A Role for the Poly(A)-binding Protein Pab1p in PUF Protein-mediated Repression

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PUF proteins regulate translation and mRNA stability throughout eukaryotes. Using a cell-free translation assay, we examined the mechanisms of translational repression of PUF proteins in the budding yeast *Saccharomyces cerevisiae*. We demonstrate that the poly(A)-binding protein Pab1p is required for PUF-mediated translational repression for two distantly related PUF proteins: *S. cerevisiae* Puf5p and *Caenorhabditis elegans* FBF-2. Pab1p interacts with oligo(A) tracts in the *HO 3'-UTR*, a target of Puf5p, to dramatically enhance the efficiency of Puf5p repression. Both the Pab1p ability to activate translation and interact with eukaryotic initiation factor 4G (eIF4G) were required to observe maximal repression by Puf5p. Repression was also more efficient when Pab1p was bound in close proximity to Puf5p. Puf5p may disrupt translation initiation by interfering with the interaction between Pab1p and eIF4G. Finally, we demonstrate two separable mechanisms of translational repression employed by Puf5p: a Pab1p-dependent mechanism and a Pab1p-independent mechanism.

Regulation of messenger RNA (mRNA) is an important part of gene expression. Each mRNA contains instructions that determine where and when it will be expressed. Many of the instructions are contained in the untranslated regions (UTRs) of the mRNA (1, 2). UTRs encode instructions for translation activation, repression, mRNA decay, or localization (3). The instructions take the form of primary sequences and structural elements that can be recognized by RNA-binding proteins (RBPs) and microRNAs (miRNAs). The combination of these factors assembled on each transcript determines the fate of each mRNA.

PUF proteins are a family of RBPs that regulate the translation and stability of mRNAs to which they bind, typically by repressing their expression (4–9). PUF proteins utilize two mechanisms to repress mRNA expression: destabilization and translational repression (8). Shortening of the poly(A) tail is the first step of mRNA decay in eukaryotes (15). In yeast, Puf5p interacts with Pop2p, a member of the Ccr4/Not deadenylase complex, facilitating deadenylation and decay of target mRNAs (11, 16). PUF-mediated recruitment of the Ccr4/Not complex has been demonstrated in *Caenorhabditis elegans*, *Drosophila*, and humans, suggesting that it is a conserved regulatory mechanism (11, 12).

PUF proteins employ several different mechanisms to disrupt translation initiation. During the first step of initiation, the mRNA is circularized by the eIF4F complex, which then facilitates ribosome recruitment (17, 18). Circularization is achieved by eIF4G binding to both the cap-binding protein eIF4E and the poly(A)-binding protein PABP, bringing both ends of the mRNA in proximity (19). *Drosophila* Pumilio disrupts circularization by recruiting two different proteins: Nanos, which recruits the eIF4E-binding protein Cup, and Brat, which recruits the cap-binding protein, 4E-HP (14, 20, 21). *Xenopus* Pum2 binds directly to the 7-methyl-G cap structure, and may compete with eIF4E although it has a relatively low affinity for the cap (22). *Xenopus* Pum1 may also recruit the eIF4E-binding protein 4E-T via CPEB (13, 23, 24). In yeast, the non-canonical PUF protein Puf6p is able to block 40 S ribosomal subunit recruitment, possibly through its interaction with eIF5B (25). Although the mechanisms vary, disrupting translation initiation is a conserved method of PUF-mediated repression.

A PUF protein can participate in multiple regulatory complexes, each with different components and outcomes. For example, the *C. elegans* PUF protein FBF promotes opposite fates for the same mRNA at different stages of development; the *glf-1* mRNA can be repressed or activated by FBF in different regions of the germ line (7). Even PUF complexes that serve the same function can vary; Pumilio forms different repression complexes in *Drosophila* depending on the target mRNA. The *cyclin B* mRNA is regulated by only Pumilio and Nanos, whereas repression of *hunchback* requires a complex of Pumilio, Nanos, and Brat (20). The combinatorial interactions between each of the components in a regulatory complex control the outcome of regulation.

We sought to characterize PUF regulation in yeast. Using the *HO 3'-UTR* as a model target, we first identified several sequence elements in the 3'-UTR, outside the PUF binding site that enhanced PUF repression. We show that Pab1p binds to these elements to promote optimal Puf5p repression and that Pab1p must be able to activate translation in order to facilitate repression. Pab1p may be part of a general mechanism of translational control by PUF proteins.
Yeast Strains—The yeast extracts used in translation assays were derived from the MB yeast strain unless otherwise specified: MATa ade2–1 his3–11,15 leu2–3,112 trpl–1 ura3–1 can1–100 [rho 5] leu, M-o, a gift from Allan Jacobson (University of Massachusetts Medical School). Both the pab1Δ strains were also provided by the Jacobson laboratory: pab1Δ/php1Δ (MATα ade2–1 his3–11,15 leu2–3,112 trpl–1 ura3–1 can1–100 pab1::HIS3 php1::LEU2) and pab1Δ/rpl39Δ (MATα ade2–1 his3–11,15 leu2–3,112 trpl–1 ura3–1 can1–100 rpl46::LEU2 pab1::HIS3). Yeast extracts for the php1Δ and comparable WT strain were derived from BY4741 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and BY4741 php1Δ::KANMX strains, obtained from Open Biosystems.

Yeast Extracts and Cell-free Translation Assays—Yeast extracts were prepared as described (26). Briefly, each yeast strain was grown to saturation (OD660 ≥ 2.0) in 1–4 liters of YPAD media at 30 °C. The yeast were washed and lysed in liquid nitrogen by grinding with a mortar and pestle. The soluble fraction of the lysate was filtered through a G25 Sephadex column and subsequently used in translation reactions.

Cell-free translation assays were performed exactly as described (26). Briefly, translation reactions were assembled using 60 μg of yeast extract, 10 ng of firefly reporter mRNA, 30 ng of Renilla reporter mRNA, 2.5 μl of 6× translation buffer, 1 μl of 4 mg/ml creatine kinase, and 0.1 μl of RNasin (Promega) in a 15 μl reaction. Reactions were incubated for 1 h at 30 °C before luciferase levels were measured using the Dual-Luciferase Reporter Assay System from Promega.

To obtain the ratio of firefly to Renilla luciferase measurements, the firefly reading was divided by the Renilla reading from each reaction. Then the basal translation level (with no added protein) was set to 1, and all ratios for the same RNA were scaled to that basal translation level. The standard deviation was calculated from 3 replicates for each sample for every experiment.

Purified recombinant Puf5p was added to extracts to a final concentration between 200–350 nM. Half-maximal repression required 45 nM protein (26). The apparent Kd of the protein for its binding site in the HO 3′-UTR is ~100 nM under these conditions (27). When noted, purified recombinant Pab1p was added to a final concentration of 300–600 nM.

Unless indicated otherwise, the yeast strains used to derive translation extracts possessed a wild-type Puf5p. We directly compared these extracts to puf5 deletion extracts by titrating in purified Puf5p into both. Puf5p activity was identical in both strains (data not shown), including the maximal level of repression observed, indicating endogenous Puf5p is either inactive at stationary phase or inactivated by the extraction process.

In Vitro Transcribed Reporter mRNAs—All reporter mRNAs were in vitro transcribed from PCR products using T3/T7/SP6 MEGAscript kits from Ambion with 20% of the suggested GTP and 6 mM m7G cap analog (New England Biolabs). PCR products were purified by phenol/chloroform extraction and ethanol precipitation prior to use as templates for transcription. The control Renilla reporters were derived from the pSP65-Ren plasmid (26). The Can1-Pgk1 firefly reporter in Fig. 1B is the same as the renilla reporter from pSP65-Ren except that it contains the firefly luciferase ORF instead of the Renilla luciferase ORF. For the 1xBS reporter, the Puf5p binding site from the Cin8 3′-UTR was inserted into the Pgk1 3′-UTR: AGTTGTAATTTAAAATAGCT. For the 3xBS reporter, the Puf5p site from the HO 3′-UTR was inserted in triplicate: AGTTGTAATTTAAT.

The HO firefly reporters were derived from the pYC2-HO plasmid as described (26). HO mutants 1–6 contained 3 nt transversion mutations as shown in Fig. 1A. Each mutation was also optimized to avoid creating new secondary structures in the 3′-UTR. The 2x(A) mutant is equivalent to mutant 2 from Fig. 1C. HO 1x(A) contains mutations in the two oligo(A) tracts downstream of the Puf5p site, converting them from AAAA to AcAA. The HO no(A) mutant combines the mutations in 2x(A) and 1x(A) such that all 3 oligo(A) tracts are disrupted. The E1 and 185 spacer mutations in the HO reporter have been described previously (26). The HO mut reporter that lacks PUF binding sites in Fig. 6B contains mutations in the Puf4p and Puf5p binding sites such that all three UGU sequences are mutated to ACA.

The other 8 reporters tested were created by ligation of the firefly luciferase ORF and the desired 3′-UTR from each gene and then PCR-selecting for the correct product. The lengths of the 3′-UTRs were based on data from a tiling array on the yeast transcriptome (28) and are as follows: HO, 67 nt; AseI, 318 nt; Cin8, 253 nt; Ddh1, 167 nt; Lrg1, 213 nt; Rax2, 89 nt; Ypp1, 112 nt; and Pgk1, 76 nt. The PCR products were cut and inserted into the pRS416-TEF vector using the HindIII and BamHI sites. The PCR template for in vitro transcription was amplified from the reporter plasmid with the T3 promoter sequence and contained the start of the luciferase ORF to directly downstream of the HindIII site. For adenylated transcripts, the reverse primer encoded an additional 50 nt poly(A) tail. For the Cin8 (A) del mutant, 13 of the 15 As downstream of the Puf5p site were deleted. The remaining 2 As were left to avoid disrupting the Puf5p binding site. The FBF target reporters, 0 FBE and 3 FBE, have previously been described (26).

Purified Proteins—The recombinant GST-tagged Puf5p and FBF-2 expression plasmids used in this study have been described previously (27, 29). The Pab1p expression plasmid was derived from pGex-6P1 and contains the full-length Pab1p coding sequence inserted into the EcoRI and NotI restriction sites, resulting in an N-terminal GST-fusion protein. The Pab1p truncation proteins contained the following deletions or mutations: ΔRRM1, 8–116 aa; ΔRRM1–2, 6–203 aa; 4G mut, D184E A185K L186M; ΔRRM3–4, 220–400 aa; ΔRRMs, 6–400 aa; ΔC-term, 414–568 aa; Δlinker, 399–436 aa; ΔMLLE, 436–568 aa. The 4G mutant was described previously as Pab1–184 (30) and specifically disrupts the interaction between Pab1p and elf4G. Proteins were purified from E. coli as described (26).

EMSA Assays—Electrophoretic mobility shift assays were performed exactly as described (31) with the exception of the binding reaction buffer, which was comprised of 10 mM HEPES (pH 7.4), 1 mM EDTA, 50 mM KCl, 2 mM DTT, 0.1 mg/ml BSA, 0.02% Tween 20, and 60 ng/μl competitor RNA. The competi-
tor RNA was yeast tRNA from Ambion. The $K_D$ values and standard errors for each RNA/protein interaction were derived from an average of at least three independent experiments.

**RESULTS**

**Oligo(A) Tracts Are Important for Puf5p-mediated Repression**—We previously developed an *in vitro* translation system that supports PUF-mediated repression (Fig. 1A) (26). The system employs two different luciferase reporter mRNAs: a firefly reporter that contains the 3'-UTR of a PUF target mRNA. The reporters were combined with purified yeast cytoplasmic extracts and purified recombinant proteins, where noted, and allowed to translate for 1 h at 30 °C. Next, the levels of luciferase activity were measured by quantifying luminescence. B, we measured the translation of several reporter mRNAs in response to Puf5p. The light gray bars in each graph indicate the basal level of translation for each mRNA while dark gray bars indicate translation in the presence of Puf5p. The HO mRNA is a target of both Puf4p and Puf5p and thus contains two PUF binding sites in its 3'-UTR (27). However, the Puf4p site does not contribute substantially to Puf5p regulation (26). A firefly reporter with the Can1 5'-UTR and the Pgk1 3'-UTR was used as a template for the insertion of Puf5p binding sites. The Pgk1, 1xBS and 3xBS reporters contain 0, 1 or 3 Puf5p binding sites in their 3'-UTR, respectively. C, to isolate sequences important in Puf5p repression, we made mutant reporters that contained 3 nt transversions across selected regions in the HO 3'-UTR. Each mutant 3'-UTR sequence was analyzed using RNAfold to avoid creating stable secondary structures. D, each of the 3 oligo(A) tracts highlighted in part C was mutated in a series of reporters. To eliminate complications from close proximity of the poly(A) tail, the poly(A) tail was removed from these reporters. E, same reporters as in part D were assayed for Puf5p repression with poly(A) tails.

**FIGURE 1.** Oligo(A) tracts in the HO 3'-UTR are important for Puf5p repression. A, diagram of the cell-free translation assay. Two different reporter mRNAs encoding firefly and Renilla luciferase were added to each translation reaction. The Renilla reporter served as a control while the firefly reporter typically contained the 3'-UTR of a PUF target mRNA. The reporters were combined with purified yeast cytoplasmic extracts and purified recombinant proteins, where noted, and allowed to translate for 1 h at 30 °C. Next, the levels of luciferase activity were measured by quantifying luminescence. B, we measured the translation of several reporter mRNAs in response to Puf5p. The light gray bars in each graph indicate the basal level of translation for each mRNA while dark gray bars indicate translation in the presence of Puf5p. The HO mRNA is a target of both Puf4p and Puf5p and thus contains two PUF binding sites in its 3'-UTR (27). However, the Puf4p site does not contribute substantially to Puf5p regulation (26). A firefly reporter with the Can1 5'-UTR and the Pgk1 3'-UTR was used as a template for the insertion of Puf5p binding sites. The Pgk1, 1xBS and 3xBS reporters contain 0, 1 or 3 Puf5p binding sites in their 3'-UTR, respectively. C, to isolate sequences important in Puf5p repression, we made mutant reporters that contained 3 nt transversions across selected regions in the HO 3'-UTR. Each mutant 3'-UTR sequence was analyzed using RNAfold to avoid creating stable secondary structures. D, each of the 3 oligo(A) tracts highlighted in part C was mutated in a series of reporters. To eliminate complications from close proximity of the poly(A) tail, the poly(A) tail was removed from these reporters. E, same reporters as in part D were assayed for Puf5p repression with poly(A) tails.

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The $K_D$ values and standard errors for each RNA/protein interaction were derived from an average of at least three independent experiments.
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To identify these sequences, we generated a series of 3 nt mutations across the 67 nt HO 3′-UTR, with the PUF binding sites intact (Fig. 1C). Mutants 2 and 4 manifested a small but significant disruption of repression (Fig. 1C). Both mutations eliminate “AAA” trinucleotides close to the Puf5p binding site. To further characterize the importance of these oligo(A) tracts, we mutated all three oligo(A) tracts in the 3′-UTR of the poly(A) tail. The results of three independent experiments are shown in Fig. 1B. 

To determine whether the oligo(A) tract enhancers in the HO 3′-UTR were a general feature of Puf5p targets, we examined six other Puf5p targets: Ase1, Cin8, Dh11, Lrg1, Rax2, and Ypp1. All six targets were significantly repressed by Puf5p, though to varying extents (Fig. 2A). Because efficient repression of HO by Puf5p requires either oligo(A) tracts or a poly(A) tail, we assayed repression of the six reporters without a poly(A) tail. Four of the seven targets tested, Ase1, Cin8, Rax2, and HO, remained repressed in the absence of a poly(A) tail (Fig. 2B). Targets that were repressed by Puf5p without a poly(A) tail contained oligo(A) tracts (of 4 or more As) within 15 nt of the Puf5p binding site (Fig. 2C).

The Cin8 3′-UTR contains a 15 nt oligo(A) tract directly downstream of the Puf5p binding site. We tested whether the oligo(A) tract was important for Puf5p regulation by exciting the oligo(A) tract from the Cin8 3′-UTR. Deletion of the oligo(A) tract slightly decreased repression of both poly(A) plus and minus mRNAs (Fig. 2D). These results mirror those of the HO reporter, where oligo(A) tracts are required for Puf5p-mediated repression, especially in the absence of a poly(A) tail.

Pab1p Is Required for PUF-mediated Repression—The importance of oligo(A) elements suggested that a protein with high affinity for oligo(A) sequences might be important for Puf5p-mediated repression. Because Pab1p binds A-rich RNA sequences (32), we tested whether it was required for Puf5p repression. We assayed Puf5p repression in yeast extracts derived from a pab1Δ/php1Δ double deletion strain; Deletion of Php1 was necessary to suppress the lethality of the Pab1 deletion (33). In extracts from pab1Δ/php1Δ strain Puf5p repression was severely compromised compared with repression in a comparable wild-type strain (Fig. 3A). To test whether this effect was due to the absence of Pab1p, we added back...
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recombinant yeast Pab1p purified from E. coli. Addition of Pab1p activated translation (supplemental Fig. S1a) and partially restored repression by PuF5 (Fig. 3A). Similarly, an HO reporter lacking a poly(A) tail was not repressed in Pab1p deficient extracts but addition of Pab1p restored repression (Fig. 3B). Addition of Pab1p also enhanced translation of the HO reporter without a poly(A) tail but to a lesser extent (supplemental Fig. S1b).

To control for the effects of pbp1Δ, we assayed repression in a pbp1Δ extract. PuF5 repression was unaffected (Fig. 3C). To further demonstrate loss of Pab1p disrupted repression, we assayed PuF5 repression in another yeast strain deficient for Pab1p: pab1Δ/pbp1Δ. PuF5 repression of HO was completely abolished in pab1Δ/pbp1Δ extracts regardless of the presence of a poly(A) tail (Fig. 3D). The requirement for Pab1p was not restricted to regulation of the HO reporter as four other PuF5 targets, Ase1, Cin8, Rax2, and Ypp1, also required Pab1p to be repressed (Fig. 3E). The C. elegans PUF protein FBF-2 was previously shown to repress a target mRNA in yeast extracts (26). We assayed repression by FBF-2 in WT and pab1Δ/pbp1Δ extracts using reporters that contained either 0 or 3 FBF binding elements (FBEs).
latory mechanism for PUF proteins, we tested whether FBF-2 could repress in the absence of Pab1p. Indeed, repression by FBF-2 was nearly eliminated in the absence of Pab1p (Fig. 3F). We conclude that Pab1p may be a part of a conserved transla-
tional control mechanism employed by PUF proteins.

Pab1p Binds to the Oligo(A) Tracts in the HO 3′-UTR—The data thus far suggested that Pab1p might bind the oligo(A) tracts in the 3′-UTR to mediate repression. To demonstrate the requirement for both oligo(A) tracts and Pab1p in Puf5p repression, we assayed whether absence of oligo(A) tracts would prevent Pab1p from facilitating Puf5p repression in pab1Δ/pbp1Δ extracts. Indeed, the ability of Pab1p to facilitate repression was proportional to the number of oligo(A) tracts in the 3′-UTR (Fig. 4). An HO reporter lacking all three oligo(A) tracts and a poly(A) tail was no longer repressed by Puf5p. Thus, Pab1p requires oligo(A) tracts or a poly(A) tail to facilitate Puf5p repression.

These findings implied that Pab1p interacts directly with the oligo(A) tracts in the HO 3′-UTR. To test this directly, we measured binding of purified recombinant Pab1p to the HO 3′-UTR in electrophoretic gel shift assays (EMSAs). Pab1p bound to the HO 3′-UTR (lacking a poly(A) tail) with an affinity of ~28 nM while mutations that eliminated the oligo(A) tracts (HO no(A)s) reduced binding at least 30-fold (Fig. 5). Pab1p bound to a poly(A) RNA with an affinity of ~1 nM (supplemen-
tal Fig. S2) demonstrating that Pab1p has a higher affinity for poly(A) versus internal oligo(A) sequences. We conclude that Pab1p binds oligo(A) sequences within the HO 3′-UTR in vitro.

RRMs 1 and 2 and the C Terminus of Pab1p Are Required for Repression—Pab1p contains four RNA-recognition motifs (RRM), a linker domain and a C-terminal helical domain known as “PABC” or the “MLLE” domain (34, 35). To identify domains necessary to facilitate Puf5p-mediated repression, we constructed a set of Pab1p mutants (Fig. 6A). Using these purified mutant proteins, we supplemented pab1-null extracts with each type of mutant Pab1p protein. We assayed whether each Pab1p mutant facilitated Puf5p repression.

FIGURE 4. Oligo(A) tracts in the HO 3′-UTR are required for Pab1p-facili-
tated repression. We assayed Puf5p repression of the HO mutant reporters shown in Fig. 1D in pab1Δ/pbp1Δ extracts. The reporters contained mutations in the indicated oligo(A) tracts and lacked a poly(A) tail. The light gray bars indicate the basal level of translation for each mRNA while dark gray bars indicate translation in presence of Puf5p. The light gray-striped bars indicate the basal level of translation with added Pab1p while dark gray-striped bars indicate translation in presence of added Puf5p and Pab1p.

FIGURE 5. Pab1p binds oligo(A) tracts in the HO 3′-UTR. A, we performed EMSAs with purified recombinant Pab1p and several radiolabeled RNA oligos. In each experiment, increasing concentrations of protein were combined with the radiolabeled RNA oligo indicated. The graph shows the averaged binding curves for Pab1p with four different RNAs. The binding curves are the result of at least three independent experiments for each RNA. The maximum percentage of shifted RNA was set to 100% except in the case of HO no(A)s oligo where a complete shift was not attained. B, sequences of the oligos used in the binding experiments as well as their apparent K_{D} values as calculated using Graphpad Prism software. The oligo(A) tracts are highlighted in black, and mutations are underlined and in lowercase. The PUF binding element (PBE) is bracketed. C–F, representative EMSA gels for the four different RNA oligos tested. The lower band is unbound radiolabeled RNA, while the upper band is RNA bound to Pab1p.
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Three mutant Pab1p proteins were capable of promoting Puf5p repression: ΔRRM3–4, Δlinker, and ΔMLLE (Fig. 6B) suggesting these domains are dispensable for repression. Simultaneous deletion of the linker and the MLLE domain eliminated repression activity (Fig. 6B, ΔC-term), indicating that the linker and the MLLE domains have redundant functions in repression. RRM1 and 2 were the only domains that were absolutely required for repression.

RNA binding activity was required for Pab1p to mediate Puf5p repression. Pab1p was only able to support Puf5p repression if two or more RRMs were present (Fig. 6B). However, the RRMs were not functionally equivalent; RRMs 1 and 2 were required for Puf5p repression while 3 and 4 were dispensable (Fig. 6B, ΔRRM1–2 versus ΔRRM3–4). RRM1s 1 and 2 are

Repression (Fig. 6B) or enhanced translation of the control Renilla reporter (Fig. 6C). Table 1 summarizes the results of these experiments.

### TABLE 1

| Pab1p     | Repression activity | Translational activation |
|-----------|---------------------|--------------------------|
| WT        | Active              | Active                   |
| ΔRRMs     | Inactive            | Inactive                 |
| ΔRRM1–2   | Inactive            | Inactive                 |
| ΔRRM1     | Inactive            | Active                   |
| 4G mut    | Inactive            | Active                   |
| ΔRRM3–4   | Active              | Active                   |
| ΔC-term   | Inactive            | Active                   |
| Δlinker   | Active              | Active                   |
| ΔMLLE     | Active              | Active                   |
thought to bind more specifically to poly(A) while RRMs 3 and 4 interact with RNA nonspecifically (36). Thus, RRMs 1 and 2 may be important for efficiently recruiting Pab1p to the RNA. However, RRM 2 is also required for the interaction with eIF4G (37). The Pab1p 4G mut cannot bind eIF4G due to a mutation in RRM 2 and therefore also lacks the ability to activate translation (30). The 4G mutant was unable to promote translation (Fig. 6B) or facilitate Puf5p repression (Fig. 6B). The same eIF4G mutant is active in stimulating cap-dependent, poly(A) tail-independent translation (30). These data suggest that the interaction between eIF4G and Pab1p may be required for Puf5p repression.

Two Distinct Repression Mechanisms—Puf5p relies on two distinct mechanisms of translational repression: a Pab1p-dependent mechanism and a Pab1p-independent mechanism. The existence of two mechanisms is evident in Fig. 7; The HO reporter was still repressed, albeit inefficiently, in the absence of Pab1p, demonstrating Puf5p can repress through an additional, Pab1p-independent mechanism. Previous experiments uncovered another mutation in the HO 3′-UTR that disrupted repression: the 185 spacer mutation increases the distance between the ORF and the PUF sites (26). In WT extracts, the 185 spacer mutation moderately decreased repression by ~20% (26). In contrast, the spacer mutation eliminated repression of HO in pab1Δ/php1Δ extracts (Fig. 7). Thus, we conclude that the effect of oligo(A) tracts and spacing are additive, indicating there are two separable mechanisms contributing to Puf5p-mediated translation repression: a Pab1p-dependent mechanism and Pab1p-independent mechanism.

DISCUSSION

We set out to understand the role of 3′-UTR regulatory elements in Puf5p regulation in vitro. We draw three main conclusions. First, oligo(A) elements in the HO 3′-UTR enhance Puf5p-mediated repression. Second, the oligo(A) elements recruit Pab1p. Third, Puf5p requires Pab1p to efficiently promote translational repression. We conclude that Pab1p bound to the 3′-UTR enhances repression by PUF proteins and Puf5p functions through Pab1p-dependent and -independent mechanisms.

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Pab1p-dependent Repression—Pab1p could be required for PUF-mediated repression either because Puf5p interferes with Pab1p activation or because Pab1p can inhibit translation. We favor the former hypothesis because Pab1p translation activation was required to observe Puf5p-mediated repression. The Pab1p 4G mutant, which cannot interact with eIF4G, could not activate translation and was incapable of facilitating Puf5p repression (Table 1, 4G mut).

Puf5p may interfere with the interaction between Pab1p and elf4G. The interaction between elf4G and Pab1p was specifically required to observe Puf5p-mediated repression (Fig. 6B, 4G mut). Furthermore, Puf5p repression did not extend beyond Pab1p ability to activate translation (supplemental Fig. S1b). The proximity of Pab1p to Puf5p also affected Puf5p ability to repress. Puf5p efficiently repressed translation when only the oligo(A) tracts were present (Fig. 1D) but repression was less effective if Pab1p was more distantly located on the poly(A) tail (Fig. 1E). In one simple model, Puf5p represses translation by disrupting the interaction between Pab1p and elf4G, an activity that might be promoted by physical proximity.

The connection between Pab1p and Puf5p activity could be direct or mediated by an as yet unidentified protein. We did not detect an interaction between the two recombinant proteins, despite each protein being able to bind RNA specifically (data not shown).

Pab1p Binding to Short Oligo(A) Tracts—Our data also imply that Pab1p may bind more promiscuously than previously thought. One reporter, Ypp1, lacked obvious Pab1p binding sites yet still required Pab1p to be repressed (Figs. 2C and 3E). It is possible that Pab1p may bind other elements in target 3′-UTRs to facilitate repression. Repression of Pab1p translation activation may be a general mechanism of PUF-mediated control; Another PUF protein, Puf3p, was recently isolated in a complex with Pab1p (38). Similarly, repression by the C. elegans PUF protein, FBE-2, was dependent on Pab1p (Fig. 3F).

Based on experiments with Pab1p mutant proteins, only two of the four RRMs (RRMs 1 and 2) were necessary for Puf5p repression (Table 1). The requirement for only these two RRMs suggests they may be important for Pab1p binding to oligo(A) tracts.

Pab1p-independent Repression—Our data show that there are two distinct mechanisms of translational repression employed by Puf5p: a Pab1p-dependent mechanism and a Pab1p-independent mechanism. The existence of two mechanisms is evident in Fig. 3, A and B; The HO reporter was efficiently repressed in WT extract, partially repressed in pab1Δ/php1Δ extract, and no longer repressed when HO lacked a poly(A) tail in pab1Δ/php1Δ extract. Thus, the Pab1p-independent mechanism relies on the presence of a poly(A) tail.

The residual repression observed in pab1Δ/php1Δ extract could be due to other poly(A) binding proteins. Nab2p and Sgn1p also interact with poly(A) sequences (39, 40) which may explain why the poly(A) tail is necessary to observe repression in the absence of Pab1p. Nab2p and/or Sgn1p may be able to facilitate repression via the poly(A) tail while only Pab1p is able to bind internal oligo(A) sequences. Alternatively, Sgn1p and Nab2p may compete with Pab1p for binding to poly(A) and oligo(A) RNA and thereby inhibit Puf5p repression.
In *pab1Δ/rpl39Δ* extracts, Puf5p repression of *HO* is entirely abolished regardless of poly(A) status (Fig. 3D), suggesting Rpl39p may be involved in Puf5p repression either directly or indirectly. Rpl39p is part of the 60 S ribosomal subunit and participates in a gating mechanism that controls release of the polypeptide chain (41, 42). In conclusion, the Pab1p-independent translational repression mechanism requires a poly(A) tail, close proximity to the ORF, and possibly Rpl39p. Elucidating the molecular mechanisms involved will require understanding how these elements combine to facilitate translational repression.

**Broad Implications; Pab1p in Repression**—PABP activates translation by enhancing translation initiation. In a prevalent model, it does so by interacting with eIF4G, which in turn binds the cap-binding protein (Fig. 8A and see Introduction). Several reports suggest that Pab1p also has roles in translational repression. In the simplest instances, repressors bound to the 3′-UTR interfere with the ability of PABP to bind eIF4G. For example, the protein GW182, which is recruited by miRNAs via Argo- nautae, binds PABP and competes with the binding of eIF4G (Fig. 8B) (34, 43–45). In this way, the miRNA bound to the 3′-UTR prevents PABP's activation function. The Musashi (MSI) repressor protein acts similarly (Fig. 8B) (46). PABP can also promote repression by binding to repressors to form complexes that interfere with initiation at a different step. For example, the protein UNR binds both SXL and PABP, and the SXL-UNR-PABP complex bound to the 3′-UTR may enhance the Puf5p ability to disrupt the PABP/eIF4G interaction. In addition, Puf5p recruits the CAF1/CCR4 deadenylase complex through an interaction with CAF1/Pop2 (11).
Similarly, a UNR-IMP1-PABP complex bound to the 5'-UTR appears to block scanning of PABP own mRNA (Fig. 8C) (47). Comparisons of the work reported here to these examples is instructive. Repression by Puf5p is Pab1p-dependent, and blocked by point mutations that disrupt the Pab1p ability to bind eIF4G (Fig. 8D). Thus the PUF protein is likely to interfere with eIF4G binding as in Fig. 8B, though this has not been demonstrated directly. Puf5p also recruits the Caf1p/Pop2p de-adenylase complex to promote mRNA deadenylation and instability (11, 16). In these respects, repression by PUF proteins parallels that by the mRNA/GW182 complex. The duality of mechanisms may ensure repression is complete.

Our work shows that Pab1p binds directly to oligo(A) segments within the 3'-UTR of the HO mRNA. These oligo(A) tracts are reminiscent of the oligo(A) segments in the 5'-UTR that PABP binds to auto-regulate its own mRNA (48–50). Similarly, PABP binds to the 3'-UTR of the non-polyadenylated Dengue Virus RNA genome, near structured elements (51). Oligo(A) segments containing as few as four contiguous aden- sine residues promoted Pab1p binding in our experiments. These data suggest that internally bound oligo(A) segments may be much more prevalent than previously thought. The nature of "non-canonical" PABP/RNA interactions and their specificities are important unsolved problems.

Our work has demonstrated a key role for Pab1p in repression by PUF proteins in vitro. In vivo, the balance between occupancy of the poly(A) tail and the oligo(A) segments in the 3'-UTR is not known. The presence of internal oligo(A) seg- ments may anchor Pab1p after a minimal poly(A) length is reached during deadenylation. In that fashion, continued repression would be ensured, and decay enhanced. Indeed, like Puf5p, yeast PuFu3p interacts with Pab1p and enhances the rate of decapping of target mRNAs (52).

Each 3'-UTR nucleates the assembly of a unique regulatory complex responsible for mRNA expression. In turn, the context of each sequence element can drastically alter the outcome of a regulatory complex; A PuFu3p binding site is not sufficient to confer repression in a non-native 3'-UTR but in its native con- text reduces translation 10-fold (Fig. 1A). It appears that regulators such as PUF proteins must employ redundant mechanisms to ensure proper expression. Deciphering the code of 3'-UTR regulation will require a better understanding of the combinatorial interactions of RNA regulators and the multiplicity of their outcomes.

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