ARE-A60 RNA or a variant that contains a mutation at every fourth position (mt ARE-A60; see Materials and Methods) were incubated in the in vitro stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The positions of deadenylated transcripts (ARE-A0 and mt ARE-A0) are indicated. Mutations in the ARE reduced the rate of deadenylation/degradation by 3.7 ± 1.4-fold compared to the wild-type ARE-A60 transcript. (*D*) The TNF-α AU-rich element mediates instability in a heterologous context. A polyadenylated 250-base RNA derived from the SV late transcription unit (SV-A60), or a variant that contains the 34-base AU-rich element from the TNF-α mRNA (SVARE-A60), were incubated in the in vitro stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The positions of deadenylated transcripts (SV-A0 and SVARE-A0) are indicated. SVARE-A60 RNA was deadenylated/degraded 3.5 ± 0.7-fold faster than SV-A60 RNA. (*E*) The AU-rich element derived from the GM-CSF mRNA functions in vitro on nearly a full-length RNA substrate. A nearly full-length version of the GM-CSF mRNA that contained an AU-rich element, GM-CSF(+ARE), or a version in which the AU-rich element was deleted, GM-CSF(–ARE), were incubated in the in vitro stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. GM-CSF(+ARE) was deadenylated/degraded 2.8 ± 0.2-fold faster than the GM-CSF(–ARE) transcript.

mRNA (SV-A60) was deadenylated but degraded inefficiently in the in vitro system. Adding the TNF-α–ARE to the 3′ portion of this RNA (SVARE-A60) resulted in an ~3.5-fold increase in the rate of turnover. Finally, we prepared a nearly full-length (~950 base) version of the human granulocyte–macrophage colony-stimulating factor (GM-CSF) mRNA, as well as one in which the ARE was deleted [GM-CSF(–ARE)]. The 3′ ends of these transcripts were polyadenylated using yeast poly(A) polymerase (Martin and Keller 1998). Gel-purified RNAs were incubated in the in vitro stability system and aliquots were removed at the times indicated. As seen in Figure 2E, the version of the GM-CSF mRNA that contains an ARE was ~2.5-fold less stable than GM-CSF(–ARE) in the in vitro system. As seen above with other transcripts, the GM-CSF transcripts were also deadenylated in the system. Deadenylation was not observable in Figure 2E because of the lack of resolution of the gel system used, but can be observed using formaldehyde–agarose gels (data not shown).

Degradation, but not deadenylation, requires ATP

Transcripts with 60 adenylates at the 3′ end were observed to undergo both deadenylation and turnover in the in vitro system. This is consistent with in vivo observations that suggest the poly(A) tail is shortened to ~30–65 bases before mRNA turnover is observed (Xu et al. 1997). Because degradation appeared to begin before the input transcript was deadenylated completely (e.g., Fig. 2), it was difficult to assess quantitatively the effects of AU-rich elements on relative deadenylation rates. To try uncoupling these processes and evaluate accurately the effect of AREs on deadenylation rates in the in vitro system, we surveyed the cofactor requirements that might be unique to either deadenylation or turnover. Both processes were inhibited by the addition of EDTA (data not shown), suggesting a role for divalent cations. Curiously, deadenylation could occur without the addition of ATP/phosphocreatine to the system (Fig. 3A). Degradation, on the other hand, required ATP/phosphocreatine as indicated by the accumulation of deadenylated intermediates in its absence (Fig. 3A, lanes –ATP). By omitting ATP from the reaction, therefore, we were able to evaluate relative deadenylation rates in the presence or absence of an AU-rich instability element. RNAs with physiologic length poly(A) tails (150–200 bases) that lack (SV-A150–200) or contain (SVARE-A150–200) an ARE were incubated in the in vitro system and aliquots were analyzed at the times indicated. As seen in Figure 3B, RNA substrates containing an ARE were deadenylated at an approximately twofold faster rate than RNAs that do not contain the instability element.

In summary, we have devised an in vitro mRNA stability system that acts on exogenous substrates and faithfully reproduces all of the known in vivo aspects of turnover. RNAs are first deadenylated before degradation of the body of the transcript. Degradation of the body of the mRNA then occurs in an apparently highly processive fashion with no discernible intermediates. Deadenylation and decay rates are increased severalfold by the inclusion of an AU-rich instability element. ARE regulation of RNA stability is sequence specific and highly reproducible, as all three of the AREs we have tested in the in vitro system function in a similar fashion. This system should provide a valuable means to elucidate

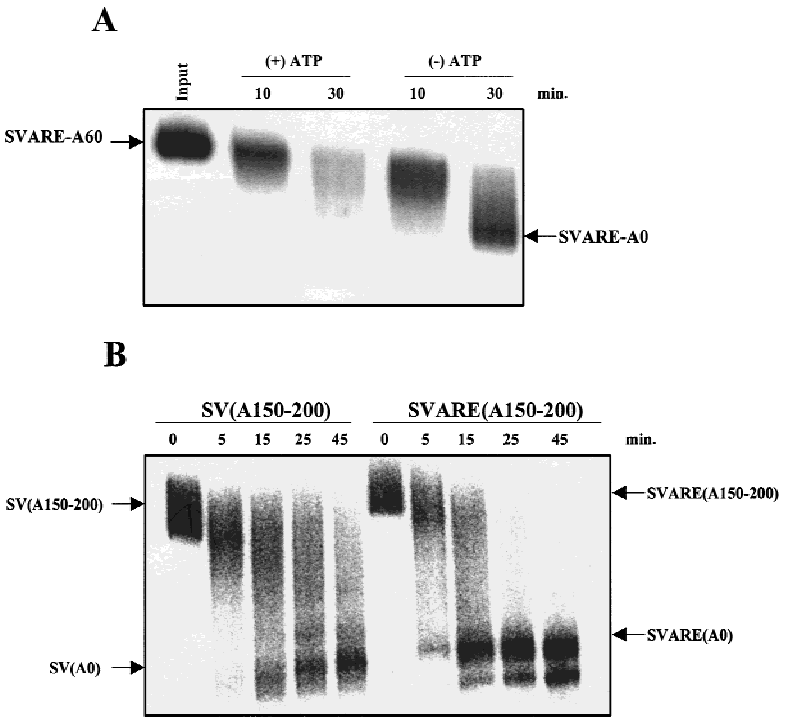

Figure 3. Deadenylation occurs in the absence of ATP and is regulated by AU-rich elements in vitro. (*A*) Degradation, but not deadenylation, requires ATP. SVARE-A60 RNA was incubated in the in vitro system in the presence, (+) ATP lanes, or absence (–) ATP lanes, for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The positions of the deadenylated SVARE-A0 transcript is indicated. (*B*) AU-rich elements regulate the rate of deadenylation on RNA substrates that carry a physiologic length poly(A) tail. SV RNA or SVARE RNA (a variant that contains an AU-rich element) were polyadenylated with yeast poly(A) polymerase and species that contained tails of ~150–200 bases were gel purified. These RNAs, SV(A150–200) and SVARE(A150–200), were incubated in the in vitro stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The positions of deadenylated transcripts (SV-A0 and SVARE-A0) are indicated. SVARE(A150-200) RNA was deadenylated 2.2 ± 0.3-fold faster than the SV(A150–200) transcript.

mechanistic aspects of regulated and general mRNA turnover pathways.

The role of ARE-binding proteins in the in vitro system

The in vitro system described here allows evaluation of the role of ARE-binding proteins in the process of RNA deadenylation/degradation. Several proteins were found to be associated with ARE-containing RNAs in our extracts. As seen in Figure 4A, a protein of ~30 kD and a group of ~40-kD proteins were specifically UV cross-linked to the short ARE-A60 transcript. A species of ~70 kD was also detected when this ARE was inserted into a larger transcript (SVARE-A60; see Fig. 5B). It is possible that this 70-kD protein was not detected on the ARE-A60 RNA because of the relatively small size of the transcript. Efforts to determine the identity of these cross-linked species using available antibodies to known ARE-binding proteins revealed the presence of an ELAV protein. As shown in Figure 4B, immunoprecipitation assays identified the 30-kD protein as HuR (a.k.a. HuA), a member of the ELAV protein family that is ubiquitously expressed in all tissues (Good 1995; Ma et al. 1996; Myer et al. 1997). Antisera against another RNA-binding protein of ~30 kD, hnRNP A1, failed to detect any cross-linked protein in our system (Fig. 4B). We tested two additional antisera to identify the 40-kD band. Antibodies to hnRNP C protein failed to detect any cross-linked protein, whereas antisera to AUF-1 (a.k.a. hnRNP D) (Brewer 1991) did precipitate a small amount of cross-linked 40-kD protein (data not shown). However, this cross-linked product was not competed by increasing amounts of the 34-base synthetic TNF-α ARE competitor RNA (data not shown). The significance of this low level of nonspecific AUF-1 cross-linking in the system is unclear. We conclude that the 30-kD species that specifically cross-links to the ARE element is HuR, a protein that has been suggested previously to play a role in ARE-mediated mRNA decay (Vakaloloupou et al. 1991; Antic and Keene 1997; Myer et al. 1997).

We then addressed whether the interaction of the cross-linked ARE-binding proteins with the element was required to mediate instability. We used synthetic ribonucleotides containing either a 34-base TNF-α ARE or randomly chosen non-ARE sequences. Synthetic competitor RNAs were added in increasing amounts to the in vitro stability system and their effect on RNA turnover was assessed. As seen in Figure 5A, the ARE competitor RNA inhibited completely deadenylation and degradation at 40 pm, whereas the nonspecific RNA had no effect at similar concentrations. The ARE competitor RNA had a similar effect on the deadenylation/degradation of RNAs whether or not they contained an ARE (data not shown). This observation suggests that factors capable of interacting with AREs are important for deadenylation, and may be a part of a multiprotein deadenylase/degradation complex.

We compared the ability of the synthetic ARE competitor RNA to block deadenylation with the ability of the RNA to compete for interaction of ARE-binding proteins with the substrate transcript. EDTA was added to cross-linking assays to inhibit RNA turnover and to evaluate the effect of various levels of competitor on cross-linking/label transfer efficiency. As shown in Figure 5B, all ARE-binding proteins (including HuR protein that could be immunoprecipitated using specific antisera before gel electrophoresis as shown in Fig. 5C) were specifically competed from the SVARE-A60 RNA substrates with the addition of 5 pm of the synthetic RNA competitor. As shown in Figure 5A, however, 5 pm of synthetic ARE competitor RNA failed to have an appreciable effect on the rate of RNA deadenylation/degradation in the system. We conclude that none of the major

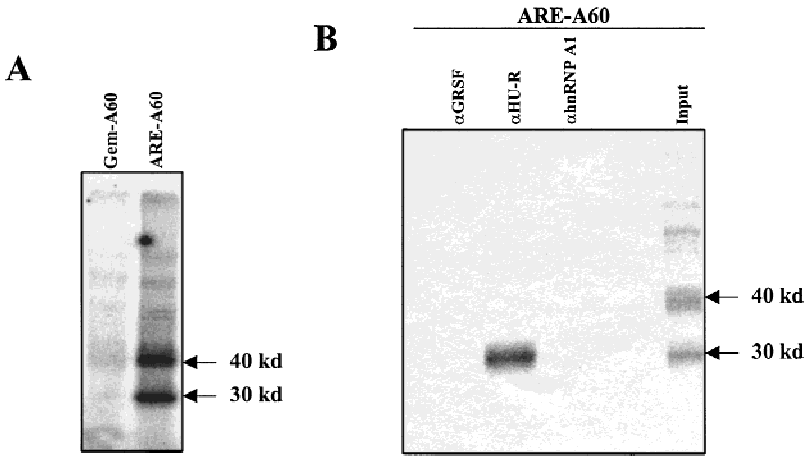

Figure 4. The HuR protein of the ELAV family specifically binds to the TNF-α AU-rich element in the in vitro system. (*A*) Two proteins specifically interact with the TNF-α AU-rich element. Gem-A60 and ARE-A60 RNAs were radiolabeled at U residues and incubated in the in vitro stability system for 5 min in the presence of EDTA (to block degradation and allow for accurate comparisons). Reaction mixtures were irradiated with UV light, cleaved with RNase A, and protein–RNA complexes were analyzed on a 10% acrylamide gel containing SDS. The approximate sizes of the cross-linked proteins indicated on the right were deduced from molecular mass markers. (*B*) The 30-kD protein is HuR. Radiolabeled ARE-A60 RNA was incubated in the in vitro RNA stability system and cross-linked to associated proteins as described above. Cross-linked proteins were immunoprecipitated using the indicated antisera before analysis on a 10% acrylamide gel containing SDS. The lane marked Input denotes total cross-linked proteins before immunoprecipitation analysis.

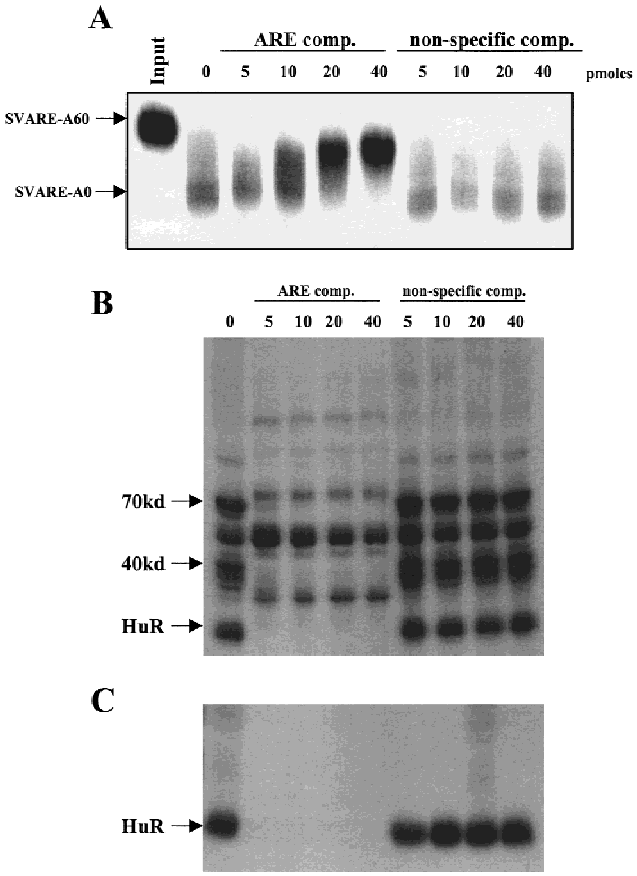

Figure 5. Although AU-rich element-binding factors are important to promote RNA deadenylation and degradation, the binding of the HuR protein to AU-rich elements is not associated with AU-rich element-mediated transcript instability. (*A*) Competition analysis suggests that AU-rich element-binding factors are required for deadenylation and degradation of transcripts. SVARE-A60 RNA was incubated in the in vitro stability system for 30 min in the presence of the indicated amounts of a synthetic RNA competitor that contained the TNF-α AU-rich element (ARE comp.) or a nonspecific sequence. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The position of deadenylated SVARE-A0 RNA is indicated. (*B*) Reaction mixtures were prepared as described in *A* with the addition of EDTA to inhibit RNA turnover. Protein–RNA interactions were analyzed by UV cross-linking analysis and analyzed on a 10% acrylamide gel containing SDS. The positions of AU-rich element-specific cross-linked species is indicated on the left. (*C*) Reactions were prepared exactly as described for *B*, except samples were immunoprecipitated using α-HuR-specific antisera before gel electrophoresis.

ARE-binding proteins that we could detect by cross-linking appear to be required for deadenylation/degradation in the in vitro system.

ELAV proteins prevent degradation of deadenylated transcripts in the in vitro system

Because the ARE-binding proteins we detected by cross-linking do not appear to be required for deadenylation/degradation, we hypothesized that they may play a role in transcript stability. Consistent with this model, recent in vivo data suggest that overexpression of Hel-N1 and HuR proteins can stabilize ARE-containing transcripts (Jain et al. 1997; Fan and Steitz 1998; Peng et al. 1998). We produced mouse recombinant HuR protein, as well as other members of the ELAV family [Hel-N1 and Hel-N2 (a.k.a. HuB)] as GST fusion proteins and added these to the in vitro stability system at a 10:1 molar ratio to substrate RNA. Similar data were obtained using any of the three recombinant ELAV family proteins, and only data with rHel-N1 are shown. As seen in Figure 6A, rHel-N1 protein failed to affect deadenylation of the SVARE-A60 RNA substrate in the in vitro system, but stabilized a deadenylated intermediate. GST alone, or another GST fusion protein that binds RNA (hnRNP H′) had no effect on transcript stability in the in vitro system (Fig. 6B). We conclude that the ELAV family of RNA-binding proteins function to protect deadenylated transcripts from the degradation enzymes.

We then tested whether the RNA substrate must contain an ARE for rELAV proteins to stabilize a deadenylated intermediate in the in vitro system. ARE-A60 RNA, or an unrelated but similarly sized and polyadenylated transcript, CX-A60, was incubated in the in vitro system in the presence or absence of rELAV proteins. As seen in Figure 6C, rHel-N2 (or other rELAV proteins; data not shown) stabilized the deadenylated intermediate only from RNAs that contain an ARE-binding site. We conclude that the stabilization of deadenylated intermediates by ELAV proteins requires an ARE. Furthermore, ELAV proteins can stabilize a deadenylated intermediate whether the ARE is located at the 3′ or 5′ positions of the 250-base SVARE-A60 RNA (Fig. 6D). These data suggest that the ARE–ELAV protein complex probably is not simply preventing turnover through steric blocking of an end of the transcript, thereby preventing exonuclease access.

Discussion

We have developed an in vitro RNA stability system that reproduces faithfully many known aspects of in vivo mRNA turnover in mammalian cells. Exogenous RNA substrates are deadenylated before degradation of the RNA body occurs in an apparently highly processive fashion without detectable intermediates. Furthermore the rates of RNA deadenylation and degradation are regulated by AU-rich elements in the system in a sequence-specific manner. We have used the system to determine a role for the ELAV family of ARE-binding proteins in the stability of deadenylated transcripts by specifically blocking the degradation step. These data illustrate the value of the system to address the mechanism of regulated mRNA turnover.

The in vitro system described in this report has several key technical advantages that significantly increase its usefulness. First, the system is highly reproducible and uses standard S100 cytoplasmic extracts from HeLa spinner cells. We have made nine independent preparations

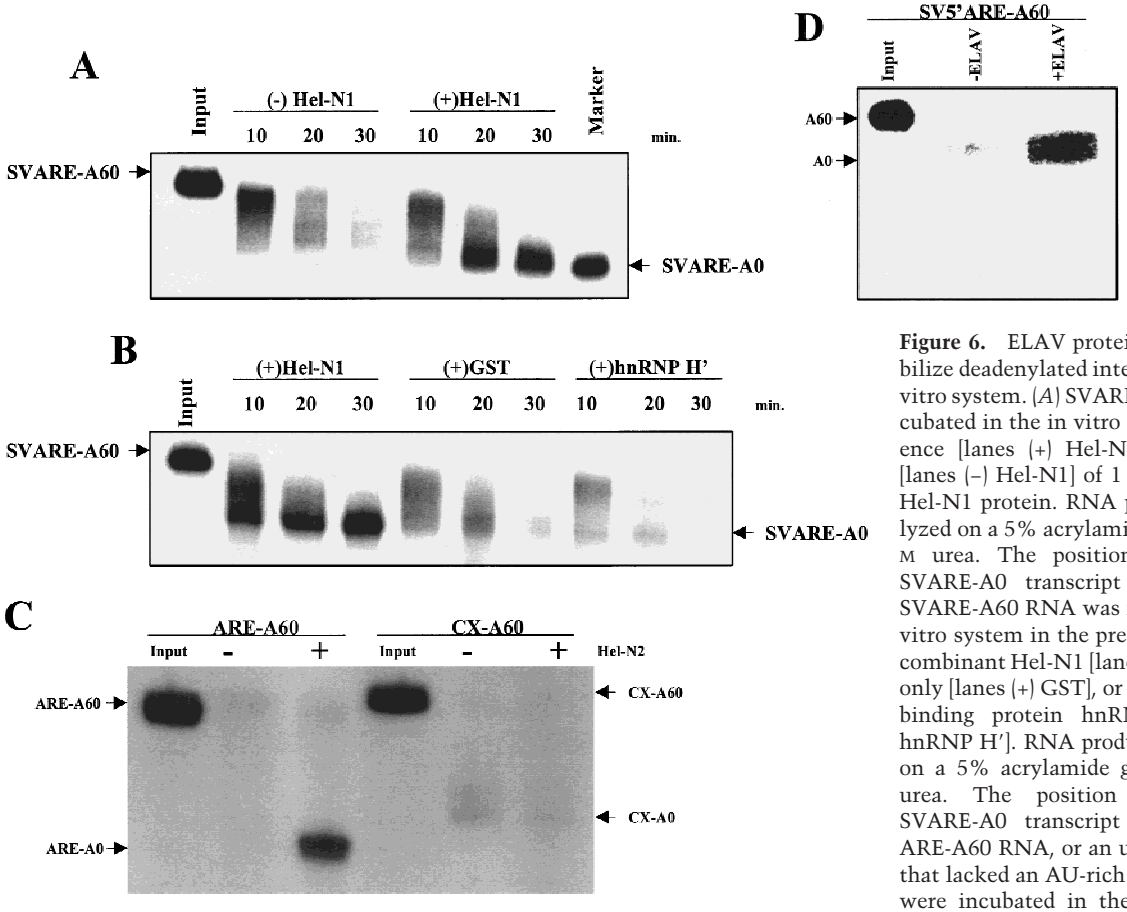

Figure 6. ELAV proteins specifically stabilize deadenylated intermediates in the in vitro system. (*A*) SVARE-A60 RNA was incubated in the in vitro system in the presence [lanes (+) Hel-N1] or the absence [lanes (–) Hel-N1] of 1 μg of recombinant Hel-N1 protein. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The position of deadenylated SVARE-A0 transcript is indicated. (*B*) SVARE-A60 RNA was incubated in the in vitro system in the presence of 1 μg of recombinant Hel-N1 [lanes (+) Hel-N1], GST only [lanes (+) GST], or an unrelated RNA-binding protein hnRNP H′ [lanes (+) hnRNP H′]. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The position of deadenylated SVARE-A0 transcript is indicated. (*C*) ARE-A60 RNA, or an unrelated transcript that lacked an AU-rich element (CX-A60), were incubated in the in vitro stability system for 30 min in the presence (+ lanes) or absence (– lanes) of ~1 μg of Hel-N2 protein. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The positions of deadenylated transcripts are indicated. (*D*) A variant of SV-A60 RNA that contained the TNF-α ARE in the 5′ portion of the transcript (SV5′ARE-A60) was incubated in the in vitro system for 50 min in the absence (– lane) or presence (+ lane) of 1 μg of Hel-N2 protein. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The positions of input and deadenylated transcripts are indicated.

of S100 extract that all function in the assay in a similar fashion (data not shown). The only difference among extracts appears to be in the kinetics of turnover (e.g., compare the slight differences in the pattern of turnover of Gem-A60 RNA in Fig. 1A with the pattern observed in Fig. 1D). Such variability in activity from extract to extract is commonly observed with other in vitro RNA-processing systems such as pre-mRNA splicing and polyadenylation. Second, our extracts exhibit minimal background degradation of RNA attributable to nonspecific nucleases. This lack of noise in the system significantly contributes to its reproducibility. Third, another key element of the system is that it uses exogenous polyadenylated RNAs as substrates. This property affords variety in RNA substrate preparation and sequence manipulation. Fourth, the system exhibits sequence-specific regulation by AU-rich elements in the absence of translation. In total, these technical advantages make the system a valuable reagent to identify components involved in mRNA turnover and address the mechanism of regulated mRNA stability.

The addition of poly(A) competitor RNA was required to activate S100 extracts to deadenylate and degrade efficiently RNAs in a regulated manner. Titration of cold poly(A) demonstrated that the extracts became activated for deadenylation/degradation when sufficient competitor was added to reduce substantially cross-linking of a 70-kD poly(A)-binding protein to the poly(A) tail of the radiolabeled substrate RNA (data not shown). The PABP-independent deadenylation observed in our in vitro system was somewhat unexpected, given the requirement for yeast PABP in mRNA deadenylation by the poly(A) nuclease (PAN) enzyme (Brown et al. 1996). The effect of PABP on deadenylation may, however, be different in mammalian systems. Partially purified deadenylase from mammalian cells is inhibited by excess PABP (Korner and Wahle 1997). In addition, Wormington et al. (1996) have shown that excess PABP inhibits deadenylation in *Xenopus* oocytes. Although PABP may not be required for deadenylation in mammalian systems, it still may play an important role in regulating the process.

Surprisingly, the deadenylation in the extracts remains active even in the presence of >500 ng of poly(A).

Commercial poly(A) preparations prepared with polynucleotide phosphorylase, therefore, do not appear to be able to interact with and sequester the deadenylase enzyme. These data suggest that the deadenylase activity is either in extraordinary concentrations in the extracts or may not have a strong affinity for its substrate. We favor the latter model. The deadenylase may rely on associated RNA-binding activities to regulate its association with an RNA substrate. In conjunction with this, we have observed an increase in deadenylation rate of ARE-containing RNAs (Figs. 2 and 3), as well as the ability of the ARE competitor RNA to inhibit deadenylation of non-ARE-containing substrates (data not shown). These data suggest that ARE-binding proteins may be associated with the deadenylase activity.

The deadenylation activity in our extracts appears to stall at variable sites on the poly(A) tail, leading to the observation of variable deadenylation intermediates in several assays (e.g., Figs. 1 and 2). Similar stalling of the deadenylase was also observed by Wahle and colleagues using a partially purified deadenylase activity, but its significance remains unclear (Korner and Wahle 1997). One observation regarding the stalling of deadenylation that is highly reproducible is that relatively stable, non-ARE-containing transcripts (e.g., Gem-A60, SV-A60) are often deadenylated to a tail of ~10–15 bases, and then appear to be generally refractory to further shortening (e.g., Figs. 1–3). Curiously, in vivo studies demonstrate that the poly(A) tail is not removed completely by the deadenylase before degradation of the RNA body (Caponigro and Parker 1996). The deadenylase enzyme may not be able to associate efficiently with RNA substrates containing short poly(A) tails. Perhaps the deadenylase delivers inefficiently these substrate RNAs to the turnover enzymes for decay of the body of the transcript. If this suggestion is correct, it implies that the deadenylase and degradation activities may assemble on RNAs as a multicomponent complex. The assembly of this complex is likely to be regulated by elements (e.g., AREs) in the body of the transcript. Alternatively, AREs may target RNAs for degradation by novel pathways. Further purification and characterization of the factors involved in general and regulated mRNA turnover will allow these possibilities to be addressed.

We have identified HuR protein, a ubiquitously expressed member of the ELAV family of RNA-binding proteins (Good 1995; Ma et al. 1996; Antic and Keene 1997; Myer et al. 1997), as one of the major ARE-binding factors in our system. We also detected weak binding to AUF-1 (hnRNP D), a protein previously speculated to be involved in regulated mRNA decay in vitro (DeMaria and Brewer 1996). We cannot, however, compete AUF-1 binding to the RNA substrate using levels of the synthetic ARE competitor RNA that will inhibit completely deadenylation and degradation in the system (data not shown). AUF-1, therefore, does not appear to play a significant role in transcript instability in our system. ELAV proteins are not required for deadenylation/degradation, but rather play a role in the stability of deadenylated RNAs that contain an ARE (Fig. 6). These data suggest that in addition to its effect on deadenylation rates (Chen et al. 1995; Xu et al. 1997), the ARE influences the efficiency of turnover of the body of the mRNA. In vivo observations (Chen et al. 1995; Xu et al. 1997; Peng et al. 1998) also support the conclusion that ARE influences mRNA degradation rates.

ELAV proteins, therefore, appear to regulate mRNA stability in vitro, an observation consistent with in vivo transfection studies. The ELAV family comprises four members, three of which are expressed in a tissue- or developmental-specific manner (for review, see Antic and Keene 1997). Tissue-specific ELAV proteins are also localized primarily to the cytoplasm, whereas the ubiquitous HuR protein is predominantly nuclear and can redistribute to the cytoplasm (Atasoy et al. 1998; Fan and Steitz 1998; Peng et al. 1998). It has been suggested that differentially expressed ELAV proteins play a role in regulating the stability of both nuclear and cytoplasmic RNA, thereby fine-tuning gene expression in specific developmental states (Gao and Keene 1996; Antic and Keene 1998).

The competition data shown in Figure 5 clearly demonstrate that factors associated with the ARE are required for deadenylation/degradation of substrate RNAs. On the basis of the kinetics of competition, these factors must either be much more abundant than the cross-linkable ARE-binding proteins like HuR, or interact with the ARE with a much lower affinity. We favor the latter model, and suggest that these factors are part of a multicomponent complex that includes the deadenylase and degradation enzymes. Through multiple cooperative interactions, these weak ARE-binding components may allow efficient assembly of the deadenylase/degradation complex on ARE-containing transcripts while still allowing the complex to assemble, albeit less effectively, on non-ARE-containing RNAs. The RNA-binding components of this proposed complex also may have affinity for other non-ARE instability elements (e.g., Peng et al. 1996).

The observation that endogenous HuR protein in our S100 extracts can be cross-linked to ARE-containing RNA substrates (Fig. 5) makes it surprising that an ARE can function as a destablizing element in the in vitro assay. Because HuR protein is predominantly nuclear, however, only low levels of the protein are likely to be present in our cytoplasmic extracts. This low level of HuR protein is probably unable to compete efficiently with destablizing factors for binding to the ARE. In fact, sequestration of the HuR protein by the addition of low levels of synthetic ARE competitor RNA does lead to an increased rate of turnover of ARE-containing RNAs in the in vitro system. As shown in Figure 5A, the amount of SVARE-A60 RNA remaining after 30 min in the system in the absence of competitor RNA (lane 0) is ~20% greater than when the assay is done in the presence of 5 pm of ARE competitor RNA (lane 5 pm). The removal or sequestration of HuR protein in S100 extracts, therefore, may be necessary to observe regulated deadenylation and degradation in some instances.

In summary, the following observations gained from

this study should be considered in a mechanistic model of mammalian mRNA stability. First, a PABP is associated with the inhibition of the deadenylation activity in vitro (Fig. 1). PABPs, therefore, may play a role in regulating transcript stability. Second, the deadenylase activity is not affected by high levels of poly(A) competitor, implying that the enzyme may not have a strong innate affinity for its substrate. Third, the differential effect of ATP levels on deadenylation and degradation suggests that different enzymes (or enzymatic sites) may be responsible for these two processes. Fourth, the synthetic ARE competitor RNA inhibits both deadenylation and degradation of all poly(A)$^+$ RNA substrates tested. These data suggest that RNA-binding proteins capable of interacting with AU-rich sequences may be an integral part or tightly associated with these enzymatic activities. Finally, ELAV proteins are involved in transcript stabilization, suggesting that independent AU-rich element-binding proteins may act in concert to promote or inhibit mRNA turnover in a tightly regulated fashion. Future fractionation–reconstitution studies with the in vitro system should continue to shed important insights into the mechanism of regulated mRNA stability in mammalian cells.

Materials and methods

Transcription templates and RNAs

RNAs were produced by in vitro transcription using SP6 polymerase (Melton et al. 1984) in the presence of ^{7m}GpppG cap analog and radiolabeled UTP or ATP as indicated. All transcripts were gel purified before use. For RNAs labeled exclusively at the 5′ cap, transcription reactions were performed in the absence of cap analog and radioactive nucleotides. Capping was then performed using guanyltransferase (BRL) and radiolabled GTP according to the manufacturer's recommendations. The sequence of short RNAs used as substrates in the in vitro system is shown in Table 1.

Transcription templates were derived as follows (all synthetic oligonucleotides used as transcription templates contain a 24-base SP6 promoter fragment at their 5′ ends): Gem-A0 RNA was produced from *Hin*dIII cut pGem4 (Promega). Gem-A60-15 RNA was produced from the PCR product used to produce Gem-A60 RNA (see below) without removing the primer binding site with *Ssp*I. Templates for ARE-A0 RNA were generated by hybridizing the synthetic oligonucleotide 5′-ATTTAGGT GACACTATAGAATACACATTATTTATTATTTATTTATT-ATTTATTTATTTA-3′ and its appropriate complement. Templates for MT-ARE-A0 RNA were generated by hybridizing the synthetic oligonucleotide 5′-ATTTAGGTGACACTATAGAA-TACACGTTAGTATTCATTTGTTTACTATTGATTTCTTT-A3′ and its appropriate complement. Templates for Fos-A0 RNA were generated by hybridizing the synthetic oligonucleotide 5′-ATTTAGGTGACACTATAGAATACACAAATTTTAT-TGTGTTTTTAATTTATTTATTAAGATGGATTCTC-3′ and its appropriate complement. The template for SV-A0 RNA was *Hin*dIII cut pSVL-Gem (Wilusz et al. 1988). Templates for SVARE-A0 RNA were generated by inserting the TNF-α ARE-containing oligonucleotide 5′-ATTATTTATTATTTATTTAT-TATTTATTATTTA and its appropriate complement between the *Pst*I and *Hin*dIII sites of pSVL-Gem (located near the 3′ end of the RNA). SVARE-A0 RNA was transcribed from *Hin*dIII linearized DNA. Templates for SV5′ARE RNA were generated by inserting the TNF-α oligonucleotides containing appropriate linkers between the *Eco*RI and *Sma*I sites of pSVL-Gem (located near the 5′ end of the RNA). The template for GM-CSF (+ARE) RNA was *Eco*RI cut pGM-CSF (Shaw and Kamen 1986). The template for GM-CSF (–ARE) RNA was *Nco*I cut pGM-CSF. Templates for CX-A0 RNA were generated by hybridizing the synthetic oligonucleotide 5′-ATTTAGGTGACACTATAGAA-TACACCCCAACGGGCCCTCCCTCCCCTCCTTGCACCA-TCATCGCATCACG and its appropriate complement.

Synthetic RNAs used in competition studies were made by the NJMS Molecular Core Facility and contained the following sequences: ARE, 5′-AUUAUUUAUUAUUUAUUUAUUAUU-UAUUUAUUUA; nonspecific competitor, 5′-GUCACGUGU-CACC.

Addition of poly(A) tails to transcripts

A template for a 60-base poly(A) tail was added to DNA templates using a ligation/PCR protocol we have recently described (Ford et al. 1997). Briefly, all of the templates described above contain a *Hin*dIII site that is used to generate the 3′ end of the RNA. The synthetic oligonucleotide 5′-AGCTA$_{60}$TATTGA-GGTGCTCGAGGT and its appropriate complement were generated, hybridized, and ligated to *Hin*dIII cut DNA templates. Ligation products were amplified using an SP6 promoter primer (5′-CATACGATTTAGGTGACACTATAG) and a primer specific for the 3′ end of the ligated oligonucleotide (5′-ACCTC-GAGCACCTC). Amplified products were purified on Centricon 100 columns, cut with *Ssp*I, and used as templates for SP6 polymerase to generate RNAs carrying the A60 designation.

Poly(A) polymerase (Amersham) was used to add 150- to 200-base poly(A) tails onto transcripts. RNAs were incubated with enzyme according to the manufacturer's recommendations on ice for 5–8 min. Following the reaction, RNAs were extracted with phenol–chloroform, ethanol precipitated, and purified on 5% acrylamide gels containing 7 M urea to obtain RNAs with the appropriate amount of poly(A) at the 3′ end.

S100 extract production

Cytoplasmic extracts were prepared from HeLa spinner cells grown in JMEM supplemented with 10% horse serum as described by Dignam et al. (1983) with the following two modifications. First, after centrifugation at 100,000*g* for 1 hr, the supernatant was adjusted to 10% glycerol before dialysis. Second, dialysis times were shortened to 30 min. Extracts were stored at –80°C.

In vitro RNA deadenylation/degradation system

Typically, ~200,000 cpm (~50 fm) of gel-purified RNA is used per reaction. In comparative studies, equal molar amounts of transcripts were used. A typical 14.25-µl reaction mixture contains 3.25 µl of 10% polyvinyl alcohol, 1 µl of a 12.5 mM ATP/250 mM phosphocreatine mixture, 1 µl of 500 ng/µl poly(A) (Pharmacia), 1 µl of RNA, and 8 µl of dialyzed extract. The poly(A) added to the reaction ranged in size from 200 to 1000 bases (average, ~500 bases). Reactions were incubated at 30°C for the times indicated and stopped by the addition of 400 µl of stop buffer [400 mM NaCl, 25 mM Tris-Cl (pH 7.6), 0.1% SDS]. Reaction mixtures were phenol extracted, ethanol precipitated, and analyzed on a 5% acrylamide gel containing 7 M urea. All

quantitation was performed using a Molecular Dynamics PhosphorImager.

Recombinant ELAV proteins (HuR, Hel-N1, and Hel-N2) were made as GST fusion proteins in *Escherichia coli* and purified using glutathione–Sepharose affinity chromatography according to the manufacturer's recommendations (Levine et al. 1993).

RNase H digestion

ARE-A60 RNA, radiolabeled at A residues, was incubated in the in vitro stability system for the times indicated. RNA products were phenol extracted and concentrated by ethanol precipitation. The sample was resuspended in a final volume of 30 µl containing 20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 100 pm of the antisense oligonucleotide 5′-AGTTAAATAAAT, and 1 unit of RNase H. Reactions were incubated at 37°C for 30 min and products were analyzed on a 5% acrylamide gel containing 7 M urea.

UV cross-linking and immunoprecipitations

UV cross-linking/label transfer experiments were performed as described previously using a Sylvania G15T8 germicidal light (Wilusz and Shenk 1988). Cross-linking experiments were done in the presence of 25 mM EDTA to inhibit RNA turnover to allow for accurate comparisons between samples. After digestion with RNases A, T1, and T2, cross-linked proteins were analyzed on 10% acrylamide gels containing SDS.

For immunoprecipitation analysis following UV cross-linking and RNase treatment, 300 µl of RIPA buffer [0.15 M NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris-Cl (pH 7.6)] was added to samples. After a brief centrifugation in a microfuge, precleared samples were incubated on ice with antibodies for 1 hr. Antigen–antibody complexes were collected using formalin fixed, washed protein A positive *Staphylococcus aureus* cells, washed five times using RIPA buffer, and analyzed on a 10% acrylamide gel containing SDS. Antibodies specific for GRSF (Qian and Wilusz 1994) and hnRNP A1 (Wilusz and Shenk 1990) have been described previously. The preparation and characterization of rabbit polyclonal antibodies specific for HuR will be described elsewhere (Atasoy et al. 1998).

Acknowledgments

We thank members of the Wilusz laboratory, especially David Fritz, for critical comments and Bob Donnelly for oligonucleotide synthesis. L.P.F. was supported by cancer training grant CA09665. Support for this work was provided in part by grants from the Pew Foundation and National Institutes of Health (NIH) (GM56434) to J. Wilusz and NIH grant CA60083 to J.D.K.

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