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Poly(A)-binding proteins regulate both mRNA deadenylation and decapping in yeast cytoplasmic extracts

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ABSTRACT

The pathway of mRNA degradation has been extensively studied in the yeast, *Saccharomyces cerevisiae,* and it is now clear that many mRNAs decay by a deadenylation-dependent mechanism. Although several of the factors required for mRNA decay have been identified, the regulation and precise roles of many of the proteins involved remains unclear. We have developed an in vitro system that recapitulates both the deadenylation and the decapping steps of mRNA decay. Furthermore, both deadenylation and decapping are inhibited by poly(A) binding proteins in our assay. Our system has allowed us to separate the decay process from translation and we have shown that the poly(A) tail is capable of inhibiting decapping in an eIF4E-independent manner. Our in vitro system should prove invaluable in dissecting the mechanisms of mRNA turnover.

Keywords: eIF4E; mRNA decay; poly(A) tail; translation

INTRODUCTION

Messenger RNA decay is an important mechanism for regulating gene expression. Many mRNAs, including several encoding cytokines, growth factors, and proto-oncogenes, are regulated at the level of mRNA stability in response to external stimuli (Chen & Shyu, 1995). Stabilization, or destabilization, of an mRNA can be achieved rapidly, allowing for massive changes in the amount of a specific transcript over a short period of time (for review, see Wilusz et al., 2001).

Virtually all mature eukaryotic mRNAs have a 5′ 7-methylguanosine cap structure and 3′ poly(A) tail that promote both translation and stability. During degradation of the mRNA, at least one of these elements must be removed to allow exonucleases access to the body of the message. The pathway of mRNA decay has been studied most extensively in the yeast *Saccharomyces cerevisiae* and several of the enzymes and regulatory factors involved have been identified genetically and biochemically. The initial step in degradation of the majority of mRNAs is shortening of the poly(A) tail (Decker & Parker, 1993). Two complexes with poly(A) shortening activity have been identified in yeast; Pan2p/Pan3p and a complex containing the Caf1 and Ccr4 proteins. The Pan2p/Pan3p poly(A) nuclease is a Pab1p-dependent activity that has been shown to process poly(A) tails in the nucleus (Brown et al., 1996; Brown & Sachs, 1998). There are two large (>1 MDa) complexes containing Ccr4p and Caf1p that were previously identified as transcriptional regulators (Bai et al., 1999). These proteins have now been shown to copurify with a 3′ → 5′ exoribonuclease activity capable of removing poly(A) tails from RNA substrates (Tucker et al., 2001). Intriguingly, strains harboring individual deletions of the *CAF1*, *CCR4*, or *PAN2* genes exhibit only minor effects on mRNA deadenylation rates in vivo. However, mRNAs in a *pan2*Δ *ccr4*Δ double mutant strain retain long poly(A) tails (Tucker et al., 2001). This suggests that there may be multiple redundant deadenylases in yeast. There are homologs of Ccr4p, Caf1p, and Pan2p in higher eukaryotes. Indeed, a mammalian deadenylase, PARN has significant homology to the exonuclease domain of Pan2p and is responsible for poly(A) shortening both in vivo and in vitro (Korner et al., 1998; Gao et al., 2000).

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Following deadenylation, the decapping enzyme, Dcp1p, rapidly removes the 5′ cap (Beelman et al., 1996; LaGrandeur & Parker, 1998). Mutations in several genes including *DCP2, VPS16, LSM1-7,* and *PAT1* affect this step of mRNA decay (Dunckley & Parker, 1999; Zhang et al., 1999a; Bonnerot et al., 2000; Tharun et al., 2000). Although these proteins are required for decapping to proceed, their exact roles in the process remain unclear.

The final step in the decay process is degradation of the body of the mRNA. In yeast, the 5′-3′ exonuclease Xrn1p is predominantly responsible for this step (Brown et al., 2000), although 3′-5′ exonucleases do play a minor role (Jacobs et al., 1998).

The mRNA decay process is very closely linked to translation. Disruption of translation elongation can lead to a general stabilization of mRNAs (Parker et al., 1991; Peltz et al., 1992), whereas disruption of translation initiation can cause destabilization (Schwartz & Parker, 1999). The association of translation initiation factors, specifically eIF4F and poly(A)-binding protein, with the mRNA has been shown to circularize the message in vitro (Wells et al., 1998). This circular conformation is thought to stimulate translation and may also prevent degradation enzymes from accessing the ends of the mRNA. The poly(A)-binding protein (Pab1p/PABP) is generally accepted as the main mediator of poly(A) tail function. However, its precise role in regulation of mRNA decay is not clear. Binding of Pab1p to the poly(A) tail might be expected to prevent deadenylation, but in a yeast *pab1*Δ strain, deadenylation actually proceeds more slowly than in a wild-type strain (Caponigro & Parker, 1995). This implies that Pab1p can promote poly(A) shortening. Alternatively, slower deadenylation could be caused indirectly by the reduction in translation rates seen in the absence of Pab1p (Sachs & Davis, 1989). In a *pab1*Δ strain, mRNAs undergo premature decapping prior to completion of deadenylation, indicating that Pab1p also modulates decapping (Caponigro & Parker, 1995).

The interplay between translation and decay in vivo makes it difficult to interpret changes in mRNA decay rates in strains that exhibit alterations in translation. Mammalian in vitro assays have allowed studies of mRNA decay to be simplified by separating mRNA turnover from translation (Brewer & Ross, 1990; Ford et al., 1999; Wang et al., 1999). Historically, mechanistic studies of cellular processes have been greatly helped by complementary biochemical and genetic approaches. For example, the yeast mRNA splicing field used genetics to identify splicing factors whereas in vitro splicing assays greatly facilitated the elucidation of the splicing mechanism (Lin et al., 1985; Lustig et al., 1986). Similarly, the yeast mRNA decay field would be greatly aided by an in vitro system in which to dissect mRNA turnover mechanisms.

We have developed a novel cell-free assay system using yeast cytoplasmic extracts that exhibits both decapping and deadenylation of exogenously added mRNAs. This system faithfully reproduces regulation of decapping by the poly(A) tail and cap-binding proteins and has given us several insights into the mechanism by which the poly(A) tail functions in modulating mRNA turnover independent of its function in translation initiation. The assays described here will likely prove invaluable in dissecting the mechanisms of mRNA decay as we can now do biochemical experiments using extracts from an organism with well-established genetics.

RESULTS

Development of a regulated mRNA decapping assay

Previously published in vitro assays for decapping were able to assess large alterations in decapping activity but did not reproduce several important aspects of regulation (Zhang et al., 1999a, 1999b). Specifically, the presence of a poly(A) tail on the substrate mRNA did not affect the rate of decapping. We therefore set out to develop an in vitro system that would exhibit regulation of decapping by the poly(A) tail. We modified the procedure of Lin et al. (1985) to produce cytoplasm-enriched extracts capable of carrying out regulated decapping and deadenylation (see Materials and Methods). To obtain extracts with maximal activity, the salt concentration was kept low (50 mM or less) throughout the procedure. This minimizes contamination of the extracts with nuclear factors.

The RNA substrates for measuring decapping activity were transcribed in vitro and cap-labeled using recombinant vaccinia capping enzyme in the presence of α-^{32}P-GTP and S-adenosylmethionine as described previously (Ford & Wilusz, 1999; Zhang et al., 1999a). The poly(A)$^-$ GemA0 RNA is a 65-nt transcript derived from pGEM4 digested with *Hin*dIII. The poly(A)$^+$ GemA60 RNA is derived from a pGEM4 plasmid with a 60-base poly(A) stretch cloned into the *Hin*dIII site. These two RNAs are therefore identical except for the presence of a 3′ poly(A) tail. We chose these substrate RNAs as they are short and contain only polylinker sequences and are therefore unlikely to include sequences that might affect mRNA decay rates. Furthermore, these RNAs have been used successfully in mammalian in vitro decay assays (Ford & Wilusz, 1999; Ford et al., 1999). We have obtained similar results with longer RNAs containing MFA2 and SV40 RNA sequences (data not shown). All results presented here were repeated multiple times with at least two different extracts.

To test whether the poly(A) tail is capable of inhibiting decapping activity in vitro, we first compared the rates of decapping of the GemA0 and GemA60 transcripts. Substrate RNAs were incubated in the extracts for the indicated periods of time and the reaction products were separated by thin-layer chromatography. As shown in

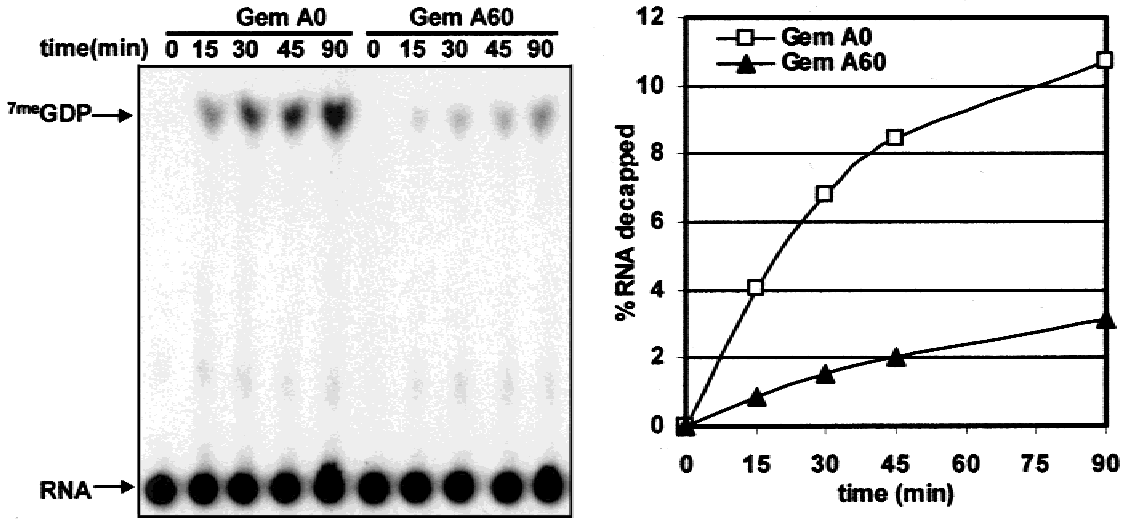

FIGURE 1. A poly(A) tail inhibits decapping. Cap-labeled GemA0 and GemA60 RNAs were incubated in extracts for the indicated times. Reaction products were separated by thin-layer chromatography. The left-hand panel shows a phosphorimage of the results. The right-hand panel graphs the quantitation of these results. The percent RNA decapped is determined by expressing the amount of ^{7me}GDP as a fraction of the total number of counts in each lane. The position of ^{7me}GDP was confirmed by UV shadowing of markers.

Figure 1, the intact RNA remained at the origin while the product of the decapping reaction (^{7me}GDP) migrated to the position indicated. The rate of decapping was estimated by quantifying the amount of radioactivity in the ^{7me}GDP spot. The results show that the polyadenylated substrate decaps four- to fivefold slower than the unadenylated RNA. This in vitro result is consistent with the poly(A) tail playing an inhibitory role as observed in vivo (Caponigro & Parker, 1995).

We compared decapping rates of GemA0 and GemA60 RNAs in the presence of increasing amounts of poly(A) competitor. As shown in Figure 2, addition of increasing amounts of poly(A) competitor to the extracts had a negligible effect on decapping of the unadenylated RNA. Significantly, the decapping of the GemA60 substrate was stimulated by addition of poly(A) competitor. In fact, in the presence of $>$250 ng of poly(A) competitor, both substrate RNAs decapped at the same rate. This effect was specific for poly(A) competitor as addition of even 500 ng of poly(C) had no effect on decapping of either substrate.

Addition of poly(A) competitor activates deadenylation

In principle, the addition of poly(A) competitor could be relieving the inhibition of decapping of polyadenylated RNA substrates in two ways. First, poly(A)-binding proteins may be inhibiting the decapping reaction directly via the Pab1p/eIF4F circularization of the mRNA. In this instance, the poly(A) competitor would compete off Pab1p and allow the decapping enzyme to attack the cap. Alternatively, addition of competitor poly(A) may result in loss of the poly(A) tail by a deadenylation process. In a mammalian in vitro decay system, addition of exogenous poly(A) results in activation of deadenylation and hence removal of the poly(A) tail (Ford et al., 1999).

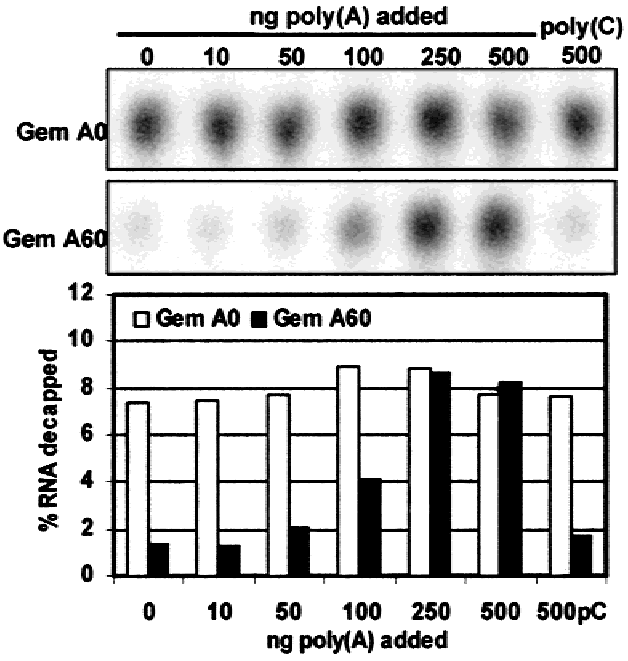

FIGURE 2. Addition of poly(A) competitor to extracts stimulates decapping of polyadenylated RNA. Cap-labeled GemA0 and GemA60 RNAs were incubated in extracts for 40 min. Reaction products were separated by thin-layer chromatography. The ^{7me}GDP products of the reaction from TLC plates are shown in the upper panels. The bar graph shows a quantitation of these results.

To distinguish between these two possibilities, we first tested whether deadenylation was occurring in our assay. We incubated cap-labeled substrate RNAs in extracts for 40 min with increasing amounts of poly(A) competitor. The reaction products were separated on a denaturing polyacrylamide gel and the results are shown in Figure 3A. The GemA0 RNA remained essentially stable in the extract both in the absence and presence of poly(A) competitor. There was only a slow shortening that occurred from the 3′ end regardless of whether poly(A) is added and this is likely due to nonspecific exonuclease activity in the extracts. The polyadenylated RNA was also slowly shortened in the absence of poly(A) competitor (compare input lane with 0 lane) but shortening was dramatically stimulated by addition of increasing amounts of competitor poly(A) but not poly(C). The more rapid, specific shortening of the GemA60 RNA is likely to be a deadenylation event as (1) it is activated by addition of poly(A) competitor similar to the mammalian deadenylating activity, (2) it does not occur on the GemA0 RNA that has no poly(A) tail, (3) it must be occurring in a 3′-to-5′ direction as the RNA is cap-labeled at the 5′ end, and (4) it is blocked by the presence of 36 non-A residues at the 3′ end of the RNA (see below and Fig. 4A). Following deadenylation, it appears that both 3′-5′ and 5′-3′ exonucleases can act on the body of the RNA as a poly(G) tract inserted into the GemA60 RNA can trap intermediates with sizes consistent with both pathways of decay (data not shown).

Poly(A)-binding protein is an inhibitor of deadenylation and decapping

We suggest that removal of Pab1p from the RNA substrate by addition of poly(A) competitor allows the deadenylase access to the poly(A) tail. Consistent with this hypothesis, addition of increasing amounts of poly(A) to the extracts results in reduced crosslinking of Pab1p to ^{32}P-ATP-labeled GemA60 RNA (Fig. 3B). In yeast extracts, there are two forms of Pab1p that migrate at 68 kDa and 55 kDa (Sachs et al., 1986). The smaller peptide is derived by proteolysis from the 68 kDa species. As seen in Figure 3B, both these proteins crosslinked efficiently only to the polyadenylated RNA and were competed specifically by poly(A). Both the 55-kDa and 68-kDa crosslinked bands could be immunoprecipitated with anti-Pab1p antibody (data not shown). The amount of poly(A) required to activate deadenylation correlates well with the amount required to compete the crosslinking of Pab1p indicating that Pab1p is a major inhibitor of deadenylation in these extracts.

As a final confirmation of this hypothesis, we added recombinant 6xHis-tagged Pab1p to the extracts after preincubation with poly(A) competitor. As can be seen in Figure 3C, addition of 15 pmol of Pab1p to the deadenylation assay resulted in complete inhibition of deadenylation. Addition of a similar quantity of 6xHis-tagged U1A protein had no effect on deadenylation, indicating that the inhibition was specific. This is the

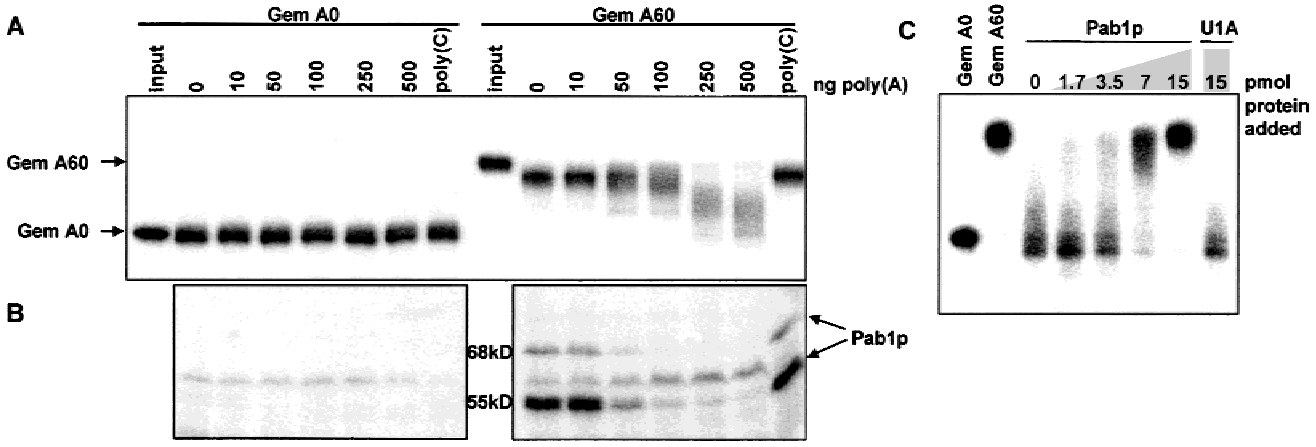

FIGURE 3. A: Addition of poly(A) competitor specifically activates a deadenylation process. Cap-labeled GemA0 and GemA60 RNAs were incubated in extracts with the indicated amounts of competitor RNAs for 40 min. The reaction products were recovered and separated on a denaturing polyacrylamide gel. The positions of the intact RNAs are indicated. Lanes marked poly(C) contain 500 ng poly(C) competitor. **B:** Activation of deadenylation correlates with a loss of binding of Pab1p to the GemA60 RNA. ^{32}P-A-labeled GemA0 and GemA60 RNAs were incubated with extracts and then crosslinked using UV light. The RNA was degraded with RNAse A and RNase One and the crosslinked proteins were separated on a 10% SDS polyacrylamide gel. The position of the two Pab1p proteins is indicated. **C:** Addition of Pab1p results in inhibition of deadenylation. Extracts were preincubated with 50 ng of poly(A) in buffer containing 20 mM Tris, pH 7.9, 50 mM NaCl for 5 min at 30 °C and then the indicated amount of recombinant Pab1p or U1A was added along with substrate RNA. Following a further 30-min incubation at 30 °C, the reaction products were recovered and separated on a 6% denaturing polyacrylamide gel. The first two lanes contain untreated Gem A0 and Gem A60 RNAs.

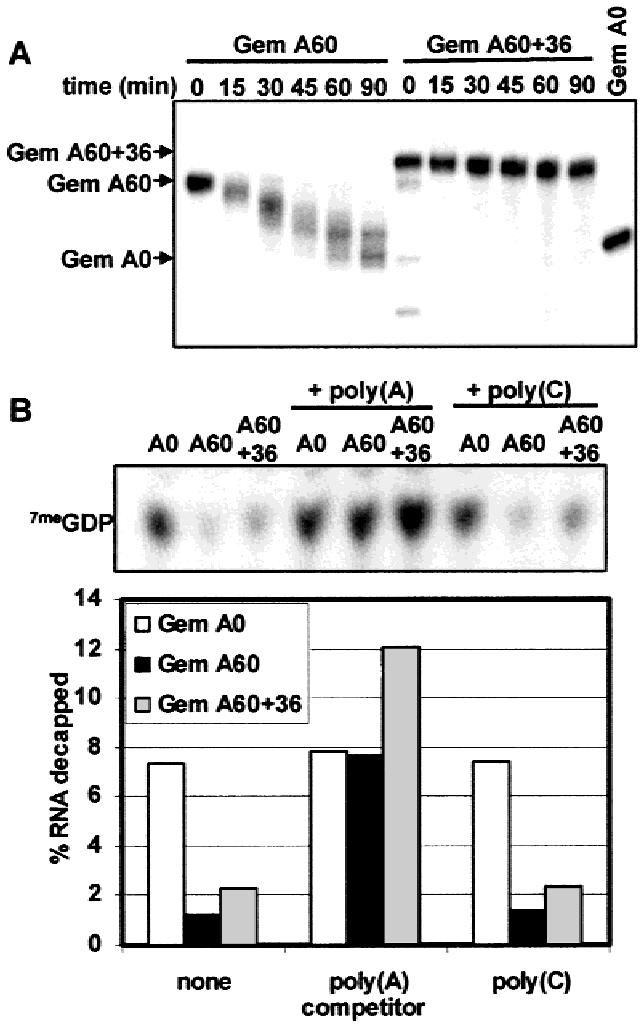

FIGURE 4. A: Deadenylation is blocked by the addition of 36 non-A residues at the 3′ end of the RNA. Cap-labeled Gem A60 and GemA60+36 RNAs were incubated in extracts containing 250 ng of poly(A) competitor for the indicated periods of time. Reaction products were recovered and separated on a denaturing polyacrylamide gel. The positions of the intact RNAs are shown. **B:** Decapping of poly(A)-containing RNA can still be activated by addition of poly(A) competitor even when deadenylation is blocked. Cap-labeled Gem A0, Gem A60, and GemA60+36 RNAs were incubated in extracts in the absence of competitors (lanes 1–3), in the presence of poly(A) competitor (lanes 4–6) or in the presence of poly(C) competitor (lanes 7–9). Reaction products were separated by TLC and the upper panel shows the ^{7me}GDP products from the TLC plate; the lower panel shows quantitation of the results.

first report of a yeast deadenylase activity that can be inhibited by poly(A)-binding proteins.

Although deadenylation was not complete until later time points, it was still formally possible that the loss of the poly(A) tail was resulting in relief of inhibition of decapping on addition of poly(A) competitor. We therefore made an RNA with an internalized poly(A) tail (Gem A60+36). This RNA is identical to GemA60 except that there are 36 non-A nucleotides 3′ of the poly(A) stretch that are designed to protect the poly(A) tail from deadenylation. Figure 4A compares deadenylation of the GemA60 and GemA60+36 RNAs in the presence of 250 ng poly(A). During the course of the experiment, the GemA60 RNA was deadenylated and a product of the same size as the GemA0 RNA accumulated. The GemA60+36 RNA remained stable over the course of the experiment, demonstrating again that the 3′-5′ exonuclease activity we observe is a deadenylase, as it is poly(A)-specific and cannot act on non-poly(A) stretches.

We next compared decapping of the GemA60+36 RNA with that of the other two substrates. As seen in Figure 4B, decapping of GemA60+36 was threefold less efficient than that of the GemA0 RNA, indicating that the poly(A) tail does not have to be at the 3′ end of the RNA to inhibit decapping (compare lane 1 with lane 3). Addition of poly(A) competitor stimulated decapping of both GemA60 and GemA60+36 RNAs approximately fivefold even though the GemA60+36 RNA cannot be deadenylated. As poly(A) binding protein can still be crosslinked to the GemA60+36 RNA (data not shown), we conclude that the interaction of poly(A) binding factors with the poly(A) tail mediates inhibition of both decapping and deadenylation. It should be noted that the GemA60+36 RNA is decapped approximately 1.5-fold more efficiently than the GemA0 RNA in the presence of poly(A). This could be due to a previously observed preference of the decapping enzyme for longer RNAs (LaGrandeur & Parker, 1998) or perhaps the additional 36 nt contain a sequence that promotes decapping.

Cap-binding proteins act as inhibitors of decapping

The results described above suggest that Pab1p is an important regulator of both mRNA decapping and deadenylation in the in vitro system. We next asked whether the inhibitory effects of Pab1p are mediated through its interaction with the cap-binding complex (eIF4F). We monitored the rates of decapping in the presence of increasing amounts of cap analog (Fig. 5A). We anticipated that the cap analog would titrate cap-binding proteins, leaving the cap accessible to the decapping enzyme. It should be noted that the decapping enzyme itself has very low affinity for cap analog (LaGrandeur & Parker, 1998). Addition of cap analog resulted in stimulation of decapping of both GemA0 and GemA60 RNAs. We found that decapping of GemA0 RNA was stimulated 1.5-fold whereas decapping of GemA60 RNA was increased 2.5-fold by addition of 25 μM cap analog (Fig. 5A). We also assayed the interaction of eIF4E with the cap by crosslinking under the same conditions (Fig. 5A, lower panel). The band shown here could be immunoprecipitated with anti-eIF4E antibodies (data not shown). There is a clear correlation between inhibition of eIF4E crosslinking and activation of decapping. It is important to note that even

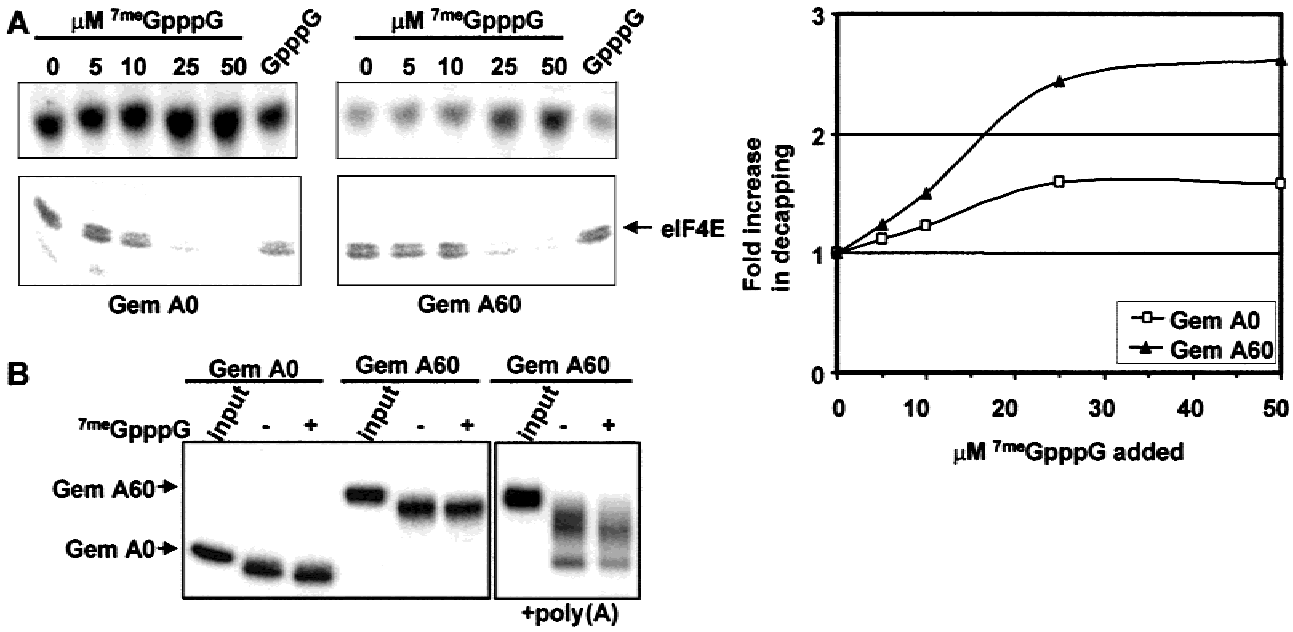

FIGURE 5. A: Cap analog competes for binding of eIF4E to the cap and stimulates decapping of both GemA0 and GemA60 RNAs. Cap-labeled GemA0 and GemA60 RNAs were incubated in extracts in the presence of the indicated concentrations of cap analog. The upper panel shows the ^{7me}GDP product of the decapping reaction after 40 min of incubation. The graph shows quantitation of the decapping results as the fold increase in decapping over the amount seen in the absence of cap analog. In the lower panel, extracts were crosslinked to cap-labeled RNA using UV light after 5 min of incubation. Following digestion of the substrate RNA, the crosslinked proteins were separated on a 12% SDS polyacrylamide gel. The eIF4E crosslinked band is shown. **B:** Addition of cap analog has no effect on deadenylation. Cap-labeled GemA0 and GemA60 RNAs were incubated in extracts for 40 min in the presence or absence of 25 μM cap analog. The reaction products were recovered and separated on a denaturing polyacrylamide gel. In the reactions shown in the right panel, 250 ng of poly(A) was also included to stimulate deadenylation.

when stimulation of decapping by cap analog is maximal, and all the eIF4E has been competed from the cap, polyadenylated RNA still decaps approximately threefold slower than unadenylated RNA. Therefore, the effects of the poly(A) tail on decapping cannot be mediated entirely through the eIF4E protein.

Finally, we investigated the effect of addition of cap analog on the deadenylation reaction. The cap analog did not stimulate deadenylation in the absence of poly(A) and the rates of deadenylation in the presence of 250 ng poly(A) were unchanged when cap analog was included in the reaction at a concentration of 25 μM (Fig. 5B). In addition, capped and uncapped substrate RNAs deadenylate at the same rate (data not shown). Experiments with longer RNAs gave similar results (data not shown). This suggests that the yeast deadenylase may differ from the mammalian deadenylase, DAN/PARN, which can be inhibited by cap analog and is stimulated by the presence of a cap on the substrate RNA (Dehlin et al., 2000; Gao et al., 2000).

DISCUSSION

We have developed a simple and reproducible in vitro yeast mRNA decay system that exhibits several aspects of regulated mRNA turnover. Our extracts demonstrate both deadenylation and decapping, the two most regulated steps of mRNA decay. They also display regulation of these processes by the poly(A) tail, poly(A)-binding proteins, and cap-binding proteins. Importantly, the system we have developed separates the decapping and deadenylation processes from translation. This allowed us to characterize the roles of cap-binding and poly(A)-binding factors in regulating mRNA decapping and deadenylation independently of their effects on translation.

The poly(A) tail is known to be influential in many events during the life of an mRNA. There is substantial evidence that the majority of its functions are mediated by the poly(A) binding protein, Pab1p. In vivo, deletion of the *PAB1* gene results in premature decapping of mRNAs prior to deadenylation indicating that Pab1p normally plays a role in inhibiting decapping (Caponigro & Parker, 1995). Our results are consistent with this hypothesis. We find that the poly(A) tail is able to inhibit decapping of an exogenous substrate RNA efficiently and removal of poly(A)-binding proteins by competition relieves this inhibition. As Pab1p forms a complex with the eIF4G protein and the cap-binding protein eIF4E, we supposed that this interaction might be circularizing the RNA and obstructing access of the decapping enzyme to the cap. Consistent with this idea,

addition of cap analog to the extracts competed eIF4E from the cap and stimulated decapping. This result extends the observation made by Schwartz and Parker (2000) that recombinant eIF4E can inhibit the activity of recombinant Dcp1p. However, if the entire inhibitory effect of Pab1p was mediated by eIF4E, one would expect addition of cap analog to completely abrogate the effect of the poly(A) tail. This was not the case. Even at high concentrations of cap analog, which prevent eIF4E crosslinking to the cap, the poly(A) tail still significantly inhibited decapping (Fig. 5). A similar observation was made with regards to a mammalian decapping activity. To observe decapping in HeLa extracts, cap-binding proteins must be removed from the RNA by competition with cap analog, yet the poly(A) tail is still able to inhibit decapping (Gao et al., 2001). These results are intriguing because they suggest a novel mode of action for Pab1p. They may also explain why a temperature-sensitive mutation in eIF4E that reduces its interaction with the cap causes accelerated mRNA turnover but does not result in premature decapping (Altmann & Trachsel, 1989; Schwartz & Parker, 1999). The eIF4E mutation may cause more rapid deadenylation because the cap/poly(A) tail interaction is reduced allowing the deadenylase access to the poly(A) tail. However, the poly(A) tail can still inhibit decapping through an eIF4E-independent mechanism even though the cap is not protected by eIF4E.

Poly(A)-binding proteins also inhibit the deadenylation process in our system. We believe that the activity we observe is a true deadenylase, as it degrades RNA in a 3′-to-5′ direction, is poly(A)-specific, and is inhibited by poly(A)-binding proteins. We therefore surmise that the poly(A) shortening activity we detect is distinct from the Pan2/Pan3 complex previously identified, as Pan2/Pan3 degrades only mRNA bound to Pab1p (Brown & Sachs, 1998). Preliminary experiments also suggest that the activity we observe is not dependent on Ccr4p or Caf1p (C.J. Wilusz and S.W. Peltz, unpubl. results). Our deadenylase activity appears similar to that seen in mammalian extracts in that it requires addition of exogenous poly(A) for activation (Ford et al., 1999). However, unlike the mammalian enzyme, the poly(A) shortening activity we observe does not appear to be stimulated by interaction with the cap (Dehlin et al., 2000; Gao et al., 2000).

Our system uncouples the various steps of mRNA decay as we observe some decapping of polyadenylated RNA. Also, the substrate RNA remains relatively stable in the reaction following deadenylation and accumulates as an intermediate of the same size as unadenylated RNA. This differs from the mammalian in vitro assay in which decapping can only be observed in the presence of cap analog (Gao et al., 2001), and deadenylated intermediates do not accumulate due to the presence of very active 3′-5′ exonucleases (Ford et al., 1999). One might expect the deadenylation process to promote decapping, but there does not appear to be a change in the rate of decapping of polyadenylated RNA at later time points as deadenylation is completed (see Fig. 1). This may be simply because the decapping activity does not survive this long in the reaction or, alternatively, the translation process may be required for such a mechanism.

Our results suggest that the circularization of the mRNA by the eIF4F/Pab1p interaction accounts for only part of the inhibition of decapping by the poly(A) tail. The poly(A) tail can inhibit decapping even in the absence of eIF4E binding to the cap, indicating that it must be functioning by some other mechanism. It seems that poly(A)-binding proteins can also inhibit decapping by an eIF4E-independent mechanism. Vilela et al. (2000) recently demonstrated an interaction between the decapping enzyme, Dcp1p, and eIF4G. They hypothesized that eIF4G recruits the decapping enzyme to the 5′ end of the RNA, as it stimulated decapping in an assay using recombinant proteins. However, we suggest that in our system, eIF4G could also act as a poly(A)-dependent inhibitor, perhaps activating decapping only when deadenylation is complete. Pab1p could also potentially be acting independently of eIF4G. Pab1p has been previously shown to function in an eIF4G-independent manner in the translation process (Otero et al., 1999). It is therefore feasible that Pab1p could be functioning similarly in our assay to inhibit degradation. A final hypothesis is that a protein other than Pab1p is interacting specifically with the poly(A) tail and promoting cap-independent inhibition of mRNA decapping. These ideas will be tested in future studies using extracts from mutant yeast strains.

In summary, we have developed an in vitro system that for the first time allows us to study both deadenylation and decapping of mRNAs in the same reaction. We have shown that poly(A)-binding proteins are an important regulator of both of these steps of mRNA decay in our system. Indeed, the poly(A) tail appears to inhibit decapping by at least two different mechanisms. The development of this system will allow us to purify and identify the yeast deadenylase activity, and to study in detail the roles of Pab1p and other translation factors in regulating the mRNA decay process. We also anticipate that the system will prove invaluable in dissecting the mechanisms of mRNA decay. The combination of a cell-free mRNA decay assay and yeast genetics is a powerful approach to study the roles of specific proteins in the mRNA turnover process.

MATERIALS AND METHODS

Plasmids and yeast strains

The yeast strain Y137 (*MAT a ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,112 can1-100*) was used for all experiments

shown here, but extracts from other strains and strain backgrounds have been used successfully.

The plasmid pGEM4 (Promega) was linearized with *Hin*dIII to make the template for transcription of GemA0 RNA. To make pGEMA60, pGEM4 was digested with *Hin*dIII and *Pvu*II and a fragment consisting of annealed oligos A60 (AGCT$(A)_{60}$TATTACCTCGAGCACTC) and T60 (GAGTGCT CGAGGTAAATA$(T)_{60}$) was cloned in. Digestion of this plasmid with *Ssp*I gave the template for transcription of GemA60 RNA. The RNA transcribed from this plasmid has a 60-base poly(A) tail with a single T residue at the 3′ end. Digestion of pGEMA60 with *Hae*III gave the template for transcription of GemA60+36 RNA. RNAs were in vitro transcribed using SP6 polymerase. Cold RNAs were cap-labeled using vaccinia capping enzyme, S-adenosyl-methionine, and α-^{32}P-GTP. RNAs for crosslinking to the poly(A) tail were internally labeled using α-^{32}P-ATP. The RNA used in the experiment shown in Figure 3C was internally labeled with α-^{32}P-UTP and capped cotranscriptionally. All RNAs were purified on denaturing polyacrylamide gels as described previously (Ford & Wilusz, 1999).

Decapping extracts

The protocol for making the decapping extracts was based on that described by Lin et al. (1985). One liter of culture was grown to mid-log phase in YPD media. The cells were pelleted and resuspended in 15 mL of spheroplast buffer (1 M sorbitol, 50 mM Tris-Cl, pH 7.9, 10 mM $MgCl_2$). DTT was added to a final concentration of 30 mM and the cells were kept at room temperature for 15 min. After spinning down and resuspending in fresh spheroplast buffer containing 3 mM DTT, 2.5 mg of Zymolase 20T(ICN) were added per gram of cells. The cells were mixed gently at room temperature and generation of spheroplasts was monitored under the microscope. The spheroplasts were spun down, washed in ice-cold spheroplast buffer (3 mM DTT) and finally resuspended in an equal volume of ice-cold buffer A (20 mM HEPES pH 7.0, 10 mM KCl, 1 mM DTT). All subsequent steps were carried out at 4 °C. The spheroplasts were lysed by Dounce homogenization, and the cell debris was removed by spinning at 10,000 × *g* for 15 min. It is necessary to keep the salt concentration low during lysis to minimize contamination with nuclear proteins. The supernatant was adjusted to 50 mM KCl, and spun at 100,000 × *g* for 1 h. The extract was finally dialyzed against 100 vol of 20 mM HEPES, pH 7.0, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 20% glycerol for 1 h and stored in aliquots at −70 °C. The extracts were unaffected by multiple freeze–thaw cycles and retained activity for at least 1 year. Protein concentrations were typically between 10 and 20 mg/mL by the BioRad Assay.

Decapping assay

Extracts were thawed on ice and reactions were set up using approximately equivalent amounts of protein (50–100 μg per assay). Each reaction contained 2 mM $MgCl_2$ and 25–50,000 cpm of cap-labeled RNA. Reaction volumes were made the same using 1× decapping buffer (20 mM HEPES, pH 7.0, 50 mM KCl, 1 mM DTT). Reactions were generally carried out at 30 °C for 40 min unless otherwise indicated. Reactions were stopped by addition of 1 μL 0.1 M EDTA and the products were separated by thin-layer chromatography as described in Zhang et al. (1999b). In some experiments, competitors (poly(A) or cap analog) were added to the and the reactions were then incubated at 30 °C for 5 min before the substrate RNA was added.

Deadenylation assay

Deadenylation reactions were generally set up exactly as described for decapping above but poly(A) competitor was included as indicated. The reactions were terminated by addition of 400 μL STOP solution (25 mM Tris-Cl, pH 7.5, 400 mM NaCl, 0.1% SDS). Following phenol extraction and ethanol precipitation, the reaction products were separated on a 6% denaturing polyacrylamide gel.

UV crosslinking

Decapping reactions were set up as above and incubated at 30 °C for 5 min. The reactions were irradiated with 254 nm UV light for 10 min. Ten micrograms of RNAse A and 1 U of RNase One (Promega) were added and the reactions were incubated at 37 °C for 10 min. Crosslinked proteins were separated on a 10% SDS polyacrylamide gel.

Recombinant proteins

Recombinant 6xHis-tagged Pab1p was expressed in *Escherichia coli* and purified as previously described (Kessler & Sachs, 1998). Recombinant 6xHis-tagged U1A protein was a gift from Dr. Sam Gunderson.

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