THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 21, pp. 15430–15438, May 25, 2007

Two Yeast PUF Proteins Negatively Regulate a Single mRNA*[S]

Received for publication, December 7, 2006, and in revised form, March 26, 2007 Published, JBC Papers in Press, March 27, 2007, DOI 10.1074/jbc.M611253200

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

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

mRNA stability and translation are regulated by protein repressors that bind 3′-untranslated regions. PUF proteins provide a paradigm for these regulatory molecules: like other repressors, they inhibit translation, enhance mRNA decay, and promote poly(A) removal. Here we show that a single mRNA in *Saccharomyces cerevisiae*, encoding the HO endonuclease, is regulated by two distinct PUF proteins, Puf4p and Mpt5p. These proteins bind to adjacent sites and can co-occupy the mRNA. Both proteins are required for full repression and deadenylation *in vivo*; their removal dramatically stabilizes the mRNA. The two proteins act through overlapping but non-identical mechanisms: repression by Puf4p is dependent on deadenylation, whereas repression by Mpt5p can occur through additional mechanisms. Combinatorial action of the two regulatory proteins may allow responses to specific environmental cues and be common in 3′-untranslated region-mediated control.

Regulation of replication, transcription, translation, and mRNA stability hinge on the assembly of multiprotein complexes. These complexes include the basal machinery for that process, such as DNA and RNA polymerases, ribosomes, or mRNA decay complexes. Commonly these large machines are recruited to specific nucleic acid sequences by interacting with regulatory proteins bound to specific sites. In this fashion, sequence specificity is provided to intrinsically non-sequence-specific enzymatic activities. The assembly of the regulatory complexes relies on combinatorial associations among protein partners and antagonists and their interactions with RNA or DNA.

Regulation of mRNA translation and stability commonly is controlled by elements in the 3′-untranslated region (UTR)[2] of the mRNA, to which specific regulatory proteins bind (1–4). In negative regulation, the resulting RNA-protein complexes either repress translation or promote mRNA turnover (2, 4). Removal of the poly(A) tail of the mRNA correlates with, and can cause, both events (3, 4). Several RNA binding regulators associate with complexes containing one or more deadenylases, although the precise molecular contacts have been identified in only a few cases (5–12).

The PUF proteins are an evolutionarily widespread family of 3′-UTR-binding proteins (13). All PUF proteins examined to date either repress translation, induce decay, or both; their activity is correlated with poly(A) shortening (13). The yeast *Saccharomyces cerevisiae* possesses six PUF proteins (13). Each PUF is associated with many mRNAs in yeast lysates, suggesting that nearly 10–15% of yeast mRNAs may be targets of PUF regulation (14).

Mpt5p, one of the yeast PUF proteins, binds to and regulates the mRNA that encodes HO endonuclease (7, 15). HO protein controls mating-type switching by causing double-strand breaks that initiate a recombination event (16). The *HO* gene is tightly controlled transcriptionally in a cell type-specific manner to prevent aberrant switching and deleterious DNA breaks (17). Recently it has become clear that *HO* is also controlled post-transcriptionally (7, 15, 18). Mpt5p contributes to this control; indeed, *mpt5* null mutants switch mating type aberrantly at high frequency (15). The observation that *HO* mRNA co-immunoprecipitated with another PUF protein, Puf4p, suggested that Puf4p might also participate in *HO* control (14).

PUF proteins from several organisms directly interact with Pop2p orthologs (7). In many organisms, Pop2p is a deadenylase, as is a related protein with which it interacts, Ccr4p (7, 19–21). In yeast, Pop2p is required for repression and deadenylation mediated by Mpt5p; however, point mutations that disrupt its active site have no effect on PUF-enhanced deadenylation *in vivo* or *in vitro* (7, 18). Instead, the Ccr4p enzyme catalyzes PUF-enhanced deadenylation; mutations in the Ccr4p active site eliminate regulated deadenylation (18). Pop2p is a bridge that links the PUF-RNA complex to the Ccr4p deadenylase. The same PUF-Pop2p connection simultaneously recruits other proteins involved in turnover and translational repression, including the decapping factor Dcp1p and the RNA helicase Dhh1p, which can repress translation (7).

In this report, we sought to determine whether Puf4p directly regulates *HO* expression. We combined *in vivo* and *in vitro* analyses to show that Puf4p and Mpt5p bind the *HO* 3′-UTR through distinct but adjacent binding sites and can do so simultaneously. Puf4p represses mRNA expression and enhances mRNA deadenylation and decay. The mechanisms of these effects overlap with, but are distinct from, those of Mpt5p. In particular, Puf4p repression activity is entirely dependent on the deadenylation activity of Ccr4p (which it recruits via Pop2p); in contrast, Mpt5p can repress by additional mechanisms. In the presence of either PUF protein, the Pop2p-Ccr4p complex is recruited to enhance deadenylation. The effects of the two proteins bound simultaneously to their cognate sites

* This work was supported by National Institutes of Health Grant GM50942. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

[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; GST, glutathione *S*-transferase; MBP, maltose-binding protein; BS, binding site; TAP, tandem affinity purification tag; RBD, RNA-binding domain.

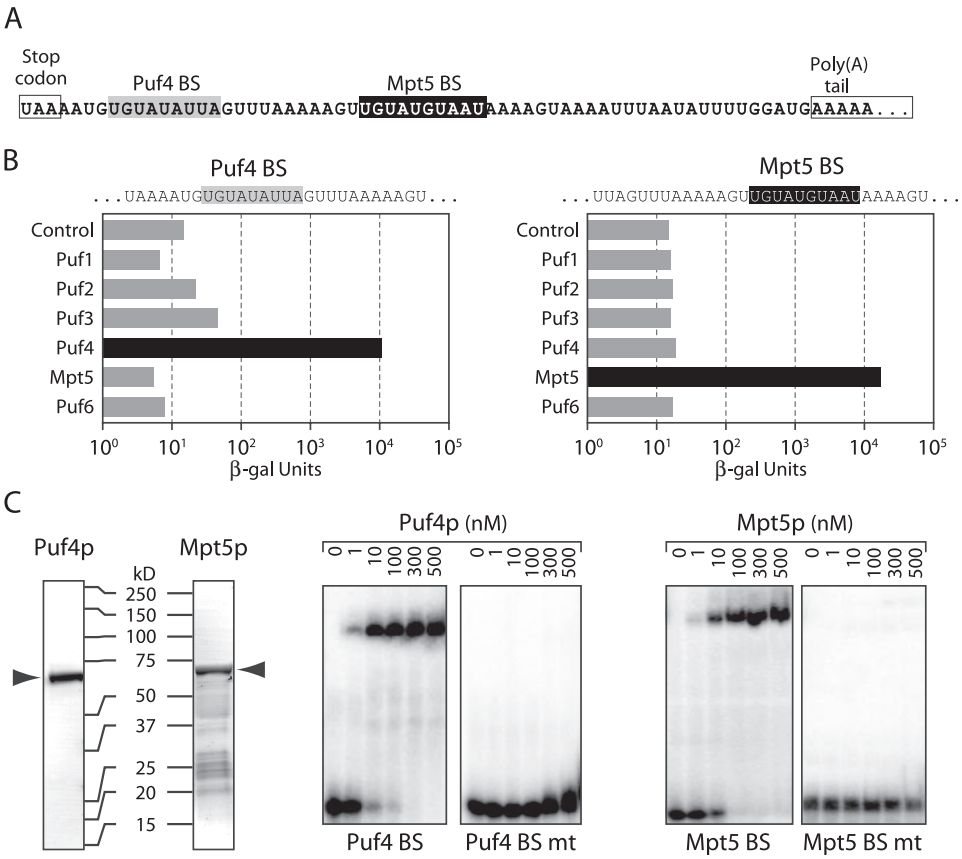


FIGURE 1. **Two PUF proteins bind the *HO* 3′-UTR.** *A*, the *HO* 3′UTR sequence, starting with the stop codon and ending at the poly(A) tail. The Puf4 binding site (*Puf4 BS*) and Mpt5 binding site (*Mpt5 BS*) are highlighted in *gray* and *black*, respectively. The 3′-end of *HO* mRNA was previously defined (15). *B*, PUF protein binding to the *HO* 3′-UTR was determined using the yeast three-hybrid system. Each PUF protein was fused to Gal4p activation domain, indicated on the *left*, and tested for interaction with the Puf4 BS (*left panel*) or the Mpt5 BS (*right panel*). Empty activation domain vector served as a negative control. The *HO* RNA sequences tested are indicated above each graph. Expression of β-galactosidase was quantitated in yeast lysates as described (22). Values correspond to relative light units, corrected for culture density. *C*, on the *left*, Coomassie-stained SDS-PAGE of purified recombinant GST-tagged Puf4p and Mpt5p with size markers. The *middle* and *right panels* show electrophoretic mobility shift assays of Puf4p and Mpt5p with wild-type Puf4 BS and Mpt5 BS or with mutant Puf4 BS and Mpt5 BS; in the mutants, the UGU trinucleotide was changed to ACA. Protein concentrations are indicated above each gel (*nM*).

are additive and greatly enhance the efficiency and rate of deadenylation and decay. Together, they provide flexible opportunities for control.

EXPERIMENTAL PROCEDURES

Strains—The yeast three-hybrid strain YBZ1 was described previously (22). The wild-type BY4742 yeast strain and isogenic strains with gene-specific deletions of *CCR4* and *POP2* were obtained from Open Biosystems. These deletion strains were created by PCR-mediated gene modification using the kanamycin/G418 resistance marker. The *MPT5*-TAP, *PUF4*-TAP, and *POP2*-TAP strains (Open Biosystems) were created in the S288C strain background by integrating a C-terminal tandem affinity purification (TAP) tag onto the coding sequence using PCR-mediated gene modification with a *HIS3* marker.

Yeast Three-hybrid Assay—Three-hybrid assays were performed as described (23). PUF proteins fused with the Gal4 activation domain were expressed from pACT2 or pGADT7 plasmids. Full-length open reading frames were used for *PUF1, PUF2, PUF3, PUF4*, and *PUF6*. The Mpt5p open reading frame starts with methionine 26. DNA oligonucleotides were designed to express either the Puf4 BS or Mpt5 BS (sequence found in Fig. 1*B*) and cloned into the XmaI and SphI sites of pIIIA/MS2-2. Assays were performed in strain YBZ1. β-galactosidase activity was measured using the Beta-Glo (Promega) substrate as described (22).

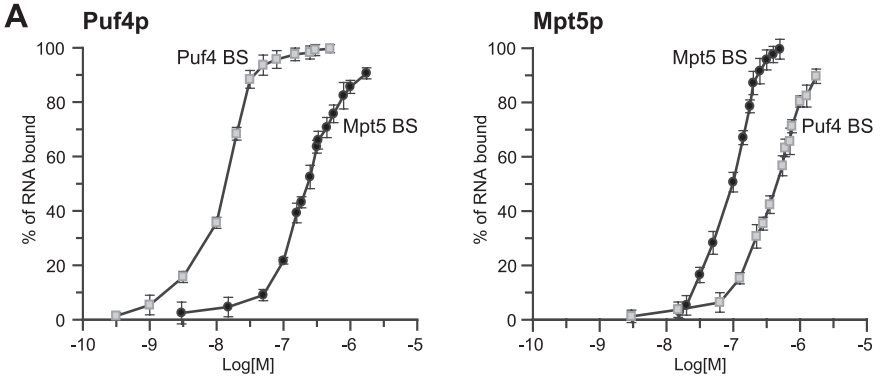
Protein Expression Constructs and Purification—Mpt5p (amino acids 126–626), Puf4p (amino acids 536–887), and Ccr4p (amino acids 319–837) were cloned into pGex6P1 (GE Healthcare) with an N-terminal TEV protease cleavage site. The resulting GST (glutathione *S*-transferase) fusion proteins were expressed in BL-21 gold *Escherichia coli* cells (Invitrogen) and purified/eluted as described (24). The recombinant Puf4p used in Fig. 2*B* was cleaved with TEV protease (Invitrogen).

Pop2p was cloned into a derivative of the pMAL vector (New England Biolabs) that contained a TEV protease cleavage site and an N-terminal hexahistidine epitope tag. The resulting maltose-binding protein (MBP)-Pop2p was purified from *E. coli* according to the manufacturer's protocol (New England Biolabs).

Electrophoretic Mobility Shift Assays—RNA binding assays were performed as described (24) using chemically synthesized RNAs (Dharmacon). The apparent K_d, the concentration of protein at which half-maximal binding occurs, was calculated using Grafit 5 (Sigma). The modest difference in Mpt5p binding affinities between Fig. 1*C* and that reported in Fig. 2*A* are likely due to different protein preparations. The data and apparent dissociation constants reported in Fig. 2*A* are the statistically determined results of three independent binding experiments.

The following synthetic RNAs were used in the assays (ones not given in figures): Mpt5p BS, ′-AGUUUAAAAAGUUGUAUGUAAUAAAAGU-3′; Mpt5p BS mt, 5′-AGUUUAAAAAGUACAAUGUAAUAAAAGU-3′; Puf4p BS, 5′-UAAAAUGUGUAUAUUAGUUUAAAAGU-3′; and Puf4p BS mt, 5′-UAAAAUGACAAUAUUAGUUUAAAAGU-3′.

mRNA Decay Analysis—Transcriptional inhibition experiments were done using 20 μg ml^{-1} of thiolutin (Pfizer) as described (7). RNA was purified by an acid-phenol-chloroform protocol at pH 4.6 and separated by 3.7% (v/v) formaldehyde, 1.0% (w/v) agarose gel electrophoresis. Transfer to nylon membrane (Ambion) was achieved by capillary action using 10×



	Puf4 BS	Puf4 BS mt	Mpt5 BS	Mpt5 BS mt
Puf4p (nM)	10±5	>1000	250±50	>1000
Mpt5p (nM)	350±50	>1000	100±25	>1000

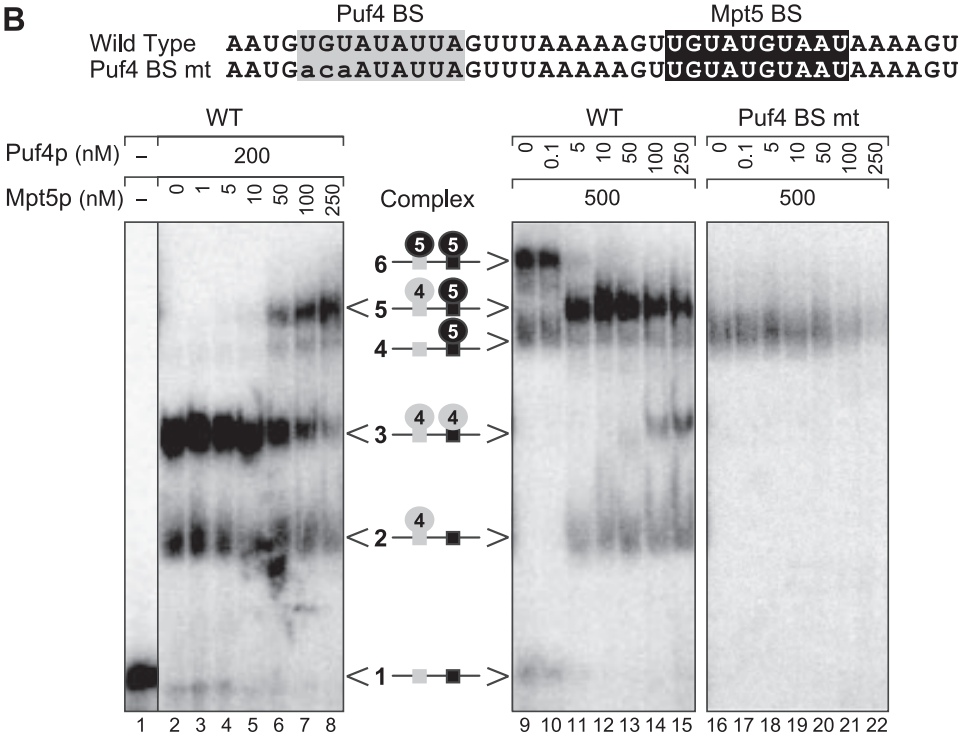


FIGURE 2. **Puf4p and Mpt5p bind the HO 3′-UTR simultaneously.** *A*, the graphs display binding curves comparing apparent affinities (apparent K_d in nM) of GST-Puf4p and GST-Mpt5p with Puf4 BS (*gray squares*) and Mpt5 BS (*black circles*). *Error bars* were derived from three different electrophoretic mobility shift assays. The *chart* on the bottom compares apparent affinities (K_d; nM) from Puf4p and Mpt5p with each wild-type or mutant PUF binding site. *B*, electrophoretic mobility shift assay with Puf4p and GST-Mpt5p together with 5′-labeled RNA containing both the Puf4 BS and Mpt5 BS. Sequences of wild-type or mutant Puf4 BS RNAs are shown at the *top*. Proteins concentrations (*nM*) are indicated above the gels. Diagrams corresponding to different mobility complexes (*Complexes 1–6*) are found between the gels, with Puf4p represented by a *gray circle* and Mpt5p by a *black circle*.

SSC. Blots were probed with radiolabeled antisense riboprobes specific to genes listed.

Repression Assays—The *HIS3-HO* 3′-UTR reporter gene contains the *HIS3* coding sequence with 2 kbp of the *HO* promoter upstream and 1 kbp of the *HO* 3′-UTR downstream in plasmid YCp33. The "*HO* mt" reporter (Fig. 4) has the UGU trinucleotides in the Puf4 and Mpt5 binding sites changed to ACA by site-directed mutagenesis of an otherwise wild-type *HO* 3′-UTR (sequence in Fig. 1). Wild-type yeast strain BY4742 or gene-specific deletion strains (*ccr4* or *pop2*) were transformed with the reporter gene plasmid and either YEp181, YEp181 *MPT5*, p415-GPD, or p415-GPD-*PUF4*. Plasmid YEp181 *MPT5* mutant was created by site-directed mutagenesis (Stratagene) of amino acids S454A and N455A in PUF repeat 7. Plasmid p415-GPD-*PUF4* mutant was derived from p415-GPD-*PUF4* by site-directed mutagenesis (Stratagene) of amino acids S799A and N800A in PUF repeat 7. *CCR4* expression plasmid was created in the high copy vector pACG1-NT as described (18). The active site mutant *CCR4* contains missense mutation E556A described by Chen *et al.* (19). For growth assay, colonies were isolated and grown to mid-log phase at 30 °C and plated on minimal medium with or without histidine. The *HIS3* competitive inhibitor 3-aminotriazole was added to increase stringency where indicated.

Immunoprecipitations—Reactions were performed with TAP-tagged *MPT5* and *PUF4* strains carrying T7-tagged versions of Pop2p, Ccr4p, or green fluorescent protein expressed from vector pACG1 NTB as described (7). Lysates and pellets were treated with 5 units of RNase A and 200 units of RNase T1 (Ambion).

GST Pulldown Experiments—GST-Mpt5p (amino acids 126–626), GST-Puf4p (amino acids 536–887), and GST-Ccr4p (amino acids 319–837) were bound to glutathione-agarose beads (Amersham Biosciences) at a concentration of 1 μg/10 μl bed volume. MBP-Pop2p was mixed to the beads at a final concentration of 500 nM in TNEMN150 (50 mM Tris-HCl, pH 8, 0.5% (v/v) Nonidet P40, 1 mM EDTA, 2 mM $MgCl_2$, and 150 mM NaCl), washed, and analyzed with monoclonal antibody to MBP (New England Biolabs). RNase one (20 units; Promega) was added to each reaction.

Deadenylation in Vitro—Deadenylation reactions were carried out with Pop2p-TAP complexes purified from yeast and recombinant PUF protein purified from *E. coli* as previously described (7). The reactions contained 10 ng of Pop2p-TAP

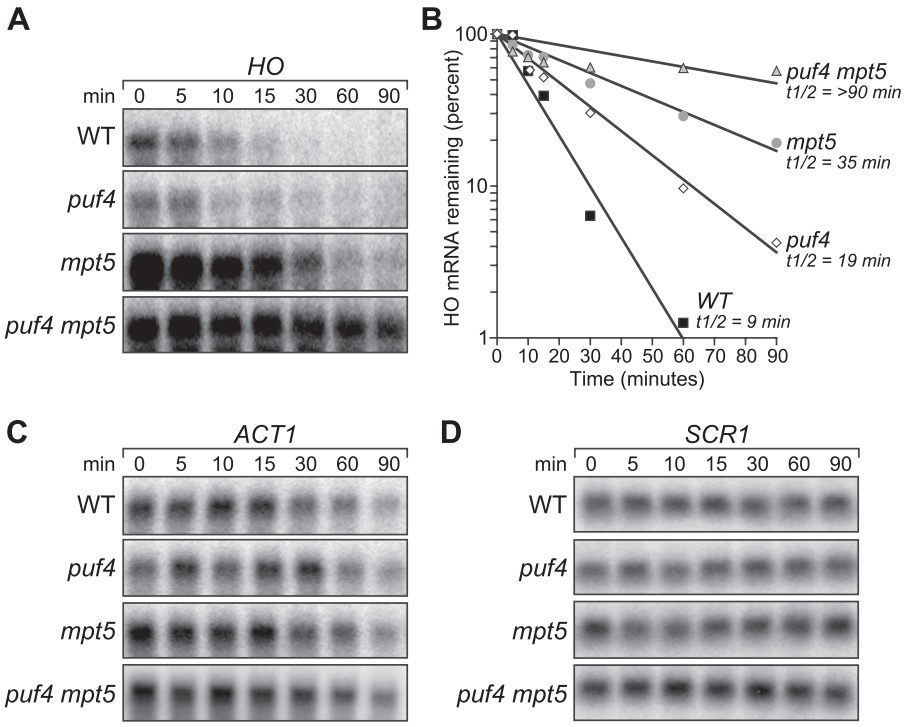


FIGURE 3. **Puf4p and Mpt5p stimulate degradation of *HO* mRNA.** Northern blot analysis of *HO*, *ACT1*, and *SCR1* RNAs in wild-type (*WT*), *puf4*, *mpt5*, and *puf4 mpt5* deletion strains after transcriptional shut off. Time points after shut off are indicated above gel. *A*, Northern blot analysis of *HO* mRNA. *B*, graph of decay rates of *HO* mRNA in each strain from *panel A*. Half-lives are indicated to the *right*. *C*, Northern blot analysis of *ACT1* mRNA. *D*, Northern blot analysis of noncoding *SCR1* RNA.

complex and 200 fmol (10 nM) substrate RNA, unless otherwise indicated. GST-Mpt5 RNA-binding domain (RBD) (250 nM) and/or Puf4 RBD (100 nM) were added to reactions as indicated.

RESULTS

Two Different PUF Proteins Bind the HO 3′-UTR—The yeast PUF protein Mpt5p regulates *HO* mRNA by binding a specific sequence in its 3′-UTR, called the Mpt5 BS (Fig. 1*A*, and Refs. 7 and 15). A second potential PUF binding site lies 20 nucleotides upstream and conforms to a sequence found in 66% of mRNAs that co-immunoprecipitated with Puf4p (14). These data suggested that both Mpt5p and Puf4p might regulate *HO* mRNA.

We first examined the binding of Mpt5p and Puf4p to their respective sites using the yeast three-hybrid system (22, 23). Each of the six yeast PUF proteins was individually fused to the Gal4p transcriptional activation domain and co-expressed with RNA "baits" containing either the predicted Puf4p binding site (Puf4 BS) or the Mpt5p binding site (Mpt5 BS). Protein-RNA interactions were detected by the production of β-galactosidase and assayed in yeast lysates (22). Only Puf4p bound the Puf4 BS (Fig. 1*B*), whereas only Mpt5p bound the Mpt5 BS (Fig. 1*B*). Mutations that changed UGU to ACA in each binding site eliminated binding (data not shown; see Fig. 1*C*).

To determine whether Puf4p and Mpt5p bind directly to the two sites, we used an electrophoretic mobility shift assay. Purified, recombinant GST-tagged Puf4p or Mpt5p was incubated with labeled RNAs comprising either the Mpt5 BS or Puf4 BS, and the resulting complexes were analyzed by electrophoresis (Fig. 1*C*). Mpt5p bound the Mpt5 BS with an apparent K_d of 100 nM, consistent with our previous findings (7), whereas Puf4p bound the Puf4 BS with an apparent K_d of 10 nM. Neither Mpt5p nor Puf4p bound its respective binding site when the critical UGU trinucleotides were mutated to ACA (Fig. 1*C*). We conclude that Puf4p specifically binds the newly defined Puf4 BS in the *HO* 3′-UTR.

Both PUF Proteins Can Bind Simultaneously—To quantify binding specificity, we compared the binding of Puf4p and Mpt5p to the two sites *in vitro*. Puf4p exhibited a 25-fold preference for its cognate site over its non-cognate site; Mpt5p displayed a 3.5-fold preference for its own site (Fig. 2*A*).

To test whether Puf4p and Mpt5p can bind simultaneously to a single RNA molecule, we prepared a 5′-end-labeled RNA containing both the Puf4 and the Mpt5 binding sites (Fig. 2*B*). To test for co-occupancy, we performed gel shift assays using a saturating concentration of one recombinant PUF protein mixed with increasing concentrations of the other. To resolve the different complexes, the GST affinity tag was removed from Puf4p but left on Mpt5p; as a result, their complexes differed in mobility.

At high concentrations of Puf4p alone (200 nM, 20-fold above its K_d for the Puf4 BS), Puf4p bound the Puf4 site to form Complex 2 and both sites to form Complex 3 (Fig. 2*B*, *lane 2*). Addition of Mpt5p to Puf4p progressively resulted in a new complex (Fig. 2*B*, *lanes 3–8*, *Complex 5*). This new complex (Complex 5) represented Puf4p and Mpt5p bound to the same RNA (see below).

We next performed the reciprocal experiment, holding Mpt5p constant and titrating Puf4p. Mpt5p was added at saturating concentrations (500 nM, 5-fold above its K_d for the Mpt5 BS); as expected, it bound the Mpt5 site to form Complex 4 and both sites to form Complex 6 (Fig. 2*B*, *lane 9*). Addition of Puf4p yielded Complex 5, containing Mpt5p, Puf4p, and the RNA (Fig. 2*B*, *lanes 10–15*). At high Puf4p concentrations, Puf4p competed with Mpt5p, ultimately displacing it from the Puf4p BS; as a result, Complexes 2 and 3 increase in abundance at the expense of Complexes 4, 5, and 6 (Fig. 2*B*, *lanes 14* and *15*). Mutation of the Puf4 BS from UGU to ACA abolishes all complexes except that of Mpt5p to the Mpt5p site (Complex 4). These data demonstrate that Mpt5p and Puf4p preferentially bind their respective sites, that they can co-occupy the *HO* 3′-UTR *in vitro*, and that they can compete for one another's binding sites.

Puf4p and Mpt5p Stimulate Degradation of HO mRNA—We analyzed the effect of Puf4p and Mpt5p on endogenous *HO* mRNA by comparing its half-life in wild-type *versus puf4*, *mpt5*,

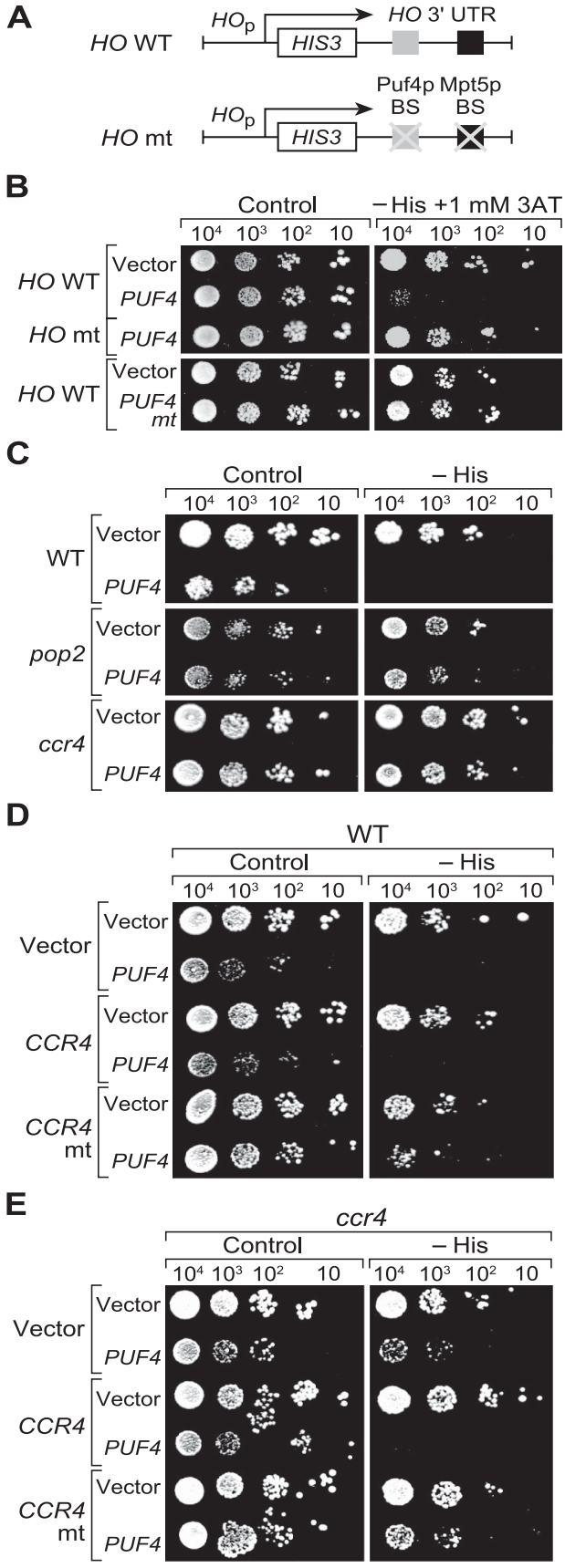


FIGURE 4. **Puf4p represses *HO in vivo* and requires Pop2p and Ccr4p.** *A*, diagrams of the *HO*p-*HIS3*-*HO* 3′-UTR reporters containing the *HO* promoter (HO_p), *HIS3* coding sequence, and *HO* 3′-UTR with Mpt5p BS and Puf4p BS. For *HO* mutant reporter (*HO mt*), mutation of the UGU trinucleotides of each PUF binding site to ACA is indicated by an "*X*". *B*, Puf4p repression assays were performed using wild-type or mutant *HO* reporter genes with plasmids expressing wild-type or mutant *PUF4* proteins or empty vector as indicated on the *left*. Yeast cells, the number of which is shown at the top, were spotted on media with (*control*) or without (−*His*) histidine. 3-Aminotriazole was added to medium lacking histidine where indicated. *C*, Puf4p repression of the wild-type *HO* reporter was tested in wild-type (*WT*), *pop2*, and *ccr4* yeast strains as indicated on the *left*. *D*, Puf4p repression of wild-type *HO* reporter gene in wild-type yeast carrying empty vector as a control or overexpressing wild-type *CCR4* or catalytically inactive mutant *CCR4* (*CCR4 mt*). *E*, Puf4p repression of wild-type *HO* reporter gene in *ccr4* deletion strain carrying empty vector as a control or overexpressing wild-type *CCR4* or catalytically inactive mutant *CCR4* (*CCR4 mt*).

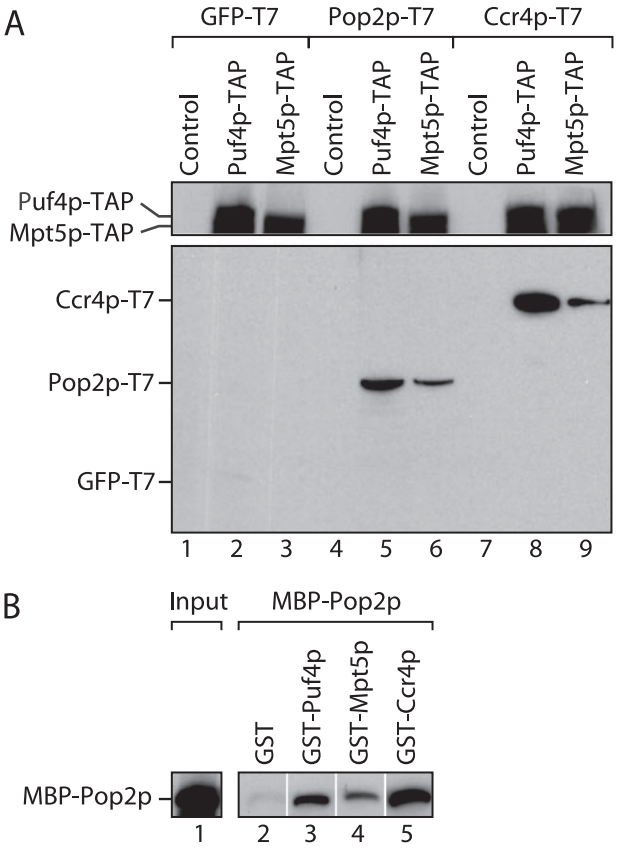


FIGURE 5. **Puf4p directly binds Pop2p.** *A*, Pop2p and Ccr4p co-immunoprecipitate with Puf4p. Western blot of T7-tagged proteins, GFP, Pop2p, or Ccr4p in Puf4p-TAP or Mpt5p-TAP immunoprecipitate pellets. Mock purification with no TAP tag served as the control. Immunoprecipitations were performed in the presence of RNases A and T1. *B*, recombinant Pop2p binds Puf4p *in vitro*. Anti-MBP Western blot of input and GST, GST-Mpt5p RBD, GST-Puf4p RBD, and GST-CCR4 pulldowns of MBP-Pop2p. Binding reactions were treated with RNase One.

and *puf4 mpt5* deletion strains (Fig. 3). Total RNA was prepared at various times after addition of thiolutin, which inhibits RNA polymerase II (25). *HO* mRNA half-life increased from 9 min in the wild-type strain, to 19 min in the *puf4* mutant, and to 35 min in the *mpt5* mutant. Most dramatically, the half-life of *HO* mRNA increased to more than 90 min in the *puf4 mpt5* mutant strain (Fig. 3, *A* and *B*). Steady state levels of *HO* mRNA increased dramatically in the *mpt5* (3-fold) and *puf4 mpt5* mutants (4-fold). As a control, we analyzed an mRNA that does not interact with Mpt5p. The half-lives of neither *ACT1* mRNA (Fig. 3*C*) nor *SCR1* RNA (Fig. 3*D*) were unaffected by removal of *PUF4*, *MPT5*, or both. These results demonstrate that Puf4p

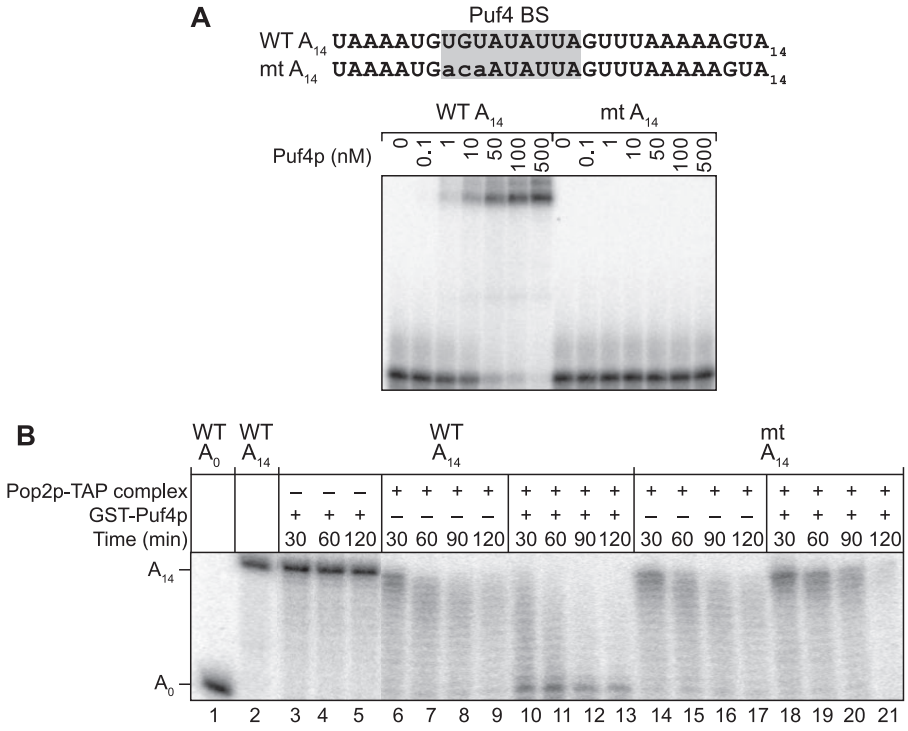


FIGURE 6. **Puf4p enhances deadenylation *in vitro*.** *A*, electrophoretic mobility shift assays of GST-Puf4p RBD binding to *HO* substrates. Sequences of wild-type (*WT* A_{14}) and mutant (*mt* A_{14}) *HO* substrate RNAs with 14 adenosines at the 3′-end are shown at the *top*. Puf4 BS is highlighted in *gray*. Puf4 protein concentrations (*nM*) are indicated above the gels. *B*, *in vitro* deadenylation reactions were performed with *HO* WT A_{14} and mt A_{14} substrate RNAs using 10 ng of Pop2p-TAP complex and GST-Puf4p RBD as indicated above the gels. (*10 ng* refers to the total protein concentration in the fraction.) Mobilities of RNA markers, without (A_0) or with (A_{14}) poly(A) tails, are indicated at *left*.

and Mpt5p specifically destabilize *HO* mRNA and that the two proteins combine to enhance destabilization.

Puf4p Represses HO in Vivo and Requires Pop2p and Ccr4p—To test whether Puf4p represses expression *in vivo*, we used a reporter gene consisting of the *HO* promoter, *HIS3* coding sequence, and the *HO* 3′-UTR (Fig. 4*A*) (7). When overexpressed, Puf4p repressed this reporter mRNA (Fig. 4*B*), largely preventing growth on selective media lacking histidine; Mpt5p behaves similarly (supplemental Fig. S1*A*) (7). Repression by Puf4p required the PUF binding sites in the RNA (Fig. 4*B*) and a functional RNA-binding domain in the protein (Fig. 4*B*). We conclude that Puf4p and Mpt5p repress the reporter mRNA *in vivo* by binding the *HO* 3′-UTR.

To determine whether repression by Puf4p requires Pop2p, we examined Puf4p-dependent repression in a *pop2* deletion mutant. Puf4p-mediated repression was dramatically reduced in the *pop2* strain (Fig. 4*C*). In this respect, Puf4p mirrors Mpt5p (supplemental Fig. S1*B*) (7).

Puf4p-mediated repression also required Ccr4p, the enzyme responsible for deadenylation of *HO* mRNA *in vivo* (18); repression was virtually abolished in a *ccr4* mutant (Fig. 4*C*). In contrast, Mpt5p-mediated repression largely persists in the *ccr4* mutant (supplemental Fig. S1*B*) (7). We conclude that Puf4p repression via the *HO* 3′-UTR requires both the Pop2p and Ccr4p subunits of the major cytoplasmic deadenylase complex.

To determine whether repression by Puf4p requires the catalytic activity of Ccr4p, we tested Puf4p-dependent repression in wild-type and *ccr4* deletion strains expressing either wild-type Ccr4p or a point mutant that disrupts its active site (Fig. 4, *D* and *E*, *CCR4 mt*). In the wild-type strain, Puf4p represses with and without overexpressed Ccr4p, as expected (Fig. 4*D*). Overexpression of the Ccr4p mutant partially inhibited repression, indicating a dominant negative effect (Fig. 4*D*). In the *ccr4* mutant strain, Puf4p repression was restored by expression of active Ccr4p, but not by the Ccr4p active site mutant (Fig. 4*E*). We conclude that Puf4p repression requires both Pop2p and an active form of Ccr4p.

Puf4p Associates with Deadenylation Factors—To determine whether Puf4p physically associates with deadenylase proteins, we performed co-immunoprecipitation experiments with yeast extracts (Fig. 5*A*). We used yeast strains expressing either TAP-tagged Puf4p or TAP-tagged Mpt5p and a T7 epitope-tagged derivative of either the deadenylase subunits Ccr4p or Pop2p or, as a control, green fluorescent protein. Mock purifications from a wild-type, untagged strain carrying each T7-tagged test protein served as negative controls.

TAP-Puf4p and TAP-Mpt5p were affinity-purified and eluted from the beads, and the immunoprecipitates were analyzed by Western blotting. Pop2p and Ccr4p were physically associated with Puf4p and Mpt5p (Fig. 5*A*, *lanes 5*, *6*, *8*, and *9*); green fluorescent protein was not (*lanes 2* and *3*). The co-immunoprecipitations of Pop2p and Ccr4p with Puf4p and Mpt5p were due to protein-protein interactions rather than co-occupancy of RNA, because they were unaffected by RNase A and T1 treatment.

Puf4p Directly Binds Pop2p—To determine whether Puf4p bound directly to Pop2p, we used purified recombinant Pop2p fused to maltose-binding protein (MBP-Pop2p). MBP-Pop2p was incubated with purified recombinant Puf4p, Mpt5p, and Ccr4p, each fused to GST and immobilized on glutathione-agarose beads. After extensive washing, the presence of MBP-Pop2p was determined using Western blotting. Immobilized Puf4p and Mpt5p bound MBP-Pop2p (Fig. 5*B*). As controls, Ccr4p bound Pop2p as expected, whereas GST alone did not. We conclude that Puf4p, like Mpt5p, binds directly to Pop2p. Because these experiments used only the RNA-binding domains of the PUF proteins, those regions are sufficient to bind Pop2p.

Puf4p Stimulates Deadenylation in Vitro—Puf4p repression requires both *CCR4* and *POP2,* implying that Puf4p repression stimulates deadenylation. Consistent with this, deadenylation of *HO* mRNA is dramatically impaired in *puf4 mpt5* double mutants compared with *mpt5* alone (7).

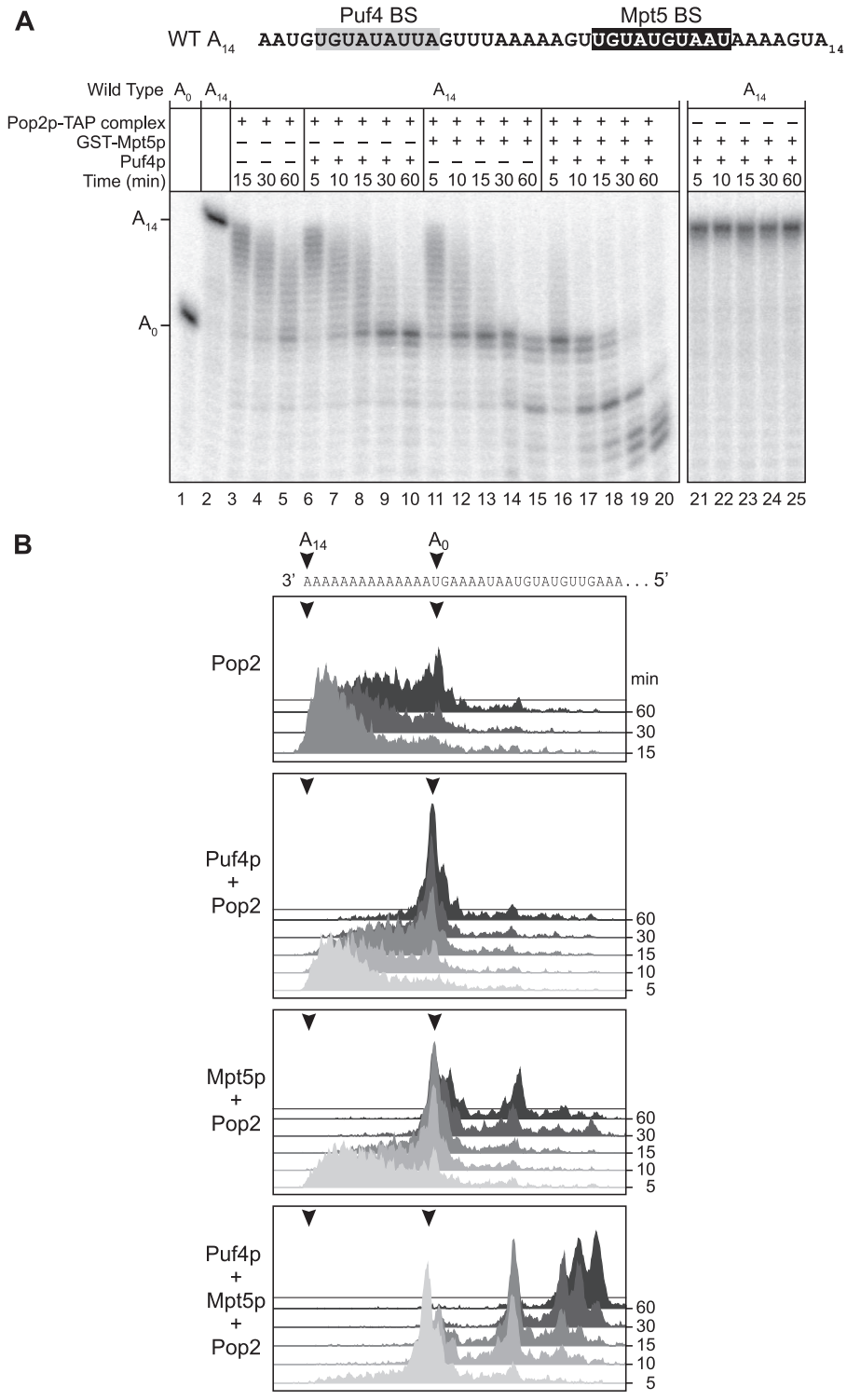


FIGURE 7. **Puf4p and Mpt5p collaborate to accelerate deadenylation *in vitro*.** *A*, *in vitro* deadenylation assays with Puf4, Mpt5p, and the Pop2p deadenylase complex. The sequence of the wild-type *HO* substrate RNA (*WT* A_{14}) with 14 adenosines at the 3′-end is given at the top. Puf4 BS and Mpt5 BS are highlighted in *gray* and *black*, respectively. Deadenylation reactions were performed using 10 ng of Pop2p-TAP complex with GST-Puf4p RBD and/or GST-Mpt5p RBD as indicated above the gels. Mobilities of RNA markers, without (A_0) or with (A_{14}) poly(A) tails, are indicated at *left*. *B*, graphs of poly(A) tail length distribution were created from phosphorimaging scans, plotting signal density for each lane in *panel A*. *Arrowheads* at the top of each graph indicate the positions of the substrate RNA with or without the A_{14} poly(A) tail.

To study Puf4p effect on poly(A) shortening in detail, we examined Puf4p-stimulated deadenylation *in vitro*. Recombinant GST-Puf4p was purified from *E. coli*. As a substrate, we created a 5′-end-labeled *HO* 3′-UTR RNA containing the Puf4 BS with fourteen adenosine residues at its 3′-end (sequences in Fig. 6*A*). As expected, Puf4p bound to the *HO* 3′-UTR substrate with high affinity (apparent K_d of 15 nM), and mutation of the UGU trinucleotide completely abrogated the interaction (Fig. 6*A*).

Pop2p complexes were purified from yeast using TAP tag affinity purification; both Pop2p and Ccr4p were enriched in this partially purified fraction as judged by Western blotting (7). The Pop2p complex alone removed the poly(A) tail in a concentration- and time-dependent manner and was capable of completely deadenylating the RNA at high concentrations (supplemental Fig. S2). Puf4p alone neither deadenylated nor destroyed the RNA (Fig. 6*B*, *lanes 3–5*). The Pop2p complex, added at low concentrations, showed modest deadenylation activity; 5% of the RNA was fully deadenylated after 120 min (*lanes 6–9*). Addition of Puf4p to the Pop2p complex resulted in rapid deadenylation; fully deadenylated RNA was observed even at 30 min and comprised 85% after 120 min (*lanes 10–13*). This Puf4p-dependent deadenylation was sequence-specific; a mutation in the *HO* RNA substrate that abolished Puf4p binding eliminated enhanced deadenylation by the two mixed components but did not affect the activity of the Pop2p complex alone (*lanes 18–21*). In conclusion, Puf4p can stimulate deadenylation of an RNA substrate by directly recruiting the Pop2p deadenylase complex.

Puf4p and Mpt5p Collaborate to Greatly Accelerate Deadenylation in Vitro—To examine the combined affects of Puf4p and Mpt5p on the Pop2p complex, we performed an *in vitro* deadenylation assay in the presence of both PUF proteins with an RNA substrate containing both the Puf4 and Mpt5 binding sites (Fig. 7*A*). We used a low concentration of Pop2p complex to yield only slow deadenylation (Fig. 7*A*, *lanes 3–5*, and *panel B*). Each PUF protein stimulated deadenylation (Fig. 7*A*, *lanes 6–15*). In the presence of both PUF

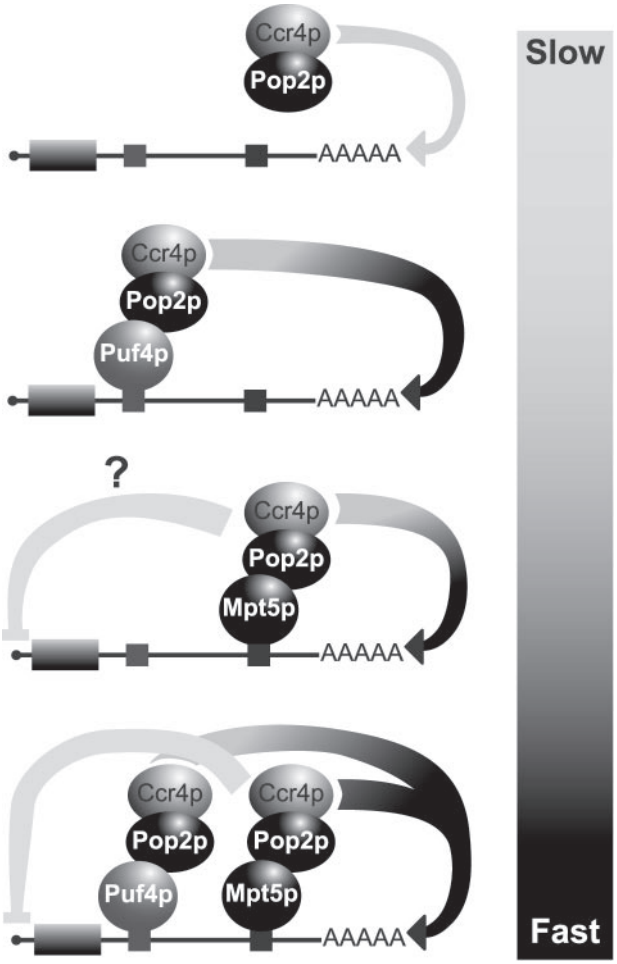


FIGURE 8. **Model of Puf4p- and Mpt5p-mediated repression of *HO* mRNA.** The target mRNA contains both the Puf4p binding site (*Puf4 BS*) and Mpt5p binding site (*Mpt5 BS*) in its 3′-UTR. Mpt5p (*black*) and Puf4p (*gray*) directly bind Pop2p, which recruits its partner Ccr4p. Puf4p requires Pop2p and Ccr4p for repression (represented as *shaded arrow*); Mpt5p enhances deadenylation via Pop2p and Ccr4p (18) and possibly represses translation through other mechanisms (represented as a *gray line*), as implied by the non-dependence of Mpt5p repression on the Ccr4p gene. PUF proteins enhance deadenylation; the *shaded box* indicates the relative rate of deadenylation from slow (*gray*) to fast (*black*).

proteins simultaneously, deadenylation was enhanced even more (Fig. 7*A*, *lanes 16–20*). For example, after 15 min the Pop2p complex alone fully deadenylated only 4% of the substrate RNA (Fig. 7*A*, *lane 3*), and Puf4p and Mpt5p individually yielded fully deadenylated 34 and 58% of the substrate RNA, respectively (Fig. 7*A*, *lanes 8* and *13*); at the same time point, the two PUFs mixed together deadenylated virtually all of the substrate and in fact progressively degraded the body of the RNA (Fig. 7*A*, *lane 18*). The mixed PUF proteins alone had no effect on the substrate in the absence of deadenylase complex (Fig. 7*A*, *lanes 21–25*). We conclude that Puf4p and Mpt5p collaborate to accelerate poly(A) removal by recruiting the Pop2p deadenylase.

DISCUSSION

Our results permit three main conclusions, summarized in Fig. 8. First, two PUF proteins, Puf4p and Mpt5p, collaborate to decrease expression of a single mRNA. The additive effects of regulation are seen both *in vivo* and *in vitro* and result from interactions with adjacent binding sites in the 3′-UTR. Second, the mechanism of repression by Puf4p involves direct binding to Pop2p, recruitment of the Pop2p-Ccr4p complex, and the enzymatic activity of the Ccr4p deadenylase. Third, the activities of the two PUF proteins differ: Puf4p, but not Mpt5p, strictly requires deadenylation.

Is repression by the two PUF proteins entirely a consequence of deadenylation? Both Puf4p and Mpt5p enhance deadenylation of *HO* mRNA *in vivo* and *in vitro* (this report and Refs. 7 and 18). The conclusion that the modes of action of the two PUF proteins differ is based on the fact that repression via Puf4p requires both Pop2p and Ccr4p whereas Mpt5p stringently requires only Pop2p. Importantly, repression via Puf4p is abolished by mutations in the active site of Ccr4p, strongly suggesting that deadenylation itself is essential for Puf4p function. Mpt5p also requires Pop2p for repression but, in contrast to Puf4p, Ccr4p is not critical for Mpt5p-mediated repression (this report and Refs. 7, 18). Yet deletion of Ccr4p stabilizes *HO* mRNA, effectively blocking deadenylation (18). Thus, in *ccr4* mutants, fully adenylated mRNA accumulates and can be repressed by overexpressed Mpt5p. These findings demonstrate that Mpt5p elicits an additional, second form of repression that is deadenylation-independent (Fig. 8). The simplest interpretation is that the complexes recruited by the two PUF proteins differ. Partners of the PUF proteins functionally analogous to Nanos, Brat, GLD-3, and CPEB in metazoa may be critical (13).

Puf4p and Mpt5p act in concert to ensure that *HO* mRNA is efficiently deadenylated and repressed. Indeed, simultaneous mutation of both PUF binding sites abolishes regulation (Fig. 4*B*). Dual regulation provides not only efficient control but new regulatory opportunities as well; differential expression or regulation of the two PUF proteins under specific conditions could selectively enhance or depress *HO* expression.

Combinatorial control by PUF proteins may be common. The 3′-UTRs of *hunchback* mRNA, a target of *Drosophila* Pumilio, and of *COX17* mRNA, a target of yeast Puf3p, both possess multiple binding sites that are important for repression *in vivo* (8, 26); *gld-1* mRNA, a target of FBF in *Caenorhabditis elegans*, possesses two FBF binding sites in its 3′-UTR (27). Twenty-eight mRNAs co-immunoprecipitate separately with Puf4p or Mpt5p; however, each of these mRNAs appears to contain a single PUF protein binding site, implying competition between the two proteins (14). Co-regulation of mRNAs by multiple PUF proteins may be common; combinations of other PUFs regulate several other mRNAs.[3]

Regulation of *HO* by Puf4p and Mpt5p demonstrates that two different PUF proteins act on a single mRNA *in vivo*. Complexes formed via a regulatory protein's interaction with a binding site in the 3′-UTR are common and a hallmark of 3′-UTR control (28). What is different here is that the different proteins bind two different sites to achieve full repression. The magnitude of repression achieved by the two proteins together (more than an order of magnitude change in mRNA half-life) is large, and greater than that achieved by either protein alone.

[3] W. Olivas, personal communication.

Puf4p and Mpt5p bind to their cognate sites with different affinities and with very different preferences for cognate over non-cognate sites (25- *versus* 3.5-fold for Puf4p and Mpt5p, respectively (Fig. 2*A*). Although it is difficult to accurately measure the low *in vivo* levels of Mpt5p and Puf4p, their intracellular concentrations were estimated to be ~29 and 17 nM, respectively (29). In this range, the cognate site of each protein would be partially occupied during standard growth conditions and neither protein would be expected to occupy the other's site. This situation provides robust opportunities for control. Under certain circumstances, one PUF protein might occupy only its own site, and in another, occupy both. Regulation could be influenced via specific protein partners as are involved in PUF regulation in *C. elegans* (NOS-3, GLD-3, and CPB-1, see Refs. 30–32) and Drosophila (NOS and Brat, see Refs. 33, 34). In addition, cell cycle and mitogen-activated protein kinases, with which Mpt5p interacts physically and genetically, could modulate repression activity (35). Importantly, the mechanism of repression will depend on which PUF is bound, providing additional versatility.

In many instances of nucleic acid polymerization or removal, the protein that possesses enzymatic activity is not intrinsically specific for a particular RNA or DNA template but gains that specificity by interacting with a separate protein. Our results show that Puf4p and Mpt5p provide sequence specificity to Ccr4p deadenylation activity. In their absence, the Ccr4p enzyme, as part of the Pop2p-Ccr4p complex, inefficiently deadenylates substrates independent of their sequence; in their presence, deadenylation becomes rapid and sequence-specific. It will be necessary to purify the relevant deadenylation complex to homogeneity to identify any essential components other than Pop2p and Ccr4p.

Deadenylation is the rate-determining step in mRNA decay, and deadenylation rates vary widely among mRNAs. Regulators such as RNA-binding proteins, and more recently small RNAs, can modulate deadenylation rates (36–38). In principle, enhancement of deadenylation can be achieved either by increasing the catalytic rate of the enzyme or by increasing its rate of association with the substrate. PUF proteins almost certainly do the latter, because their effect on deadenylation is entirely dependent on the presence of a cognate binding sequence in the RNA (this study; Fig. 6*B*; Ref. 7). Once bound by a PUF protein, an RNA molecule is rapidly deadenylated. Pab1p, which is bound to poly(A) tails *in vivo*, may also influence the character of deadenylation by Ccr4p *in vivo*.

The off-rates of PUF proteins from RNA and from Pop2p are predicted to determine the rate and extent of deadenylation *in vivo*. Because repression by Puf4p strictly requires deadenylation, these parameters will also determine the efficiency of repression.

Acknowledgments—We thank members of the Wickens laboratory for discussions and suggestions. We appreciate the work of L. Vanderploeg in preparing figures.

REFERENCES

1. Colegrove-Otero, L. J., Minshall, N., and Standart, N. (2005) *Crit. Rev. Biochem. Mol. Biol.* **40,** 21–73
2. Gebauer, F., and Hentze, M. W. (2004) *Nat. Rev. Mol. Cell. Biol.* **5,** 827–835
3. Gray, N. K., and Wickens, M. (1998) *Annu. Rev. Cell Dev. Biol.* **14,** 399–458
4. Wickens, M., Goodwin, E. B., Kimble, J., Strickland, S., and Hentze, M. W. (2000) in *Translational Control* (Mathews, M., ed) 2nd Ed., pp. 295–370, Cold Spring Harbor Press, Cold Spring Harbor, NY
5. Aviv, T., Lin, Z., Lau, S., Rendl, L. M., Sicheri, F., and Smibert, C. A. (2003) *Nat. Struct. Biol.* **10,** 614–621
6. Chang, T. C., Yamashita, A., Chen, C. Y., Yamashita, Y., Zhu, W., Durdan, S., Kahvejian, A., Sonenberg, N., and Shyu, A. B. (2004) *Genes Dev.* **18,** 2010–2023
7. Goldstrohm, A. C., Hook, B. A., Seay, D. J., and Wickens, M. (2006) *Nat. Struct. Mol. Biol.* **13,** 533–539
8. Jackson, J. S., Jr., Houshmandi, S. S., Lopez Leban, F., and Olivas, W. M. (2004) *RNA* **10,** 1625–1636
9. Lykke-Andersen, J., and Wagner, E. (2005) *Genes Dev.* **19,** 351–361
10. Semotok, J. L., Cooperstock, R. L., Pinder, B. D., Vari, H. K., Lipshitz, H. D., and Smibert, C. A. (2005) *Curr. Biol.* **15,** 284–294
11. Chou, C. F., Mulky, A., Maitra, S., Lin, W. J., Gherzi, R., Kappes, J., and Chen, C. Y. (2006) *Mol. Cell. Biol.* **26,** 3695–3706
12. Moraes, K. C., Wilusz, C. J., and Wilusz, J. (2006) *RNA* **12,** 1084–1091
13. Wickens, M., Bernstein, D. S., Kimble, J., and Parker, R. (2002) *Trends Genet,* **18,** 150–157
14. Gerber, A. P., Herschlag, D., and Brown, P. O. (2004) *PLoS Biol.* **2,** 0342–0354
15. Tadauchi, T., Matsumoto, K., Herskowitz, I., and Irie, K. (2001) *EMBO J.* **20,** 552–561
16. Herskowitz, I. (1988) *Microbiol. Rev.* **52,** 536–553
17. Haber, J. (1998) *Annu. Rev. Genet.* **32,** 561–599
18. Goldstrohm, A. C., Seay, D. J., Hook, B. A., and Wickens, M. (2007) *J. Biol. Chem.* **282,** 109–114
19. Chen, J., Chiang, Y. C., and Denis, C. L. (2002) *EMBO J.* **21,** 1414–1426
20. Thore, S., Mauxion, F., Seraphin, B., and Suck, D. (2003) *EMBO Rep.* **4,** 1150–1155
21. Tucker, M., Staples, R. R., Valencia-Sanchez, M. A., Muhlrad, D., and Parker, R. (2002) *EMBO J.* **21,** 1427–1436
22. Hook, B., Bernstein, D., Zhang, B., and Wickens, M. (2005) *RNA* **11,** 227–233
23. Bernstein, D. S., Buter, N., Stumpf, C., and Wickens, M. (2002) *Methods* **26,** 123–141
24. Bernstein, D., Hook, B., Hajarnavis, A., Opperman, L., and Wickens, M. (2005) *RNA* **11,** 447–458
25. Duttagupta, R., Vasudevan, S., Wilusz, C. J., and Peltz, S. W. (2003) *Mol. Cell. Biol.* **23,** 2623–2632
26. Murata, Y., and Wharton, R. P. (1995) *Cell* **80,** 747–756
27. Crittenden, S. L., Bernstein, D. S., Bachorik, J. L., Thompson, B. E., Gallegos, M., Petcherski, A. G., Moulder, G., Barstead, R., Wickens, M., and Kimble, J. (2002) *Nature* **417,** 660–663
28. Kuersten, S., and Goodwin, E. B. (2003) *Nat. Rev. Genet.* **4,** 626–637
29. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003) *Nature* **425,** 686–691
30. Luitjens, C., Gallegos, M., Kraemer, B., Kimble, J., and Wickens, M. (2000) *Genes Dev.* **14,** 2596–2609
31. Eckmann, C. R., Kraemer, B., Wickens, M., and Kimble, J. (2002) *Dev. Cell* **3,** 697–710
32. Kraemer, B., Crittenden, S., Gallegos, M., Moulder, G., Barstead, R., Kimble, J., and Wickens, M. (1999) *Curr. Biol.* **9,** 1009–1018
33. Sonoda, J., and Wharton, R. P. (1999) *Genes Dev.* **13,** 2704–2712
34. Sonoda, J., and Wharton, R. P. (2001) *Genes Dev.* **15,** 762–773
35. Chen, T., and Kurjan, J. (1997) *Mol. Cell. Biol.* **17,** 3429–3439
36. Roush, S. F., and Slack, F. J. (2006) *ACS Chem. Biol.* **1,** 132–134
37. Wu, L., Fan, J., and Belasco, J. G. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 4034–4039
38. Giraldez, A. J., Mishima, Y., Rihel, J., Grocock, R. J., Van Dongen, S., Inoue, K., Enright, A. J., and Schier, A. F. (2006) *Science* **312,** 75–79