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PUF Protein-mediated Deadenylation Is Catalyzed by Ccr4p*[S]

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Aaron C. Goldstrohm, Daniel J. Seay, Brad A. Hook, and Marvin Wickens[1]

From the Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706

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)[2] 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 His_6 and T7 epitope tags that could be cleaved off using TEV prote-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

[1] To whom correspondence should be addressed: Dept. of Biochemistry, 433 Babcock Dr., University of Wisconsin-Madison, Madison, WI 53706-1544. Tel.: 608-262-8007; Fax: 608-262-9108; E-mail: wickens@biochem.wisc.edu.

[2] The abbreviations used are: UTR, untranslated region; TAP, tandem affinity tag.

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ase. 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.

High Resolution Northern Blotting—RNA was extracted from samples using the hot acidic phenol method. Cell pellets were resuspended in 500 ul of TENS (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.3 M NaCl, 0.2% SDS) and extracted with 500 μl of 1:1 phenol pH 4.6:chloroform by incubating at 70 °C for 5 min followed by vigorous vortexing for 2 min. RNA was then extracted a second time with chloroform, precipitated, and resuspended in sterile water.

The decay of *HO* mRNA poly(A) tails was analyzed over the indicated time course following addition of the transcriptional inhibitor thiolutin (gift from Pfizer) at 20 μg/ml as previously described (24). After RNA isolation, 10 μg of each total RNA sample was cleaved with ribonuclease H (Promega) and an antisense *HO* oligonucleotide (5′-GGACAGCATCAAACTG-TAAGATTCCGCCAC-3′) (Integrated DNA Technologies). When indicated, oligo(dT)$_{15}$ was added to the ribonuclease H reactions. The RNA was then analyzed by 6% polyacrylamide-urea-denaturing PAGE and transferred to nylon membranes using 1× TBE (Tris borate-EDTA) in a Bio-Rad Trans-Blot apparatus. Blots were probed with an antisense *HO* 3′-UTR probe or an antisense *SCR1* probe. The blots were imaged using a Typhoon PhosphorImager (GE Healthcare).

Co-immunoprecipitation Analysis—Co-immunoprecipitations were performed as described by Goldstrohm *et al.* (13), using the *MPT5*-TAP strain with plasmids expressing the indicated T7 epitope-tagged *POP2* or *CCR4* proteins.

PUF Repression Growth Assays—Mpt5p-mediated repression assays were performed using the reporter gene construct YCp33 *HO* promoter-*HIS3-HO* 3′-UTR and YEp181 *MPT5*, described by Goldstrohm *et al.* (13), in either wild-type BY4742 or *pop2* strains. Wild-type or catalytically inactive mutant *POP2* were expressed from vector pACG1-NT. Colonies from each test strain were isolated and grown to mid-log phase at 30 °C, and the indicated number of cells was plated on minimal medium with or without histidine in the presence of 300 μg/ml of Zeocin (Invivogen). The His3p competitive inhibitor 3-aminotriazole was added to medium lacking histidine at a concentration of 1 mM to increase stringency of the growth assay.

Purification of Deadenylase Complexes—Wild-type or catalytically inactive mutant Pop2p or Ccr4p complexes were purified from *pop2* or *ccr4* deletion strains as indicated in Fig. 4 and supplemental Fig. S1. Conditions identical to the TAP-*POP2* purification (13) were used except that the target proteins had a T7 affinity tag at the N terminus with a TEV protease site to allow elution of the isolated complexes. All steps were done on ice or at 4 °C. Cell extracts were prepared from 1-liter cultures grown to OD$_{660\ nm}$ of 1.5 in YPAD with 300 μg/ml Zeocin (Invivogen). Cells were washed and lysed by glass bead bashing for 15 min in 1 packed cell volume of TNEMN150 (50 mM Tris-HCl, pH 8.0, 0.5% Nonidet P40, 1 mM EDTA, 2 mM $MgCl_2$, and 150 mM NaCl) containing Complete protease inhibitors mixture (Roche Applied Science). The lysate was then centrifuged 10 min at 16,000 × *g*, and the supernatant was removed and incubated with T7 monoclonal antibodies covalently linked to agarose beads (Novagen) for 4 h. Beads 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 $MgCl_2$, 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 $MgCl_2$, 10% (v/v) glycerol) and 10 μM nonspecific competitor RNA (synthetic oligoribonucleotide with sequence 5′-UCUAAUCGGGG-UACAAUUAUAUAAUAUA-3′). *HO* substrate RNA (Integrated DNA Technologies) with sequence 5′-AGUUUAAA-AAGUUGUAUGUAAUAAAGUA$_{14}$-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, *pop2*, and *ccr4* 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. 1*A*, *lanes 1–7*). The long *HO* poly(A) tails were removed rapidly; the oligo-adenylated (A_{5-15}) form disappeared more slowly, most likely via the well established deadenylation-dependent decapping pathway (21).

In *pop2* cells, the distribution of poly(A) ranged form 20 to 60 nucleotides; poly(A) lengths below 20 nucleotides were not detected (Fig. 1*A*, *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-

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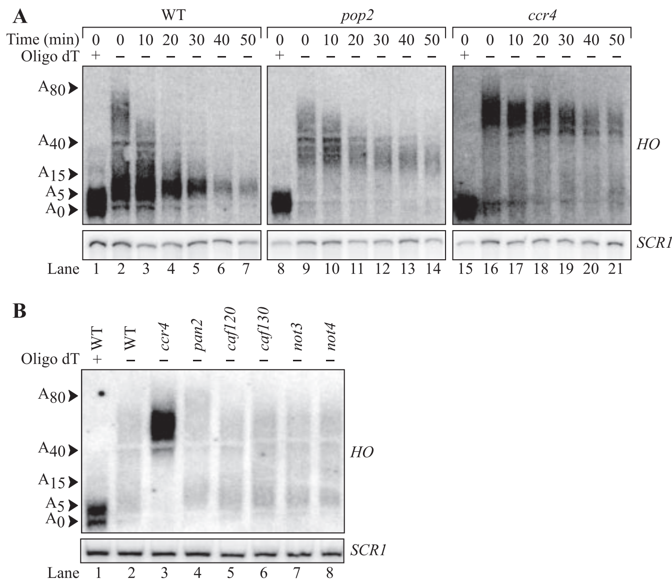

FIGURE 1. ***CCR4* and *POP2* are required for deadenylation of *HO* mRNA *in vivo*.** *A*, *HO* mRNA poly(A) tail length was measured by denaturing polyacrylamide gel electrophoresis and Northern blotting of the *HO* 3′-UTR in wild-type yeast strain (*WT*), *pop2* deletion strain, or *ccr4* deletion 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 were used to cleave the 3′-UTR of *HO* mRNA. Poly(A) tail lengths, indicated at the *left* of the figure, were determined based on migration of bands compared with an RNA molecular weight marker (Century Plus, Ambion). Where indicated by a "+" 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. Northern blots of *HO* mRNA from wild-type (*WT*) or deletion strains indicated at the *top*. As described above, a 3′-UTR fragment of *HO* mRNA was generated by ribonuclease H cleavage. *HO* poly(A) tail lengths, on the *left* of the panel, were determined based on RNA molecular weight markers. Oligo(dT) treatment is indicated on the top by the "+". *SCR1* served as a loading control.

bution, the apparent rate of removal of the poly(A) tail was slower in *pop2* cells (compare *lanes 2–4* with *lanes 9–11*).

In *ccr4* cells, *HO* mRNA possessed long 60–80-nucleotide poly(A) tails (Fig. 1*A*, *lanes 15–21*). Very slow deadenylation was detected, likely catalyzed by the Pan2p-Pan3p complex (15). In both *pop2* and *ccr4* mutants, *HO* mRNA slowly disappeared without complete deadenylation, consistent with previously observed deadenylation-independent decay of other mRNAs in *pop2* and *ccr4* cells (15, 19).

We conclude that both Pop2p and Ccr4p are required for deadenylation of *HO* mRNA *in vivo* but that their roles differ. Ccr4p is required for initial deadenylation. Ccr4p is unable to remove *HO* poly(A) tails in the range of 5 to 20 nucleotides in the absence of Pop2p. Pop2p facilitates deadenylation from 20 to 60 nucleotides and is most critical for removal of shorter poly(A)$_{5-20}$ tails.

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. 1*B*). Deletion of *CCR4* again caused a complete block in deadenylation (Fig. 1*B*, *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. 1*B*, *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).

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. 2*A*). 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. 2*A*, *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 Pop2p still associated with Mpt5p, we determined whether Pop2p co-immunoprecipitated with Mpt5p (Fig. 2*B*). Both mutant and wild-type forms of Pop2p interact with Mpt5p in yeast extracts (Fig. 2*B*). We conclude that the *POP2* protein is important for normal deadenylation of *HO* mRNA *in vivo* but its enzymatic activity is not.

Ccr4p 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. 2*C*). *CCR4* deletion mutants exhibited a severe defect in deadenylation that was not rescued by empty vector (Fig. 2*C*, *lane 2 versus 3*). The wild-type distribution of poly(A) 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 nearly the same levels (data not shown) and co-immunoprecipitated with Mpt5p (Fig. 2*D*). We conclude that Ccr4p is the main deadenylase responsible for *HO* mRNA deadenylation *in vivo*.

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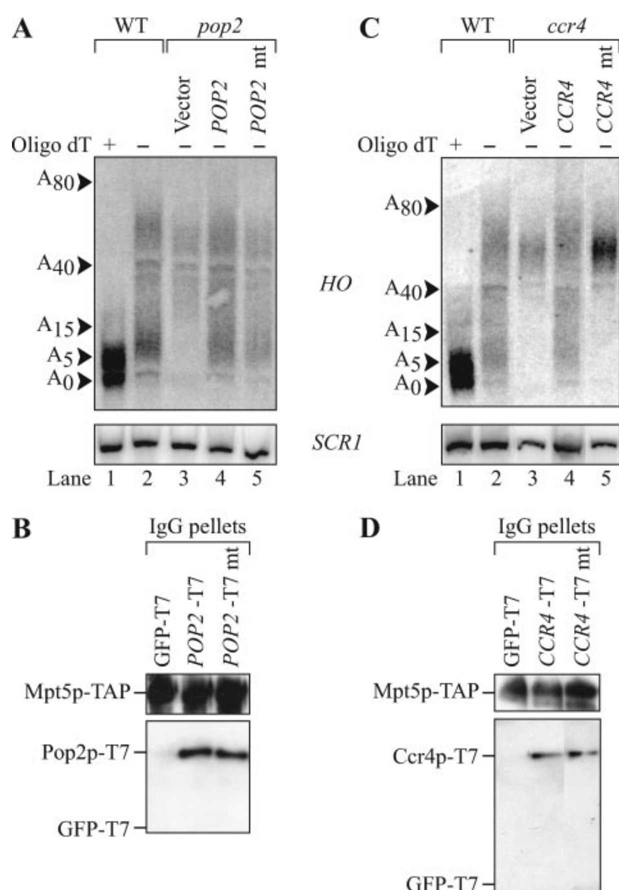

FIGURE 2. **Deadenylation of *HO* mRNA *in vivo* is dependent on the deadenylase activity of Ccr4p, but not that of Pop2p.** *A*, Pop2 protein, but not its enzymatic activity, is required for *HO* deadenylation *in vivo*. Poly(A) tail distribution of *HO* mRNA was measured by denaturing polyacrylamide gels and Northern blotting of RNA from wild-type (*WT*) and *pop2* deletion cells as indicated at the *top*. Empty plasmid vector, wild-type *POP2,* or catalytically inactive *POP2* mutant (*POP2 mt*) were expressed in *pop2* yeast cells. Poly(A) tail lengths are indicated on the *left*. Northern blot of *SCR1* RNA served as a loading control. *B*, wild-type (*POP2-T7*) and catalytically inactive *POP2* mutant (*POP2-T7 mt*) proteins co-immunoprecipitate with Mpt5p. Mpt5p was Tandem Affinity Tagged (*TAP*), immunoprecipitated using rabbit IgG agarose beads (Sigma), eluted with TEV protease, and Western blotted with anti-T7 monoclonal antibodies to detect Pop2 proteins. Mpt5p-TAP was detected in a Western blot using Peroxidase/anti-Peroxidase (Sigma). T7-tagged green fluorescent protein served as a negative control. *C*, Ccr4p deadenylase activity is critical for *HO* deadenylation *in vivo*. *HO* poly(A) tails were measured in wild-type (*WT*) and *ccr4* deletion strains on denaturing polyacrylamide gels and Northern blotting. Poly(A) tail length is indicated on the *left*. Empty plasmid vector or plasmids expressing wild-type *CCR4* or a catalytically inactive mutant form of *CCR4* (*CCR4 mt*) were introduced in the *ccr4* cells. *D*, T7-tagged wild-type *CCR4* (*CCR4-T7*) and catalytically inactive *CCR4* mutant (*CCR4-T7 mt*) proteins co-immunoprecipitate with Mpt5p. Mpt5p was Tandem Affinity Tagged (*TAP*), immunoprecipitated using rabbit IgG-agarose beads, eluted with TEV protease, and Western blotted with anti-T7 monoclonal antibodies to detect Ccr4 proteins. Mpt5p-TAP was detected in a Western blot using Peroxidase/anti-Peroxidase (Sigma). T7-tagged green fluorescent protein served as a negative control.

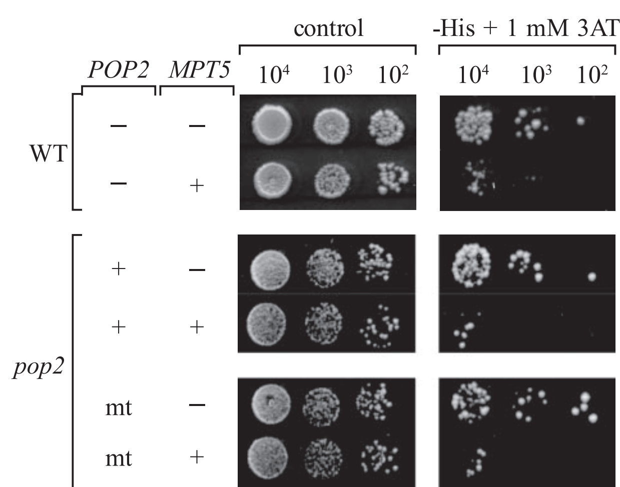

FIGURE 3. **Mpt5p repression is not disrupted by Pop2p active site mutations.** *MPT5*-induced repression of the *HIS3-HO* 3′-UTR reporter gene was assayed in wild-type (*WT*) or *POP2* deletion (*pop2*) strains by growth assay. Wild-type *POP2* or catalytically inactive *POP2* (*mt*) were expressed in the *pop2* cells as indicated on the *left*. The number of cells spotted on control medium, *left panels*, or on medium lacking histidine, *right panels*, is indicated on the *top*. The His3p competitive inhibitor 3-amino-triazole was added at 1 mM to the medium lacking histidine to increase the stringency of the growth assay. Repression by Mpt5p results in inhibition of growth on the medium lacking histidine. Mpt5p-mediated repression is abrogated by deletion of the *POP2* gene (13).

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-aminotriazole, 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 Deadenylase 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 disrupt 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 deadenylase activity was minimal under the conditions used (Fig. 4*A*, *lanes 3–5* and *9–11*; supplemental Fig. 1*A*). Recombinant Mpt5p stimulated deadenylation by both the wild-type Pop2p and mutant Pop2p complexes and yielded a fully deadenylated product (Fig. 4*A*, *lanes 6–8* and *12–14*). Therefore, Pop2p deadenylase 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

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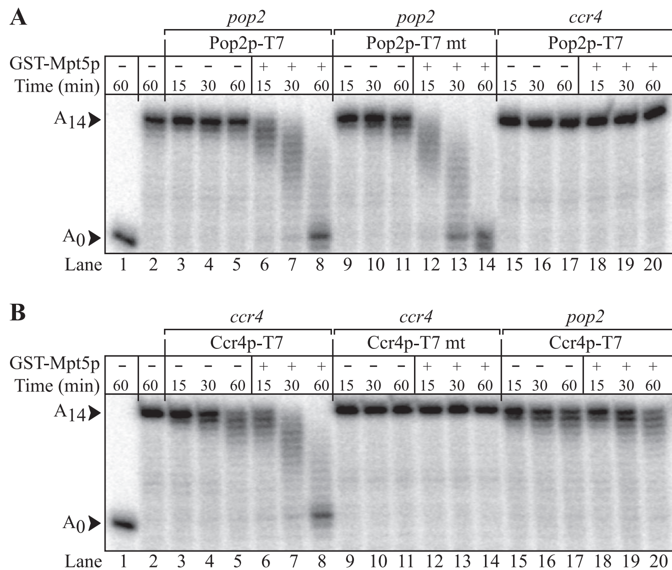

FIGURE 4. **Mpt5p-stimulated *in vitro* deadenylation requires the enzymatic activity of Ccr4p and the presence of Pop2p.** *A*, *in vitro* deadenylation assays using T7-tagged, purified Pop2p complexes. Wild-type (*Pop2p-T7*) or catalytically inactive mutant Pop2p (*Pop2p-T7 mt*) complexes was isolated 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 (A_0) or with a 14-adenosine poly(A) tail (A_{14}) 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*). These Ccr4p complexes were isolated from a *CCR4* deletion strain (*ccr4*) or a *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 (A_0) or with a 14-adenosine poly(A) tail (A_{14}) were included in *lanes 1* and *2*, respectively.

Ccr4p. This Pop2p complex had no detectable deadenylase activity, even at high concentrations (Fig. 4*A*, *lanes 15–17*; supplemental Fig. S1*A*). Moreover, this complex, lacking Ccr4p, exhibited no deadenylation activity when mixed with Mpt5p (Fig. 4*A*, *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. 4*B*, *lanes 3–8*; supplemental Fig. S1*B*). In contrast, purified mutant Ccr4p complexes, bearing a catalytically inactive Ccr4p subunit, were totally inactive for deadenylation (Fig. 4*B*, *lanes 9–11*; supplemental Fig. S1*B*) and remained inactive in the presence of Mpt5p (Fig. 4*B*, *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. 4*B*, *lanes 15–17*; supplemental Fig. S1*B*), the Ccr4p complex purified from *pop2* mutants did not support Mpt5p-stimulated deadenylation (Fig. 4*B*, *lanes 18–20*). This result contrasts dramatically with the ability of Ccr4p complexes to promote deadenylation when Pop2p was present (Fig. 4*B*, *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 deadenylase 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-Pop2-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.[3] 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* blocked 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. 2*b* 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. 1*A*). The Ccr4p-Pop2p-Not complex remains func-

[3] A. Goldstrohm and M. Wickens, unpublished data.

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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 non-adenylated 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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Aaron C. Goldstrohm, Daniel J. Seay, Brad A. Hook and Marvin Wickens

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