3'-UTR-dependent deadenylation by the yeast poly(A) nuclease

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Poly(A) tail removal is the first step in the degradation pathway for some mRNAs. The purified poly(A)-binding protein (PAB)-dependent poly(A) nuclease (PAN) from yeast removes mRNA poly(A) tails in vitro by a process similar to that observed in vivo. The exonucleolytic PAN degrades poly(A) and RNA bound by PAB, and can be activated by spermidine to degrade poly(A) in the absence of PAB. The shortening of the poly(A) tail down to 10–25 nucleotides and the terminal deadenylation of this short adenine tract are kinetically distinct reactions. Poly(A) shortening rates are stimulated by the yeast *a*-mating factor (*MFA2*) RNA 3' UTR sequence, and this occurs by switching PAN from a distributive to a more processive enzyme. Terminal deadenylation rates are also stimulated to different extents by various RNAs. Inversion of the *MFA2* 3' UTR sequence completely inhibits the terminal deadenylation reaction owing to the presence of an inhibitory element 70 nucleotides from the poly(A) tail. Other sequence elements inserted at a similar distance from the poly(A) tail also interfere with the reaction. These data suggest that the two phases of poly(A) degradation can be regulated by mRNA sequences, and they provide a mechanistic description of how this regulation could occur in vivo.

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The degradation of mRNA in eukaryotic cells is a regulated process. Different mRNAs have different decay rates, and these rates are determined by sequence elements within the mRNA. For instance, the stability of transferrin mRNA is regulated by a 3'-sequence element that responds to the availability of iron to the cell (Klausner and Harford 1989). Similarly, the instability of tubulin mRNA in some species results from the appearance of a specific amino-terminal peptide during translation (Gay et al. 1989). More generally, the instability of some mRNAs can result from the presence of sequences within the open reading frame (ORF), the presence of nonsense mutations, or the presence of destabilizing sequences in untranslated regions (for review, see Peltz et al. 1991).

Each of these sequence elements could stimulate different degradation pathways. Some may initiate degradation by an endonucleolytic cleavage within the body of the mRNA (Stoeckle and Hanafusa 1989; Brown and Harland 1990; Stoeckle 1992). Others may initiate degradation by activating 5' → 3' or 3' → 5' exonucleases. The degradation pathway of a group of RNAs containing a destabilizing AU-rich sequence element (ARE) (Shaw and Kamen 1986) within their 3'-untranslated regions (3' UTRs) is the most well characterized. This element in-

duces degradation by stimulating poly(A) tail removal (e.g., see Wilson and Treisman 1988; Shyu et al. 1991). Following this, the element may stimulate a second, coupled reaction that could either be an endonucleolytic or exonucleolytic cleavage.

The observation that the decay of an mRNA is initiated by the stimulated removal of its poly(A) tail raised the possibility that the enzyme responsible for this deadenylation step is identical to the enzyme required for the slow poly(A) tail shortening seen on all mRNAs, the poly(A) nuclease (PAN) (Sachs and Deardorff 1992). In yeast, PAN requires the poly(A)-binding protein (PAB) for activity (Sachs and Davis 1989). PAN has been purified from yeast extracts deficient in PAB based on this requirement, and the essential gene encoding it has been cloned. Conditional mutations in PAN lead to both alterations in poly(A) tail lengths and an arrest of translation initiation, suggesting that these two reactions are coupled (Sachs and Deardorff 1992).

Using purified PAN, we have investigated the mechanism of poly(A) tail removal in vitro and the effects of different yeast mRNA 3' sequences on this mechanism. PAN normally shortens mRNA poly(A) tails by a distributive mechanism, but sequence elements within the 3' UTR of the yeast *MFA2* RNA stimulate this shortening process by increasing the processivity of the enzyme. PAN removes the adenines from a short poly(A) tail in a kinetically distinct reaction called terminal deadenyla-

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tion. This reaction is also differentially inhibited by sequences within the 3' ends of different mRNA. These data suggest that the enzyme responsible for slow poly(A) tail shortening can be stimulated by mRNA to rapidly remove the tail. They also suggest that the stimulation of deadenylation on specific mRNAs *in vivo* results from a mechanism of activation similar to that observed *in vitro*.

Results

Characterization of PAN

The mechanism of poly(A) tail degradation by PAN was investigated in detail using PAN purified from yeast (Sachs and Deardorff 1992). As reported previously, PAN will not digest poly(A) tails in the absence of PAB (Fig. 1A). The presence of PAB in the reaction leads to the appearance of poly(A) tail shortening activity. PAN is unable to digest the final 20 adenines of the tail on the RNA shown in Figure 1, suggesting that complete deadenylation by PAN requires more than a poly(A) tail preceded by a short (25 nucleotide) tract of RNA.

To analyze the mechanism of poly(A) removal, the products of reactions containing various dilutions of PAN incubated with identical poly(A) concentrations were examined (Fig. 1B). The appearance of all intermediate sizes of products suggests that PAN is a distributive ribonuclease. This is in contrast to the predicted

findings for a highly processive enzyme, which would exhibit a PAN concentration-independent rate of terminal product formation.

PAN releases trichloroacetic acid (TCA)-soluble nucleotides as a reaction product (Sachs and Deardorff 1992). To examine the structure of the product, aliquots of a PAN-poly(A) digestion reaction were separated by thin-layer chromatography (data not shown). Only single mononucleotides were detected in this experiment, and the migration of this product was identical to 5'-AMP. Both 3'-AMP, as well as 3'5'-cAMP, 2'3'-cAMP, and 2'-AMP migrated more quickly than 5'-AMP under these solvent conditions. Less pure preparations of PAN were also unable to degrade poly(A) tails containing a terminal phosphate group (data not shown). From these data, we conclude that PAN is a distributive exonuclease that requires a free 3'-hydroxyl group and releases 5'-AMP as a product.

The TCA release assay was used to optimize the reaction conditions for PAN activity. PAN has an absolute requirement for Mg^{2+} and exhibits a broad pH dependence between 7.0 and 8.5 (data not shown). PAN also shows a broad temperature dependence, with optimum temperature between 30°C and 37°C (data not shown), and is completely inactivated after preincubation at 55°C for 10 min. The ionic strength dependence of PAN was quite dramatic, with near complete inactivation of the enzyme at ionic strengths >50 mM (Fig. 1C). At much higher ionic strengths (>300 mM) or in the presence of 10

Figure 1. PAN is an ionic strength-dependent distributive exoribonuclease requiring PAB for activity. (A) PAN requires PAB for activity. Homogeneously labeled ^{32}P -labeled poly(A) (pAS200) was incubated in the presence (+) or absence (–) of PAB for the indicated times. Reaction products were visualized by polyacrylamide gel electrophoresis and autoradiography. (B) PAN is a distributive enzyme. Increasing concentrations of PAN (0.15, 0.44, 1.33, or 4.0 μ l per reaction) were incubated with ^{32}P -labeled poly(A) (pAS200) for 40 min, and the reaction products were visualized as described above. (C) PAN activity is ionic strength dependent. Fixed amounts of ^{32}P -labeled poly(A) (pAS200) were incubated in a standard reaction at the indicated ionic strengths for 10 min. Following the addition of PAN, the reaction was monitored by the TCA precipitation assay (see Materials and methods). The release of TCA-soluble nucleotides by PAN after 20 min of incubation (cpm released) is shown.

mm MgCl_2 at low ionic strength, a weak PAB-independent nucleolytic activity could be detected in the PAN preparations (data not shown). This effect is presumably analogous to the effects of spermidine, described below.

The poly(A) substrates used in the previous experiments contained 6/7 nonadenine residues at their 3' end (pAS200), and these were still substrates for PAN. This suggested that PAN could have a nonspecific nucleolytic activity that was directed to any 3' end bearing a PAB molecule. To investigate this further, poly(A) tails with an additional 27 or 154 nucleotides of random RNA at their 3' ends were incubated with PAN and PAB, and the products of the reaction were analyzed by either a TCA release assay or by gel electrophoresis. Consistent with the TCA data (not shown), poly(A) tails with either 7- or 32-nucleotide 3' extensions were removed efficiently by the enzyme, whereas poly(A) tails containing a 162-nucleotide extension were not metabolized (Fig. 2A).

To examine the nucleotide specificity of the enzyme in more detail, several other RNA substrates were investigated. Most mRNAs examined (see below) could not be digested past their poly(A) tails, yet an RNA substrate whose 3' end was AU-rich over a region of 25 nucleotides, and which was shown to contain a PAB-binding site by nitrocellulose filter binding (Sachs et al. 1987; data not shown), provided an adequate substrate for the PAB-dependent reaction (Fig. 2B). The accumulation of distinct intermediate-sized fragments during the digestion probably reflects some nucleotide preference for the enzyme, with pausing occurring at nucleotides that are not removed efficiently.

These data lead to the conclusions that PAN will degrade 3' ends bound by PAB and that PAN is not absolutely specific for adenine. Short extensions of RNA 3' to a poly(A) tract may present an efficient substrate for PAN owing to the binding of PAB, and this binding could be induced by a slight cooperativity in the binding properties of PAB (Sachs et al. 1987). Extensions well beyond the end of the poly(A) tract, as well as the 3' ends of most other RNAs tested, will not be degraded because they do not provide an adequate PAB-binding site. One prediction from these data is that the 3' UTRs of some mRNAs could be degraded by PAN after their deadenylation if they provide a functional PAB-binding site.

PAB-independent PAN activity was detected under two different conditions. First, an mRNA substrate bearing an $(\text{A})_{25}$ tail (pAS364) could be slowly deadenylated in the absence of PAB (Fig. 3A). This activity was present in PAN preparations derived from yeast extracts missing or containing PAB, indicating that the activity was not the result of a slight contamination by endogenous PAB. The PAB-independent decay pattern revealed all intermediate lengths of RNA, and TCA-soluble nucleotides were released during the reaction (data not shown). This suggests that the terminal deadenylation reaction is also occurring through an exonucleolytic mechanism. PAN was also able to degrade an $(\text{A})_{25}$ tail attached to the inverted *MFA2* 3' UTR in the absence of PAB (see below), indicating that the inhibitory properties of this RNA on terminal deadenylation requires a ribonucle-

Figure 2. PAB-dependent cleavage of RNA by PAN. (A) 3'-Terminal extensions of the poly(A) tail can be degraded by PAN. The ^{32}P -labeled poly(A) (pAS200) substrate (containing 6/7 nonadenine residues) was extended at its 3' end by cleaving the DNA template for the synthesis reaction either 34 or 162 nucleotides from the terminal adenine with *HindIII* or *BanI*, respectively. The RNAs were added to a standard PAN digestion reaction in the absence (–) or presence (+) of PAB for 60 min. Reaction products were visualized by autoradiography following gel electrophoresis. (B) AU-rich 3' ends of RNA can be degraded by PAN. The DNA template containing the *MFA2* 3'UTR (pAS263) was linearized at the internal *NdeI* site, and radioactive RNA transcribed from this template was subject to a standard PAN digestion reaction in the absence (–) or presence (+) of PAB. Reaction products were visualized by autoradiography following gel electrophoresis.

oprotein substrate. Why the enzyme exhibits a poor but detectable PAB-independent activity on short, but not long, poly(A) substrates remains to be explored.

The second condition that elicited PAB-independent activity was when poly(A) was incubated first with sper-

Figure 3. PAB-independent cleavage of poly(A) by PAN. (A) Synthetic RNA substrate containing only 25 adenine residues at the 3' end (pAS364) was subject to a standard PAN digestion reaction in the presence (+) or absence (–) of PAB for the indicated time. Reaction products were visualized by autoradiography following gel electrophoresis. (B) Spermidine activates poly(A) tail degradation. 32 P-labeled poly(A) synthesized by T7 RNA polymerase (see Materials and methods) was incubated with spermidine and subsequently with PAN for 24 min. The extent of digestion was monitored by the release of TCA-soluble nucleotides. The concentration of spermidine represents the final concentration in the reaction, and the fractional activity is derived from dividing the amounts of released radionucleotides in the presence of spermidine by that released in the presence of PAB for the same length of time.

midine and then with PAN (Fig. 3B). The poly(A) substrate used in this experiment contained only adenines at its 3' ends, in contrast to the poly(A) preparations used in the other experiments. Approximately 80% of the PAB-activated PAN activity could be achieved under optimal conditions. Equivalent concentrations of spermine led to no activation, and the addition of PAB to the spermidine-containing reaction did not lead to a greater activation of the enzyme than with PAB alone (data not shown). The ionic strength-dependent inhibition profile of PAN with spermidine was nearly identical to that seen with PAB (data not shown). Poly(A) substrates bearing nonadenine 3' residues and all other RNAs tested

were not substrates for the spermidine-stimulated reaction (data not shown). These data indicate that spermidine can only activate poly(A) tail degradation and that PAN requires PAB for the degradation of all other substrates. Together with the PAB-independent terminal deadenylation activity, these data also show that PAN is the nuclease in the reaction mixture and not an activator of a latent nucleolytic activity in PAB.

PAN shortening activity is regulated by 3' UTR sequences

The possibility that yeast PAN could be activated by 3' UTR sequences was investigated by examining the poly(A) shortening rates on a series of synthetic 3' polyadenylated RNA fragments from the yeast genes *MFA2* (pAS263), *RP29* (pAS342), *ACT1* (pAS340), *CYH2* (pAS341), and *HIS3* (pAS343). These RNAs were chosen because their *in vivo* stabilities reflect the range found for most yeast mRNAs (Herrick et al. 1990). Compared with the other RNAs tested, the yeast *MFA2* RNA clearly stimulated its poly(A) tail shortening rate (Fig. 4). The mechanism of shortening also seemed to be different. First, terminally deadenylated reaction products were detectable before all of the precursor had been utilized. Second, the deadenylation intermediates had a broad size distribution. In contrast, the other RNAs went through a slow poly(A) tail shortening reaction, with a narrower size range of intermediates and no product appearance before all of the usable precursor had been utilized. Increasing the concentration of *ACT1* RNA fourfold did not affect this pattern, suggesting that the observed differences in shortening rates result from differences between the RNAs and not the specific reaction conditions chosen (data not shown). Taken together, these data indicate that the *MFA2* RNA stimulates poly(A) tail shortening by switching PAN from a distributive to a more processive enzyme. An analysis of the extent of *MFA2* and *ACT1* 3' UTR deadenylation as a function of PAN concentration, similar to the experiment shown in Figure 1B, supports this conclusion (data not shown). The sequence elements within the *MFA2* 3' UTR that are responsible for this change in processivity are currently being identified.

PAN terminal deadenylation activity is regulated by 3' UTR sequences

A kinetic distinction between the shortening of poly(A) tails down to a length of 10–25 nucleotides and the terminal deadenylation of the tail was observed on several different substrates. A clear example of the differences between the two reactions is shown in Figure 5. For this RNA (pAS321), the shortening of its poly(A) tail is almost complete by 20 min, whereas its terminal deadenylation to a poly(A)[–] form occurs slowly over the next 160 min. The temporal separation of the two reactions, together with the ability of the enzyme to slowly remove terminal adenines in the absence of PAB (see above), suggests that terminal deadenylation is mechanistically dis-

Figure 4. Poly(A) tail shortening rates are influenced by mRNA sequences. ³²P-labeled RNA containing the *MFA2* 3' UTR (pAS263) (A) or the final 165 nucleotides of the *RP29* (pAS342) (B), *HIS3* (pAS343) (C), *ACT1* (pAS340) (D), or *CYH2* (pAS341) (E) transcripts attached to an (A)₁₀₀ tail were incubated in a standard PAN digestion reaction with 0.5 μ l of PAN for the indicated times. Poly(A)⁻ RNA was prepared by incubation of the substrate with 2.5 μ l of PAN for 180 min. RNA was resolved by polyacrylamide gel electrophoresis and visualized by autoradiography.

tinct from poly(A) shortening. Interestingly, the substrate for the terminal deadenylation reaction is equal to the size of one PAB-binding site (Sachs et al. 1987), suggesting that dissociation from this last site could be a rate-limiting step in the reaction.

Terminal deadenylation of RNA 3' fragments from different RNAs by PAN also occurred at different rates (Fig. 4). While the *MFA2* RNA rapidly appeared in the terminally deadenylated form, *HIS3* and *CYH2* RNAs exhibited an accumulation of RNA containing ~25 adenine residues, which was subsequently slowly removed. *ACT1* RNA slowly lost the final adenines from its poly(A) tail, but a clear reaction intermediate containing 25 residues was not identifiable.

Instead of decreasing the rate of the terminal deadenylation reaction, inversion of the *MFA2* 3' UTR (pAS264) inhibited it completely (Fig. 6A,B). The migration of a poly(A)⁻ form of this RNA was identical to that of poly(A)⁻ *MFA2* RNA, indicating that the decreased mobility of the terminal reaction product in these experiments is the result of the presence of residual adenines and not an aberrant migration of the RNA on polyacrylamide gels. We note that the inverted *MFA2* RNA may be shortened more quickly than the *MFA2* RNA, and

future work will attempt to address this observation more thoroughly. In contrast to the *MFA2* RNA data, inversion of either the *ACT1* or *HIS3* 3' fragments did

Figure 5. Terminal deadenylation is kinetically distinct from poly(A) tail shortening. ³²P-Polyadenylated RNA (pAS321) was incubated in the standard PAN digestion reaction for the indicated times. Products were visualized by autoradiography following gel electrophoresis.

Figure 6. Inversion of the *MFA2* 3' UTR inhibits the terminal deadenylation reaction. (A) ^{32}P -Polyadenylated RNA containing the *MFA2* 3' UTR in the sense (pAS263) or inverted orientation (pAS264) was incubated in the standard PAN digestion reaction for the indicated times. Products were visualized by autoradiography following gel electrophoresis. (B) ^{32}P -Polyadenylated RNA containing the *MFA2* 3' UTR (pAS263), the inverted *MFA2* 3' UTR (MFA2') (pAS264), the inverted *MFA2* 3' UTR

UTR containing a linker substitution between -56 and -67 (*MFA2'* $\Delta 56-67$) (pAS366) or -68 and -79 (*MFA2'* $\Delta 68-79$) (pAS367) nucleotides from the poly(A) tail was incubated in the standard PAN digestion reaction for the indicated times. The lower of the two products is poly(A) deficient. Products were visualized by autoradiography following gel electrophoresis.

not inhibit the terminal deadenylation reaction (data not shown). Together with the above data, this suggested that 3' UTR sequences were capable of regulating both the shortening and the terminal deadenylation reaction.

To define more precisely the location of the putative inhibitory element on the inverted *MFA2* RNA, a limited linker scanning substitution analysis was performed (Fig. 6B). Although the replacement of 12 nucleotides from the poly(A) tail (pAS366) did not relieve the inhibitory effect, the replacement of 12 nucleotides 68 nucleotides from the tail did (pAS367). These data confirm the hypothesis that inversion of the *MFA2* 3' UTR introduces an inhibitory element and does not remove a specific activation element, and they indicate that a similar region in other 3' UTRs could contain regulatory sequences for the deadenylation reaction. The size of the inhibitory element and whether it is affecting the terminal deadenylation reaction through alterations in RNA structure are unknown at this time.

The basic sequence requirements for terminal deadenylation of the *MFA2* RNA were determined by a 5'-end deletion analysis. This experiment indicated that 3' UTR fragments shorter than 90 nucleotides (pAS315) could not be terminally deadenylated (Fig. 7). The addition of 25 nucleotides to this short fragment resulted in its complete deadenylation (pAS316). This region is referred to as ARE3 owing to its overall high content of adenine and uridine nucleotides. Interestingly, the distance between the poly(A) tail and the ARE3 element (60 nucleotides) is similar to the distance between the poly(A) tail and the inhibitory element on the inverted *MFA2* RNA (68 nucleotides).

To examine whether other sequences in this region could regulate the terminal deadenylation reaction, the ARE3 region was substituted with other regions of the *MFA2* 3' UTR and with synthetic sequences. Substitution of the ARE3 with the first of two AU-rich elements (ARE1) found at the 5' end of the *MFA2* 3' UTR (pAS345) also allowed terminal deadenylation (Fig. 7). Substitution of the second AU-rich elements (ARE2) (pAS344) partially inhibited the reaction. Replacing each of the uridines with cytosines in the ARE2 (pAS346) also partially inhibited the reaction. Similar results were obtained when the entire ARE3 region was replaced with

15 cytosines (pAS363). In contrast, replacing ARE3 with 15 adenines (pAS361) allowed the completion of the terminal deadenylation reaction.

In summary, these data indicate that an RNA molecule must be >90 nucleotides in length to be deadenylated, regions within the 3' UTR can inhibit PAN activity, and one location for an inhibitory element is between 55 and 80 nucleotides from the poly(A) tail. The complexity of the results make it difficult to define at the nucleotide level what allows or disallows terminal deadenylation. Future work examining the affects of these mutations on the rates instead of the extent of terminal deadenylation may help in defining the sequence requirements for the reaction more completely.

Discussion

Purified PAN from yeast exhibits two changes in its deadenylation properties that depend on the 3' UTR sequence. These are the change from a slow, distributive poly(A) shortening enzyme to a rapid, processive one and a change in the ability to terminally deadenylate a residual poly(A) tail. Each of these changes relies on the presence of a ribonucleoprotein substrate, suggesting that regulation could be occurring through ribonucleoprotein recognition and not just RNA sequence recognition.

PAN represents the first RNase known to require a ribonucleoprotein for activity. The ribonucleoprotein is probably required for at least two different reasons. First, PAB could protect the phosphate backbone of poly(A), thereby providing a proper substrate for the enzyme. This property is inferred from the ability of spermidine to replace PAB for poly(A) degradation (Fig. 3B). The protection by PAB presumably occurs through lysine or arginine residues. The sequence of the carboxyl terminus of PAB is not conserved between organisms, whereas the sequences of its RNA-binding domains are (for review, see Burd et al. 1991). If the function of the carboxy-terminal domain is conserved, it is probably through the presence of a small number of key residues. The working hypothesis is that the carboxy-terminal domain of PAB is responsible for providing the positively charged residues necessary for phosphate backbone protection. Preliminary data examining the nonstimulatory effects of

Figure 7. Terminal deadenylation is sensitive to internal RNA sequences. Diagram of synthetic polyadenylated RNA constructs that were subject to the standard PAN digestion reaction for up to 180 min and visualized by autoradiography following gel electrophoresis. The residual length of the poly(A) tail (nucleotides remaining) was calculated by subtracting the calculated length of the deadenylated form of the RNA from the measured length of the reaction product. The plasmids encoding the RNAs (pAS) are listed. (ARE) AU-rich element; (U/C) U → C substituted ARE2.

carboxy-terminal truncations of PAB (Sachs et al. 1987) on PAN activity in vivo and in vitro support this view. PAB also seems to be required to target PAN to the correct substrate because the presence of a PAB-binding site appears to be sufficient to allow degradation of RNA by PAN (Fig. 2). The absence of a PAB requirement for poly(A) in the presence of spermidine and the inability of spermidine to activate the degradation of RNA other than poly(A) are consistent with the model that the affinity of PAN for the RNA may be modulated by PAB. For instance, PAN could degrade poly(A) coated with spermidine because its intrinsic affinity for poly(A) is sufficient to allow its degradation in the absence of PAB. This predicts that at high enough concentrations, PAN should be able to degrade all mRNA in the presence of spermidine because binding to the mRNA should be saturated at these concentrations. A specific region of PAB probably interacts with PAN to increase its time spent bound to the mRNA, and we assume that this interacting region is present within a highly conserved region of PAB. The potential ability of PAB to modulate the affinity of PAN for the RNA molecule may help in understanding the ability of the 3' UTR of *MFA2* to stimulate the shortening reaction. The switch in the mechanism of poly(A) shortening from distributive to processive is consistent with the model that PAN contains a higher affinity for this RNA than for poly(A) or the other RNAs examined. This increase in affinity would result in PAN degrading

the same 3' end as a result of it being bound to the same RNA, thereby giving the appearance of an increase in the processivity of the enzyme. The increase in the affinity of PAN for the RNA could be mediated by PAB binding to 3' UTR sequences, thereby offering a distal site of interaction for PAN that would keep it localized on the same mRNA molecule. Alternatively, these 3' UTR sequence elements may contain a high affinity binding site for PAN, which would also result in a decrease in its dissociation rate. Future work on identifying the sites on the mRNA that stimulate the shortening, and an evaluation of their interactions with PAN or PAB, will help to distinguish between these possibilities. The regulation of the terminal deadenylation reaction by inhibitory sequences at least 55 nucleotides away from the poly(A) tail in vitro clearly shows the potential for the regulation of this reaction in vivo. The terminal deadenylation reaction appears to be distinct from the shortening reaction by the criteria that it is kinetically separable and that it can occur slowly in the absence of PAB. Because PAB movement from the residual oligo(A) tail may be a prerequisite for the reaction to proceed, internal sequence elements that either stimulate or inhibit this movement could control this phase of deadenylation. The presence of PAB-binding sites within the mRNA would allow this movement because PAB can readily hop between binding sites without having to dissociate from the RNA molecule (Sachs et al. 1987; Bernstein and Ross 1989). The minimum length of 90 nucle-

otides of RNA needed to see the terminal deadenylation reaction in vitro may reflect a constraint on the distance between two PAB-binding sites to allow hopping, and the effects of sequences between 55 and 80 molecules on the rates and extents of the reaction may reflect the relative affinity of PAB for this second binding site. Whether these putative binding sites would also control the rate of the shortening reaction remains to be explored.

The similarities of the deadenylation reaction observed in vitro for PAN with that seen in vivo for the group of highly unstable mRNAs containing an AU-rich destabilizing sequence element in their 3' UTR are striking. First, the observation that ARE stimulates the shortening of the poly(A) tail on these mRNAs to rates that far exceed those found in other cellular mRNAs (Shyu et al. 1991) is analogous to what is observed for the *MFA2* RNA shortening with PAN in vitro. Second, the appearance of semistable oligoadenylated intermediates on mRNAs containing partially mutated AREs (Shyu et al. 1991), as well as the apparent stability of many oligoadenylated RNAs that have undergone the poly(A)-shortening process (for review, see Sachs 1990), is analogous to the appearance of the precursors of the terminal deadenylation reaction performed by PAN in vitro.

A recent detailed characterization of the *MFA2* degradation pathway in vivo shows that deadenylation of this mRNA is rate limiting for its degradation, the deadenylation occurs in two distinct steps, and specific sequence elements in the 3' UTR of the mRNA are responsible for these effects (Muhlrads and Parker, this issue). On the basis of our data and these results, we conclude that the *MFA2* 3' UTR contains sequence elements that are analogous to the AREs of higher cells and that the target of these elements is the PAB-dependent poly(A) nuclease. Future work examining the effects of *PAN1* and *PAB1* mutations on *MFA2* RNA stability in vivo will help to confirm this.

If AREs on unstable mRNAs act by enhancing PAN activity, then the function of the specific mRNA-binding proteins bound to them (Bohjanen et al. 1991; Brewer 1991; Gillis and Malter 1991; Vakalopoulou et al. 1991; You 1992) can be limited to several possibilities. If these elements are PAB recognition sites, then these proteins can either be functionally substituting for PAB or inhibiting the access of PAB to these sites. If these elements are PAN-binding sites, then the proteins are either necessary cofactors for PAN binding, serving a function that PAB cannot provide, or they are inhibiting PAN binding. Discerning whether these AU-rich binding proteins are actually inhibitors or activators of the mRNA degradation process, as well as understanding the role of PAB and PAN in recognizing these elements, will help to clarify these issues. It is important to note that the PAN fractions utilized in these assays do contain other polypeptides (Sachs and Deardorff 1992); therefore, the non-nucleolytic attributes assigned to PAN in this discussion could actually result from the activity of other factors within this fraction.

The two phases of deadenylation observed in this re-

port are reminiscent of the two phases of polyadenylation that occur in the nucleus (Sheets and Wickens 1989). For polyadenylation, the slow addition of the first 10 adenine residues is a kinetically distinct reaction from the subsequent rapid polymerization of the remainder of tail. The transition between these two steps requires the binding of a nuclear PAB, and it is the presence of this protein that ultimately stimulates the poly(A) polymerase to elongate the tail (Wahle 1991). For deadenylation, the transition between the two phases could require the movement of PAB from the oligo(A) tail, thereby leading to the kinetically distinct terminal deadenylation step. It is interesting that the enzymes involved in polyadenylation and deadenylation have such a strict requirement for PABs. This suggests that there may be other similarities between the mechanisms of regulating polyadenylation and deadenylation within the cell. A recent report identifying a single 3' UTR element that can regulate both deadenylation and polyadenylation in the mouse oocyte (Huarte et al. 1992) is consistent with this proposal.

The reconstitution of the poly(A) shortening reaction in the absence of translation suggests that some phases of mRNA degradation may be translation independent. The recent identification of the involvement of PAN in translation (Sachs and Deardorff 1992), together with the availability of yeast strains that are deficient in PAN shortening activity but still viable (Sachs and Davis 1989), argues that poly(A) shortening in vivo may be tightly coupled to translation but is not required for it. The localization of PAN to the ribosome, mRNA, or the cytosol may help in elucidating the relationship between the translational requirement of PAN and poly(A) tail shortening. Future work will aim at unraveling the links between translation and mRNA degradation by using both a biochemical and genetic approach to evaluate the unique role of PAN in both of these reactions.

Materials and methods

Proteins

PAN fractions from the poly(U)-Sepharose column (Sachs and Deardorff 1992) and recombinant PAB (Sachs et al. 1987) were used in all experiments described. PAN extracts were derived from yeast strains containing a deletion of *PAB1* (YAS 1008) or strains containing *PAB1* and a galactose-inducible *PAN1* gene (YAS1064). The standard PAN reaction mix was prepared by mixing PAN (1–6 μ l) at 4°C in 10 μ l of 50 mM K acetate, 10 mM HEPES (pH 7.5), 1 mM $MgCl_2$ (0.5 $\times$ buffer A) with 1,000–10,000 cpm of RNA and 2 μ g of yeast tRNA (Sigma) with or without 200 ng of PAB in 10 μ l of 0.5 $\times$ buffer A, diluting to 200 μ l final with 5 mM HEPES (pH 7.5) and 2 mM $MgCl_2$ (LS buffer) at room temperature, and incubation at 30°C. Reactions were monitored by the TCA release assay by adding 200 μ l of 20% TCA at the indicated time, precipitation at 4°C for 10 min, and microcentrifugation for 10 min. Two hundred microliters of the supernatant containing the soluble radionucleotide was added to 200 μ l of unbuffered IM Tris base and 4 ml of Aquasol (New England Nuclear) and subject to scintillation counting. Ionic strengths in the reaction mixture were increased by the addition of 3 M K acetate. RNA samples were prepared for electrophoresis by the

addition of 50 μ l of 3 M NaCl, 100 mM EDTA, 200 mM Tris (pH 8.0), 50 μ l of 10% SDS and H₂O to the reaction mixture to a final volume of 500 μ l, extraction with one volume of phenol-chloroform-isoamyl alcohol (25 : 24 : 1), precipitation with ethanol, and resuspension in 7 M urea, 10 mM Tris at pH 8.0 containing bromophenol blue and xylene cyanol (0.05%). Following electrophoresis on 9% acrylamide/7 M urea/0.5 $\times$ TBE sequencing gels [Sambrook et al. 1989] run in 1 $\times$ TBE, RNA was visualized by directly exposing the gel to autoradiographic film under an intensifying screen at -70°C . Size standards were *Msp*I-digested pBR322 (New England Biolabs) DNA fragments end-labeled with [³²P]dCTP (New England Nuclear) and the Klenow fragment of *Escherichia coli* DNA polymerase.

For examination of the rates of poly(A) tail shortening on different mRNAs (Fig. 4), approximately one-tenth of the PAN used in the other experiments was used per point, and all final RNA concentrations were made equal. For PAB-independent activation, spermidine and poly(A) were first mixed at room temperature in 70 μ l of LS buffer, LS buffer (at room temperature) was added to 180 μ l, and PAN in 20 μ l of LS was added last to initiate the reaction, which was then incubated at 30 $^{\circ}\text{C}$.

The ability of PAN to use all of the input RNA changes between RNA preparations, and this probably reflects the variation in the method of RNA purification from the polyacrylamide gels.

Nucleic acids

The 5' and 3' ends of the *MFA2* RNA were mapped by reverse transcription and S1 nuclease protection respectively [Sam-

brook et al. 1989] using RNA prepared from YAS306 (a *ade2 his3 ura3 leu2 trp1*) [Sachs and Davis 1989], the reverse transcription primer OAS1, or the S1 protection probe derived from 3'-end labeling of *Bam*HI-linearized pSM29 ([Michaelis and Herskowitz 1988]) with [³²P]dCTP (New England Nuclear) and T4 DNA polymerase [Sambrook et al. 1989]. The cDNA corresponding to the mapped RNA ends was amplified from the pSM29 genomic clone by polymerase chain reaction (PCR) using oligonucleotides OAS6 and OAS7 (Table 1) and *Taq* DNA polymerase (Cetus) under conditions suggested by the manufacturer to yield the following DNA fragment (the ORF is underlined; the cDNA sequence includes the *Eco*RI site at the ends):
gaattcCAGCGAGCTATCATCTTCATACAACAATAACT-
ACCAACCTTAATGCAACCGATCACCCTGCTTCCACA-
CAAGCCACTCAGAAGGATAAATCCTCTGAAAAGAAAG-
ACAACCTATATAATCAAGGGCCTCTTCTGGGATCCCGC-
CTGTGTTATCGCTTAATTTTTGACGACAACCAAGAGG-
TCAAATCAATATCTACCTTTTCATTTATTACGTGTTGC-
TGGCAACTAATTTATTCCAATTCTCTCATCATTAGCT-
CACCCTTTCCATACTTTTTCATACTTTTATACATATGT-
ATTTGTAGTTATCACTTTAACAGATTTTTTCATGaggaa-
tc. This amplified fragment was digested with *Eco*RI and ligated into the *Eco*RI site of pAS200 to yield pAS225.

PCR amplification of most of the 3' UTR with oligonucleotides OAS7 and OAS78 from pAS225 with *Taq* DNA polymerase (Cetus) under the manufacturer's recommended conditions yielded the DNA insert in pAS263. Table 2 lists the other constructions derived from this parent plasmid.

Plasmid clones containing the terminal 165 nucleotides of the *ACT1* (pAS340), *CYH2* (pAS341), *RP29* (pAS342), or *HIS3*

Table 1. Oligonucleotides

Oligonucleotide (OAS)	Sequence
1	GGCTTGTTGTGGAAGCAGTGGT
2	TAATACGACTCACTATA
4	T ₅₀ CCTATAGTGAGTCGTA TTA
6	CCCGGGAATTCCAGCGAGCTATCATCTTCATACAAC
7	CCCGGGAATTCCCTCATGA AAAAAATCTGTAAAGTGATA
78	CCGGAATTCCCTTTTCAT(GT)TATTACGTGTTGCTGGCCAACTAAT(GT)TA
80	GACTAGTCCAATTCTCTCATCATTAGC
81	GACTAGTTCATTAGCTCA CCCTTTTC
82	GACTAGTCCTTTCCATAC TTTTTTCATA
83	GACTAGTTTTTTCATACT TTTATACATA
84	GACTAGTGGTACCTGCCAGCAACACGTAATAAA
85	GACTAGTGGTACCAATAA ATTAGTTTGCCAGCA
86	GACTAGTGGTACCTGAGAG AATTGGAATAAATT
87	GACTAGTGGTACCGTGAG CTAATGATGAGAGAA
90	GGGCCCTTTTCATTTATTG GTAC
91	CAATAAATGAAAGGGCCC
92	GGGCAAACCAACCCACCG GTAC
93	CGGTGGGTTGGTTTTGCC
108	TATGTTTTTTTTTTTTTT T TGGTAC
110	TATGGGGGGGGGGGGGGGG GGGTAC
113	GGAATTCCTTTTCTTAA AAATACT
114	GGAATTCCTCTCAAAACA A GAATACG
115	GGAATTCGAAATAATACA GAAGTAG
116	GGAATTCCTGTCAAAGCTA GATTTCGT
117	GGAATTCCTAATGATAGAG AAGTGGA
118	GGAATTCCTGCTTTTCCAA A AGGTTG
119	GGAATTCCTCGTTTCAGAA TGACACG
120	GGAATTCCTCAAGGTGTT TCTTATGT
122	GATCGTACCAAAAAAAAAA AAAAAACA
124	GATCGTACCCCCCCCCC CCCCCCA

Table 2. *MFA2-derived plasmids and RNA transcripts*

pAS200 (125)
Poly(A) ₁₀₀ transcript. (A) ₁₀₀ inserted at <i>Sma</i> I site of pSp65 (Varnum and Wormington 1990) (ggaatacacggaattcgagctcgcccA ₁₀₀)
pAS263 (167)
<i>MFA2</i> 3' UTR <i>Eco</i> RI linked and inserted at <i>Eco</i> RI site of pAS200. PCR product of OAS7 and OAS78 with pSM29 (ggaatacacggaattcCCTTTTCATTTATTACGTGTTGCTGGCcAAACTAATTTATTCCAATTCTCTCATCATT AGCTCACCCCTTCCATACTTTTTCATACTTTTATACATATGTATTTGTAGTTATCACTTTAACAGATTTTTT CATGaggaattcgagctcgcccA ₁₀₀)
pAS264 (167)
Inverted form of pAS263 insert (ggaatacacggaattcctCATGAAAAAATCTGTAAAGTGATAACTACAAATACATATGTATAAAAGTATGAAA AAGTATGGAAGGGTGAGCTAATGATGAGAGAATTGGAATAAATTAGTTTGCCAGCAACACGTAATAAATG AAAGGgaattcgagctcgcccA ₁₀₀)
pAS366 (167)
pAS264 with linker substitution at –56 to –67 from poly(A) tail (OAS81, OAS85) (ggaatacacggaattcctCATGAAAAAATCTGTAAAGTGATAACTACAAATACATATGTATAAAAGTATGAAA AAGTATGGAAGGGTGAGCTAATGAactagtgtaccAATAAATTAGTTTGCCAGCAACACGTAATAAATGAAA GGaattcgagctcgcccA ₁₀₀)
pAS367 (167)
pAS264 with linker substitution at –68 to –79 from poly(A) tail (OAS82, OAS86) (ggaatacacggaattcctCATGAAAAAATCTGTAAAGTGATAACTACAAATACATATGTATAAAAGTATGAAA AAGTATGGAAGGactagtgtaccTGAGAGAATTGGAATAAATTAGTTTGCCAGCAACACGTAATAAATGAAA GGaattcgagctcgcccA ₁₀₀)
pAS364 (118)
<i>MFA2</i> 3' UTR blunted at <i>Nde</i> I and joined to (A) ₂₅ tail (ggaatacacggaattcCCTTTTCATTTATTACGTGTTGCTGGCcAACTAATTTATTCCAATTCTCTCATCATT AGCTCACCCCTTCCATACTTTTTCATACTTTTATACATAtcgaccA ₂₅)
pAS321 (127)
<i>MFA2</i> 3' UTR fragment to –71 (ggaatacacggaattcgagctcgcccgggtagccactagtCCTTTCCATACTTTTTCATACTTTTATACATATGTATT TGAGTTATCACTTTAACAGATTTTTTTCATGaggaattcgagctcgcccA ₁₀₀)
pAS316 (115)
<i>MFA2</i> 3' UTR ARE3 at 5' end (ggaatacacggaattcgagctcgcccgggtagccactagtTTTTCATAC TTTTATACATATG- <i>Nde</i> I---)
pAS345 (111)
<i>MFA2</i> 3' UTR ARE1 at 5' end (OAS90/91) (ggaatacacggaattcgagctcgcccgggCCCTTTTCATTTATTggtaccactagTATG- <i>Nde</i> I---)
pAS344 (115)
<i>MFA2</i> 3' UTR ARE2 at 5' end (ggaatacacggaattcgagctcgcccgggtagccAACTAATTTATTggtaccactagTATG- <i>Nde</i> I---)
pAS346 (111)
<i>MFA2</i> 3' UTR ARE2 U → C substitution at 5' end (OAS92/93) (ggaatacacggaattcgagctcgcccgggCAAAccAAccAccggtaccactagTATG- <i>Nde</i> I---)
pAS363 (112)
<i>MFA2</i> 3' UTR C15 at 5' end (OAS110/124) (ggaatacacggaattcgagctcgcccgggtagctacccccccccccccCATATG- <i>Nde</i> I---)
pAS361 (112)
<i>MFA2</i> 3' UTR A15 at 5' end (OAS108/122) (ggaatacacggaattcgagctcgcccgggtagctacccccccccccccCATATG- <i>Nde</i> I---)
pAS315 (90)
<i>MFA2</i> 3' UTR to –41 (ggaatacacggaattcgagctcgcccgggtagctTATG- <i>Nde</i> I---)

Plasmid numbers are listed (pAS); RNA transcript lengths are in parenthesis. Distances from the poly(A) tail are shown (–N), oligonucleotides used in the construction are indicated (OAS), and the entire RNA transcript sequence is listed [uppercase letters indicate *MFA2*-derived sequences, A₁₀₀ marks the site of the poly(A) tail; *Nde*I indicates that sequences past this region are identical to those in pAS263 after the *Nde*I site].

(pAS343) RNA transcripts were prepared by PCR amplification of genomic DNA clones of these genes with oligonucleotides OAS113/114, OAS115/116, OAS117/118, and OAS119/120, respectively, using *Taq* DNA polymerase (Cetus) and the conditions suggested by the manufacturer. Amplified DNA fragments were digested with *Eco*RI and cloned into *Eco*RI-digested pAS200. RNA was prepared from *Xba*I-linearized template as described below.

SP6 RNA polymerase transcription reactions (Yisraeli and Melton 1989) were performed in 24 μ l with *Bam*HI-, *Xba*I (pAS316, pAS321, pAS340–344)-, or *Pst*I (pAS364)-linearized template (4 μ g) using either [α - 32 P]ATP or [α - 32 P]UTP (New England Nuclear) (60 μ Ci per reaction) with 0.1 mM of the unlabeled nucleotide, 0.05 mM GTP, 0.5 mM each of the other two nucleotides, 0.5 mM of the cap analog diguanosine triphosphate (Pharmacia), 12 units of RNA polymerase, and 24 units of RNasin (Promega). Following electrophoresis of the reaction mixture on 7 M urea–polyacrylamide gels and visualization by autoradiography, RNA was isolated by crushing the gel slice in 0.5 ml of TE buffer [10 mM Tris (pH 7.5), 0.1 mM EDTA] with 20 μ g of yeast tRNA (Sigma), elution overnight at 37°C, and precipitation of the RNA in the eluate made 0.3 M NaCl with 2.5 volumes of ethanol (Sambrook et al. 1989). RNA was resuspended and reprecipitated and, finally, resuspended in water and stored at –20°C.

Synthetic 32 P-labeled poly(A) prepared from a T7 RNA polymerase reaction was as described in Milligan and Uhlenbeck (1989) in 100 μ l using oligonucleotides OAS2 and OAS4, 250 units of T7 RNA polymerase, 24 units of RNasin (Fisher), and the nucleotide mixture described above without the cap analog and with 0.5 mM GTP. Following purification by gel electrophoresis, the major products were excised and purified by the procedures described above.

For thin-layer chromatographic analysis of the PAN reaction products, 2 μ l of a standard PAN digestion reaction with 32 P-labeled poly(A) (pAS200) was spotted onto cellulose F plates (EM Science), dried, and chromatographed in isobutyric acid/ $\text{NH}_4\text{OH}/\text{H}_2\text{O}$ (577 : 38 : 385) solvent (Filipowicz et al. 1985). The radioactive material was visualized by autoradiography. The positions of the unlabeled 3' and 5' AMP nucleotides were determined by UV shadowing.

DNA cloning methods were as described in (Sambrook et al. 1989), and all plasmid constructions were sequenced (Sanger et al. 1977) to confirm their structure.

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