PUF proteins control gene expression by binding to the 3′-untranslated regions of specific mRNAs and triggering mRNA decay or translational repression. Here we focus on the mechanism of PUF-mediated regulation. The yeast PUF protein, Mpt5p, regulates HO mRNA and stimulates removal of its poly(A) tail (i.e. deadenylation). Mpt5p repression in vivo is dependent on POP2, a component of the cytoplasmic Ccr4p-Pop2p-Not complex that deadenylates mRNAs. In this study, we elucidate the individual roles of the Ccr4p and Pop2p deadenylases in Mpt5p-regulated deadenylation. Both in vivo and in vitro, Pop2p and Ccr4p proteins are required for Mpt5p-regulated deadenylation of HO. However, the requirements for the two proteins differ dramatically: the enzymatic activity of Ccr4p is essential, whereas that of Pop2p is dispensable. We conclude that Pop2p is a bridge through which the PUF protein recruits the Ccr4p enzyme to the target mRNA, thereby stimulating deadenylation. Our data suggest that PUF proteins may enhance mRNA degradation and repress expression by both deadenylation-dependent and -independent mechanisms, using the same Pop2p bridge to recruit a multifunctional Pop2p complex to the mRNA.

Regulation of mRNA stability, translation, and localization ensure that a given mRNA produces the right amount of protein at the proper time and place. These events are often controlled by elements in the 3′-untranslated region (3′-UTR) of the mRNA (1, 2). mRNA stability and translational regulation are linked to cytoplasmic changes in poly(A) tail lengths (3, 4). In particular, poly(A) shortening (deadenylation) is correlated with translational repression and mRNA decay (1, 2). Specific regulatory proteins and micro-RNAs bind to 3′-UTR elements to promote poly(A) shortening and either repress translation or destroy the mRNA, or both (2, 5–7).

The *Saccharomyces cerevisiae* protein Mpt5p is a member of one such family of regulatory proteins, the so-called PUF proteins (8). These proteins promote deadenylation, decay, and translational repression. Mpt5p binds to the 3′-UTR of multiple target mRNAs (9–11) and stimulates their deadenylation and decay. In particular, Mpt5p binds a regulatory element in the 3′-UTR of HO mRNA and causes rapid deadenylation and decay of that mRNA (12, 13). The HO endonuclease is tightly controlled at multiple levels to prevent inappropriate mating-type switching and aberrant double-stranded DNA breaks (14). The Mpt5p repressor contributes to that regulation; in its absence, aberrant switching occurs at high frequency (12).

Recently, using a genetic assay, we showed that repression by Mpt5p requires the POP2 gene and that PUF proteins, including Mpt5p, bind directly to Pop2p (13). Pop2p is a subunit of the major cytoplasmic deadenylase complex, the Ccr4p-Pop2p-Not complex (15, 16), thus providing a direct link between PUF proteins and the deadenylation machinery. Two of the Ccr4p-Pop2p-Not complex subunits, Pop2p and Ccr4p, bear sequence similarity to nucleases, and both proteins have been reported to possess deadenylase activity in vitro (17–20). However, Ccr4p is thought to be the predominant deadenylase in yeast, at least under standard growth conditions (17, 18, 21). The finding that Pop2p was critical for PUF-mediated regulation of HO mRNA suggested that it might act as a deadenylase on that mRNA (13); it is controversial whether it contributes general deadenylase activity in vivo (15, 17, 19, 20, 22, 23). The Pop2p deadenylase may be regulated (20, 21, 23), and Mpt5p might stimulate its enzymatic activity as well as target it to specific mRNAs.

The individual roles of the Pop2p and Ccr4p deadenylases in regulated mRNA decay are not understood. We sought to determine which deadenylase was responsible for PUF-stimulated deadenylation and to delineate the roles of Pop2p and Ccr4p. Our data suggest that Pop2p acts as a bridge through which the PUF protein recruits the Ccr4p enzyme to the mRNA. Additional data suggest that PUF proteins may repress expression by deadenylation-dependent and -independent mechanisms, using the same Pop2p bridge.

**EXPERIMENTAL PROCEDURES**

*Strains and Plasmids*—The wild-type BY4742 yeast strain and isogenic strains with gene-specific deletions of POP2, CCR4, PAN2, CAF120, CAF130, NOT3, and NOT4 were obtained from Open Biosystems. These deletion strains were created by PCR-mediated gene modification using the kanamycin/G418 resistance marker. The *MPT5*-TAP strain (Open Biosystems) was created in the S288C strain background by integrating a C-terminal TAP tag onto the coding sequence of *MPT5* using PCR-mediated gene modification with a HIS3 marker.

*POP2 and CCR4* expression plasmids were created in the high copy vector pACG1-NT and contained N-terminal His6 and T7 epitope tags that could be cleaved off using TEV prote-
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The ADH1 promoter and 3'–UTR were used for expression, and the plasmids carried the Zeocin resistance marker. The active site mutant POP2 contains two missense mutations, S188A/E190A, described by Thore et al. (20). The active site mutant CCR4 contains missense mutation E556A described by Chen et al. (18). Both mutants were created by QuikChange (Stratagene) site-directed mutagenesis. Deadenylation assays were performed using the reporter gene construct described by Goldstrohm et al. (13). In either wild-type BY4742 or deletion strains as indicated in Fig. 4 and supplemental Fig. S1. Conditions identical to the TAP-grown to OD660 nm of 1.5 in YPAD with 300 µg/ml Zeocin (Invivogen). Cells were washed four times with 150 bed volumes of TNEMN150 and then one time with 150 bed volumes of deadenylation buffer (50 mM Tris–HCl, pH 8.0, 20 mM NaCl, 0.1 mM MgCl2, 10% glycerol) (25). Bound proteins were eluted from beads in 1 bed volume of deadenylation buffer using 8 units of AcTEV protease (Invitrogen) for 12 h at 4°C. Purified complexes were evaluated by silver staining and by deadenylation assays.

In Vitro Deadenylation Assays—Deadenylation reactions were carried out in a 20-µl volume in deadenylation buffer (50 mM Tris–HCl, pH 8, 20 mM NaCl, 0.1 mM MgCl2, 10% (v/v) glycerol) and 10 µM nonspecific competitor RNA (synthetic oligoribonucleotide with sequence 5′-UCUAAUCGGGG-UACAAUUAAUAAUAUAA-3′). HO substrate RNA (Integrated DNA Technologies) with sequence 5′-AGUUUAAA-AAGUUGUAUGUAAAAAGAUAU-3′ was radioactively labeled with T4 polynucleotide kinase (Promega) at the 5′ end and added to the reactions at a final concentration of 10 nM. Recombinant purified GST-Mpt5p RBD (250 nM) was added to reactions where indicated. Purified Pop2p or Ccr4p complexes (10 ng each) were added to their respective reactions. In initial experiments, we carefully titrated these complexes to measure their deadenylase activity (supplemental Fig. S1), and then balanced the amount and activity used in the final experiments shown in Fig. 4.

RESULTS

CCR4 and POP2 Are Required for Deadenylation of HO mRNA in Vivo—Mpt5p stimulates deadenylation of HO mRNA in vivo and in vitro (13). We sought to determine the contributions of Pop2p and Ccr4p to PUF-stimulated deadenylation. We first analyzed the length of poly(A) on endogenous HO mRNA in wild-type cells and in strains with deletions of genes encoding subunits of Ccr4p-Pop2p-Not complex. We focused initially on a comparison of wild-type, pop2Δ and ccr4Δ deletion strains. We used thiolutin to inhibit transcription and then collected samples over a time course to observe the degradation of the poly(A) tails. Total RNA was extracted from each sample and cleaved with ribonuclease H and a specific antisense oligonucleotide to produce a short HO 3′-end fragment. To measure the poly(A) length of this 3′-fragment, we performed high resolution polyacrylamide electrophoresis and Northern blotting. The noncoding SCR1 RNA served as a loading control.

In wild-type cells, HO poly(A) tails displayed a broad distribution of poly(A) lengths, from 5 to 80 nucleotides (Fig. 1A, lanes 1–7). The long HO poly(A) tails were removed rapidly; the oligo-adenylated (A₅₋₁₅) form disappeared more slowly, most likely via the well-established deadenylation-dependent decapping pathway (21).

In pop2Δ cells, the distribution of poly(A) ranged from 20 to 60 nucleotides; poly(A) lengths below 20 nucleotides were not detected (Fig. 1A, lanes 8–14). Over the time course, the longer tails were shortened to 20–40 adenosines (lane 14) and the mRNA disappeared slowly, without further deadenylation (see below). In addition to this effect on steady-state mRNA distri-
HO mRNA poly(A) tail length was measured by denaturing polyacrylamide gel electrophoresis and Northern blotting of the HO 3′-UTR in wild-type yeast strain (WT), pop2 deletion strain, or ccr4 deletion strain. Total RNA samples were collected over a time course, indicated at the top of each panel in minutes (min), following addition of the transcription inhibitor Thiolutin. To measure poly(A) tails accurately, ribonuclease H and a specific antisense oligonucleotide (Ambion) were added to the RNA and digested using ribonuclease H to remove the poly(A) tail, to provide a marker for deadenylated mRNA. Endogenous SCR1 RNA, detected by hybridization to the same blot, served as a loading control. A, HO mRNA poly(A) tail lengths (A80, A40, A15, A5) were determined based on migration of bands compared with an RNA molecular weight marker (Century Plus, Ambion). Where indicated by an “+” at the top, oligo(dt) was added to the RNA and digested using ribonuclease H to remove the poly(A) tail, to provide a marker for deadenylated mRNA. Endogenous SCR1 RNA, detected by hybridization to the same blot, served as a loading control. B, HO mRNA poly(A) tail lengths in yeast strains with gene-specific deletions of mRNA decay factors were required for deadenylation of HO mRNA. Deletions of these same genes also did not disrupt repression by Mpt5p in vivo (13).

FIGURE 1. **CCR4 and POP2 are required for deadenylation of HO mRNA in vivo.** A, HO mRNA poly(A) tail length was measured by denaturing polyacrylamide gel electrophoresis and Northern blotting of the HO 3′-UTR in wild-type yeast strain (WT), pop2 deletion strain, or ccr4 deletion strain. Total RNA samples were collected over a time course, indicated at the top of each panel in minutes (min), following addition of the transcription inhibitor Thiolutin. To measure poly(A) tails accurately, ribonuclease H and a specific antisense oligonucleotide (Ambion). Where indicated by an “+” at the top, oligo(dt) was added to the RNA and digested using ribonuclease H to remove the poly(A) tail, to provide a marker for deadenylated mRNA. Endogenous SCR1 RNA, detected by hybridization to the same blot, served as a loading control. A, HO mRNA poly(A) tail lengths (A80, A40, A15, A5) were determined based on migration of bands compared with an RNA molecular weight marker (Century Plus, Ambion). Where indicated by an “+” at the top, oligo(dt) was added to the RNA and digested using ribonuclease H to remove the poly(A) tail, to provide a marker for deadenylated mRNA. Endogenous SCR1 RNA, detected by hybridization to the same blot, served as a loading control. B, HO mRNA poly(A) tail lengths in yeast strains with gene-specific deletions of mRNA decay factors were required for deadenylation of HO mRNA. Deletions of these same genes also did not disrupt repression by Mpt5p in vivo (13).

**CCR4 Deadenylase Activity Is Crucial for HO Deadenylation in Vivo.**—To determine whether Ccr4p nuclease activity is required for deadenylation of HO mRNA, we introduced a wild-type or mutant CCR4 gene on an episome into ccr4 mutant cells and again assayed the length of poly(A) on HO mRNA (Fig. 2C). CCR4 deletion mutants exhibited a severe defect in deadenylation that was not rescued by empty vector (Fig. 2C, lane 2 versus 3). The wild-type distribution of poly(A) tail lengths was restored by episomal CCR4 (lane 4). A missense mutation in CCR4 gene (CCR4 mt), bearing a single amino acid substitution (E556A) that inactivates Ccr4p nuclease activity (18), completely abrogated the rescue of activity (lane 5). Both wild-type and mutant Ccr4p were expressed at the same levels. We conclude that the CCR4 nuclease activity is crucial for normal deadenylation of HO mRNA in vivo but its enzymatic activity is not.

**POP2 Protein, but Not Its Enzymatic Activity, Is Required for HO Deadenylation in Vivo.**—The dependence of deadenylation and repression on Pop2p suggested that its deadenylase activity might be essential for PUF action. To test this idea, we determined whether Pop2p enzymatic activity was required for PUF-stimulated deadenylation in vivo. We analyzed HO poly(A) tails in pop2 cells carrying a wild-type copy (POP2) or mutant form of POP2 (POP2 mt) on an episome (Fig. 2A). The mutation in POP2 comprised two missense substitutions (S188A/E190A) that inactivate its deadenylase activity in vitro (20). HO mRNA in these strains was compared with the wild-type strain (Fig. 2A, lane 2) and to a pop2 mutant containing an empty vector (lane 3). pop2 mutant cells displayed the expected deadenylation defects, accumulating mRNAs with tails between 20 and 60 nucleotides in length (lane 3). The wild-type POP2 gene restored deadenylation fully (lane 4). The missense mutant form of POP2 also yielded a wild-type HO poly(A) pattern (lane 5). Both wild-type and mutant Pop2p were expressed at the same level (data not shown). To determine whether the mutant form of POP2 still associated with Mpt5p, we determined whether Pop2p co-immunoprecipitated with Mpt5p (Fig. 2B). Both mutant and wild-type forms of Pop2p interact with Mpt5p in yeast extracts (Fig. 2B). We conclude that the POP2 protein is important for normal deadenylation of HO mRNA in vivo but its enzymatic activity is not.

**Deletion of Other Deadenylase Complex Components Does Not Affect HO Poly(A) Tails.**—To determine whether other mRNA decay factors were required for deadenylation of HO mRNA, we analyzed steady-state mRNA from a series of mutant strains, each lacking various turnover-related components. The same high resolution Northern blotting method as above was used to determine HO poly(A) tail lengths accurately (Fig. 1B). Deletion of CCR4 again caused a complete block in deadenylation (Fig. 1B, lane 2 versus 3). Mutants lacking Pan2p, the catalytic subunit of the Pan2p-Pan3p deadenylase complex, showed little effect on HO poly(A) tail lengths; a slight increase in the length of the longest poly(A) tails was observed (Fig. 1B, lane 4) and is consistent with a role for the Pan2p-Pan3p complex in initial poly(A) tail trimming (26). Deletions of components of the Ccr4p-Pop2p-Not complex, including Caf120p, Caf130p, Not3p, and Not4p, had no apparent effect on HO poly(A) tail length (lanes 5–8). Defects in other Ccr4p-Pop2p-Not complex components were not tested because those mutants were inviable.

We conclude that the Pan2p deadenylase has very little effect on removal of HO poly(A) tails and that at least four proteins in the described Ccr4p-Pop2p-Not complex are not necessary for HO mRNA deadenylation. Deletions of these same genes also did not disrupt repression by Mpt5p in vivo (13).
PUF Protein-mediated Deadenylation

**Mpt5p**

**Repression Is Not Affected by Pop2p Active Site Mutations**—To determine whether the enzymatic activity of Pop2p was required for PUF repression, we used a HIS3 reporter gene containing the **HO** 3′-UTR (13). Mpt5p binds to the **HO** 3′-UTR and specifically represses the HIS3-HO mRNA, which can be assayed on medium lacking histidine and containing 3-amino triazole, a competitive inhibitor of the HIS3-encoded protein (Fig. 3). When Mpt5p is overexpressed in wild-type cells, the reporter mRNA is repressed and the cells no longer grow on medium lacking histidine (Fig. 3, WT strain). In pop2 mutant cells, Mpt5p-mediated repression was restored by either the wild-type Pop2p or the catalytically inactive Pop2p mutant (Fig. 3, pop2 strain). Thus, we conclude that whereas POP2 protein is necessary, its deadenylase activity is not. Because deletion of **CCR4** has only a modest effect on Mpt5p repression (13), these findings suggest that deadenylation may not be the only mechanism involved in Mpt5p-mediated repression of the reporter mRNA (see “Discussion”).

**Pop2p Deadenylation Activity Is Not Required for PUF-stimulated Deadenylation in Vitro**—To further examine the requirements for POP2 and **CCR4** proteins, we exploited the in vitro system we recently developed that recapitulates PUF-mediated deadenylation (13). In this assay, deadenylation of synthetic RNAs containing a PUF binding site is catalyzed by Pop2p complexes purified from yeast, mixed with purified recombinant Mpt5p. We used this assay to determine whether the enzymatic activity of Pop2p was required for PUF-stimulated deadenylation. Either wild-type or mutant **POP2** genes, with T7 epitope tags, were introduced into **pop2** cells. The mutant Pop2p carried the two missense substitutions (S188A/E190A) that disrupted its enzyme activity (20). The **POP2** proteins, with associated factors, were purified from yeast and assayed for deadenylation using **HO** 3′-UTR RNA substrates with 14 adenosines at their 3′-end. We titrated the amount and activity of these Pop2p complexes so that deadenylation activity was minimal under the conditions used (Fig. 4A, lanes 3–5 and 9–11; supplemental Fig. 1A). Recombinant Mpt5p stimulated deadenylation by both the wild-type Pop2p and mutant Pop2p complexes and yielded a fully deadenylated product (Fig. 4A, lanes 6–8 and 12–14). Therefore, Pop2p deadenylation activity is not essential for PUF-stimulated deadenylation in vitro.

We also purified Pop2p complexes from a **ccr4** strain to determine whether Pop2p had any activity in the absence of...
In contrast, purified mutant Ccr4p complexes, bearing a catalytically inactive mutant Pop2p (Pop2p-T7 mt) complexes was isolated from POP2 (pop2) or CCR4 (ccr4) deletion strains. Following the indicated incubation times, the reaction products were separated by denaturing polyacrylamide electrophoresis. The HO substrate RNA, which contained 14 adenosines at its 3'-end, was radioactively labeled at its 5'-end and incubated with 10 ng of each Pop2p complex for the indicated amount of time, in minutes, without (-) or with (+) recombinant, purified Mpt5p fused to glutathione S-transferase (GST-Mpt5p). Marker RNAs with no poly(A) tail (A₀) or with a 14-adenosine poly(A) tail (A₁₄) were included in lanes 1 and 2, respectively. In vitro deadenylation assays with T7-tagged, purified wild-type (Pop2p-T7), or catalytically inactive mutant Pop2p (Pop2p-T7 mt) complexes was isolated from POP2 (pop2) or CCR4 (ccr4) deletion strains. Following the indicated incubation times, the reaction products were separated by denaturing polyacrylamide electrophoresis. The HO substrate RNA, which contained 14 adenosines at its 3'-end, was radioactively labeled at its 5'-end and incubated with 10 ng of each Pop2p complex for the indicated amount of time, in minutes, without (-) or with (+) recombinant, purified Mpt5p fused to glutathione S-transferase (GST-Mpt5p). Marker RNAs with no poly(A) tail (A₀) or with a 14-adenosine poly(A) tail (A₁₄) were included in lanes 1 and 2, respectively.

Ccr4p. This Pop2p complex had no detectable deadenylase activity, even at high concentrations (Fig. 4A, lanes 15–17; supplemental Fig. S1A). Moreover, this complex, lacking Ccr4p, exhibited no deadenylation activity when mixed with Mpt5p (Fig. 4A, lanes 18–20). Thus, Mpt5p does not activate Pop2p deadenylase activity and Ccr4p is critical for the deadenylase activity of the purified Pop2p complex.

Ccr4p Deadenylase Is Required for PUF-stimulated Deadenylation in Vitro—To test whether the enzymatic activity of Ccr4p was essential for PUF-stimulated deadenylation, we purified T7 epitope-tagged Ccr4p from ccr4 cells. As expected, purified wild-type Ccr4p complexes supported Mpt5p-stimulated deadenylation (Fig. 4B, lanes 3–8; supplemental Fig. S1B). In contrast, purified mutant Ccr4p complexes, bearing a catalytically inactive Ccr4p subunit, were totally inactive for deadenylation (Fig. 4B, lanes 9–11; supplemental Fig. S1B) and remained inactive in the presence of Mpt5p (Fig. 4B, lanes 12–14). These data demonstrate that Ccr4p is responsible for deadenylase activity of the Ccr4p-Pop2p-Not complex and for PUF-stimulated deadenylation.

Because PUF proteins directly bind the Pop2p subunit (13), we tested whether Pop2p was required for Ccr4p-catalyzed deadenylation. We purified T7-tagged Ccr4p from pop2 cells and assayed its activity. Though the Ccr4p complexes from pop2 mutants and wild-type cells were equally active in nonspecific deadenylation assays (Fig. 4B, lanes 15–17; supplemental Fig. S1B), the Ccr4p complex purified from pop2 mutants did not support Mpt5p-stimulated deadenylation (Fig. 4B, lanes 18–20). This result contrasts dramatically with the ability of Ccr4p complexes to promote deadenylation when Pop2p was present (Fig. 4B, lanes 6–8). Thus Pop2p is required for PUF-stimulated deadenylation in vitro.

**DISCUSSION**

In this report, we set out to discover how PUF proteins enhance deadenylation of target mRNAs. Mpt5p associates with the Ccr4p-Pop2p-Not complex through a direct protein-protein interaction with the Pop2p subunit (13). By so doing, the deadenylase complex acts preferentially on those mRNAs to which Mpt5p is bound. We have shown here that Pop2p and Ccr4p are required for PUF-mediated deadenylation, both in vivo and in vitro. However, the roles of the two proteins differ dramatically.

The deadenylation activity of Pop2p is dispensable for PUF repression in vivo, for deadenylation of HO mRNA in vivo, and for PUF-stimulated deadenylation in vitro. Furthermore, purified, recombinant, yeast Pop2p, which was competent for binding to Mpt5p, possessed no deadenylase activity in vitro with or without Mpt5p (not shown). From these findings, we conclude that Pop2p does not catalyze PUF-stimulated deadenylation.

Although the enzyme activity of Pop2p is not required, the protein itself is essential for PUF-mediated deadenylation, both in vivo and in vitro. What role does Pop2p play? Ccr4p isolated from pop2 cells possesses nonspecific deadenylation activity, consistent with previous results (17, 18). However, without Pop2p, Ccr4p is not stimulated by the PUF protein. We infer that Pop2p recruits the Ccr4p enzyme to the RNA. This may be achieved through a direct Pop2p-Ccr4p contact or could be indirect, through other subunits of the Ccr4p-Pop2p-Not complex (16). It is unlikely that contacts between the PUF protein and other subunits of the Ccr4p-Pop2p-Not complex are critical, because deletion of Pop2p alone is sufficient to disrupt PUF-mediated deadenylation (13). We do not detect direct contacts between Mpt5p and several other subunits of the Ccr4p-Pop2p-Not complex. Furthermore, deletions of other subunits of the complex do not significantly influence HO deadenylation or repression (this study and Ref. 13). The most parsimonious interpretation is that Pop2p is physically required to mediate recruitment of Ccr4p to HO mRNA by Mpt5p.

Ccr4p is the enzyme responsible for PUF-stimulated deadenylation; deletion of CCR4 blocks deadenylation in vivo and in vitro, as did mutation of the Ccr4p active site. Further support for the role of Ccr4p in PUF-stimulated deadenylation comes from our previous analysis of HO poly(A) tails in a strain deleted of both PUF proteins that regulate HO (see Fig. 2b in Goldstrohm et al.; Ref. 13). When MPT5 is deleted (along with the redundant PUF4), the poly(A) tails on HO mRNA are removed very slowly, an effect that mirrors deletion of CCR4 (see Fig. 1A). The Ccr4p-Pop2p-Not complex remains func-

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**FIGURE 4.** Mpt5p-stimulated in vitro deadenylation requires the enzymatic activity of Ccr4p and the presence of Pop2p. A, in vitro deadenylation assays using T7-tagged, purified Pop2p complexes. Wild-type (Pop2p-T7) or catalytically inactive mutant Pop2p (Pop2p-T7 mt) complexes was isolated from POP2 (pop2) or CCR4 (ccr4) deletion strains. Following the indicated incubation times, the reaction products were separated by denaturing polyacrylamide electrophoresis. The HO substrate RNA, which contained 14 adenosines at its 3'-end, was radioactively labeled at its 5'-end and incubated with 10 ng of each Pop2p complex for the indicated amount of time, in minutes, without (-) or with (+) recombinant, purified Mpt5p fused to glutathione S-transferase (GST-Mpt5p). Marker RNAs with no poly(A) tail (A₀) or with a 14-adenosine poly(A) tail (A₁₄) were included in lanes 1 and 2, respectively. B, in vitro deadenylation assays with T7-tagged, purified wild-type (Ccr4p-T7) or catalytically inactive mutant (Ccr4p-T7 mt) complexes was isolated from a CCR4 deletion strain (ccr4). These Ccr4p complexes were isolated from a CCR4 deletion strain (ccr4) or POP2 deletion strain (pop2). The 5'-end labeled HO substrate RNA with 14 adenosines at the 3'-end and was incubated with 10 ng of each Ccr4p complex for the indicated amount of time, in minutes, without (-) or with (+) recombinant, purified GST-Mpt5p. The reaction products were analyzed by denaturing polyacrylamide electrophoresis. Marker RNAs with no poly(A) tail (A₀) or with a 14-adenosine poly(A) tail (A₁₄) were included in lanes 1 and 2, respectively.

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3 A. Goldstrohm and M. Wickens, unpublished data.
tional in this double PUF deletion strain, but the deadenylase is no longer efficiently recruited to the mRNA. Therefore, poly(A) removal proceeds at a slow basal rate.

Our findings suggest that deadenylation is not the only way in which Mpt5p can reduce expression of mRNAs. The importance of poly(A) tails for efficient translation and mRNA stability is well documented (1, 3, 4). PUF repression correlates with enhanced deadenylation and degradation of target mRNAs in a number of biological systems (i.e. Xenopus, Drosophila, Caenorhabditis elegans, and yeast; reviewed in Ref. 8). Ccr4p is stringently required for deadenylation, but ccr4 mutants exhibit only a modest effect on repression by Mpt5p in reporter gene repression assays (13). In contrast, pop2 mutants are impaired both in deadenylation and repression.

One possible explanation for these findings is that Mpt5p may repress translation independent of deadenylation. The Drosophila PUF protein, Pumilio, which normally stimulates deadenylation of target mRNAs, can repress translation of nonadenylated mRNAs, albeit with reduced efficiency (27). We suggest that PUF proteins recruit multiple activities to repress target mRNAs. Because Pop2p is required for PUF repression, we suggest that it serves as a bridge to recruit the multifunctional Ccr4p-Pop2p-Not complex. The complex not only removes poly(A) tails via its Ccr4p subunit but also may elicit translational repression through other mechanisms. Dhh1p is an intriguing candidate to mediate this repression: it co-immunoprecipitates with Mpt5p and has a well established role in inhibiting translation (28–31). Co-recruitment of decapping factors Dcp1p and Dcp2p by Mpt5p (13), which associate with Pop2p (29), would further seal the fate of the target mRNA.

Pop2p, Ccr4p, and their orthologs are implicated in regulated mRNA decay and repression by several 3′-UTR-binding proteins and by miRNAs (5, 17, 32–36). In these systems, the relative biochemical roles of the two deadenylases have not yet been delineated. PARN, another deadenylase, also is recruited by certain 3′-UTR-borne factors (37, 38). The opportunity for diversity in regulated deadenylation and repression is clear.

The versatility of regulatory mechanisms is echoed even within a single regulatory family, the PUF proteins. The interaction between PUF and Pop2 proteins is conserved through evolution (13). Yet not all PUF proteins necessarily repress in precisely the same manner as Mpt5p. Moreover, the human Pop2p ortholog, CNOT8, which can interact with Ccr4p (39), is active as a deadenylase (23) and binds to a human PUF protein (13). Thus, a single human PUF protein may recruit two active deadenylases, CNOT8 and human CCR4, to degrade target mRNAs. It will be of interest to determine whether the requirements for the Ccr4p deadenylase and a Pop2p protein bridge are universal among PUF proteins or are a theme with many variations.

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