Translational repression by PUF proteins in vitro

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ABSTRACT

PUF (Pumilio and FBF) proteins provide a paradigm for mRNA regulatory proteins. They interact with specific sequences in the 3′ untranslated regions (UTRs) of target mRNAs and cause changes in RNA stability or translational activity. Here we describe an in vitro translation assay that reconstitutes the translational repression activity of canonical PUF proteins. In this system, recombinant PUF proteins were added to yeast cell lysates to repress reporter mRNAs bearing the 3′UTRs of specific target mRNAs. PUF proteins from *Saccharomyces cerevisiae* and *Caenorhabditis elegans* were active in the assay and were specific by multiple criteria. Puf5p, a yeast PUF protein, repressed translation of four target RNAs. Repression mediated by the *HO* 3′UTR was particularly efficient, due to a specific sequence in that 3′UTR. The sequence lies downstream from the PUF binding site and does not affect PUF protein binding. PUF-mediated repression was sensitive to the distance between the ORF and the regulatory elements in the 3′UTR: excessive distance decreased repression activity. Our data demonstrate that PUF proteins function in vitro across species, that different mRNA targets are regulated differentially, and that specific ancillary sequences distinguish one yeast mRNA target from another. We suggest a model in which PUF proteins can control translation termination or elongation.

Keywords: translational control; PUF protein; RNA-binding protein; mRNA control

INTRODUCTION

The lives of mRNAs are regulated from birth to death. In the cytoplasm, their activity depends on proteins and RNAs that control mRNA localization, translation, and decay. The 3′ untranslated region (3′UTR) of the mRNA recruits these factors and thereby determines the mRNA’s fate (Kuersten and Goodwin 2003). Understanding how 3′-UTR-borne complexes exert their effects is an important challenge.

PUF proteins (Pumilio and FBF) are a paradigm for protein-mediated control of mRNA fate. PUF proteins are required for stem cell self-renewal in a variety of species and cell types (Wickens et al. 2002). PUFs also have acquired idiosyncratic functions, including roles in body patterning in *Drosophila*, mating-type switching and mitochondrial activity in *Saccharomyces cerevisiae*, and cell fate determination in *Caenorhabditis elegans* (Wickens et al. 2002; Wharton and Aggarwal 2006). As judged by RNP immunoprecipitation and microarray (“RIP-chip”) analysis, each PUF protein interacts with a large group of mRNAs, ranging from tens to

thousands in yeast, *Drosophila*, and human cells (Gerber et al. 2004, 2006; Galgano et al. 2008). To date, all PUF proteins bind specific sequences in the 3′ UTR, termed the PBE, or PUF binding element (Wickens et al. 2002). Canonical PBEs contain a “UGUN₃₋₅AU” motif (“N” is any nucleotide), although variations exist (Wang et al. 2002, 2009; Gerber et al. 2004; Miller et al. 2008; Koh et al. 2009). PUF proteins usually repress the mRNAs they bind, although several recent reports demonstrate activation as well (Pique et al. 2008; Kaye et al. 2009; Suh et al. 2009).

PUF proteins decrease the amount of protein produced either by repressing translation or enhancing mRNA decay (Wickens et al. 2002). mRNA destabilization often is correlated with deadenylation of the target mRNA (Chagnovich and Lehmann 2001; Wickens et al. 2002; Goldstrohm et al. 2007). PUF proteins directly bind Pop2p, which recruits the Ccr4/Pop2/Not deadenylase complex (Goldstrohm et al. 2006; Kadyrova et al. 2007). Ccr4p is the major cytoplasmic deadenylase in yeast (Collart 2003; Denis and Chen 2003; Goldstrohm et al. 2006, 2007). PUFs may also enhance decay through Pop2p’s indirect recruitment of Dhh1p or Dcp1p, both of which enhance decapping (Hata et al. 1998; Collier et al. 2001; Maillet and Collart 2002). Recruitment of the Ccr4p/Pop2/Not complex may mediate translational repression as well (Goldstrohm et al. 2006). Indeed, Dhh1p can

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.2070110>.

repress translation (Coller et al. 2001; Minshall et al. 2001). PUF proteins also can act by sequestration of the m7GpppG cap that is required for translation of most mRNAs. For example, the *Drosophila* PUF protein, Pumilio, recruits Brat, which, in turn, binds d4EHP. The latter protein binds the cap and so inhibits translation (Wickens et al. 2002; Cho et al. 2006).

PUF repression complexes vary for different transcripts even in the same organism. In *Drosophila*, repression of the *cyclin B* mRNA requires only Pumilio and Nanos, whereas repression of *hunchback* requires a complex of Pumilio, Nanos, and Brat (Sonoda and Wharton 2001). The outcome of PUF regulation is also developmentally controlled: In the *C. elegans* germline, FBF first represses and later activates the *gld-1* mRNA (Suh et al. 2009). Thus, a single PUF protein can act through multiple mechanisms and binds multiple protein partners.

S. cerevisiae possesses five canonical PUF proteins: Puf1p/Jsn1p, Puf2p, Puf3p, Puf4p, and Puf5p/Mpt5p. mRNAs that associate with all five yeast PUF proteins have been identified by “RIP-chip” (Gerber et al. 2004). Here, we chose to analyze mRNAs that not only interacted with Puf5p, but whose mRNA stability and/or translation were affected in vivo by Puf5p. *HO*, *CIN8*, and *RAX2* mRNAs met these criteria (Gerber et al. 2006; Seay et al. 2006; Hook et al. 2007). Our aim was to establish a set of targets whose regulation was recapitulated in vitro, enabling us to uncover mRNA-specific variations in regulation and dissect their mechanisms.

PUF-mediated control of *HO* mRNA is particularly well documented. HOp is the endonuclease responsible for mating type switching, a key event in yeast biology. Not surprisingly, HOp expression is regulated at multiple levels (Nasmyth 1993; Broach 2004; Cosma 2004; Klar 2007). *HO* mRNA is a target of both Puf4p and Puf5p, which mediate repression by interacting with distinct sequences in the *HO* 3'UTR (Goldstrohm et al. 2006, 2007; Hook et al. 2007). Repression by Puf5p decreases mating-type switching specifically in daughter cells (Tadauchi et al. 2001).

CIN8, *RAX2*, and *DHH1* were initially identified as candidate Puf5p targets in a RIP-chip experiment and later in a yeast three-hybrid screen for RNA sequences that interact with Puf5p (Gerber et al. 2004; Seay et al. 2006). Cin8p is a kinesin-related motor protein involved in spindle assembly during mitosis (Gardner et al. 2008; Fridman et al. 2009). The *CIN8* 3'UTR binds to Puf5p with high affinity (~50 nM), and its half-life nearly doubles in the absence of Puf5p (Seay et al. 2006). Rax2p is a membrane protein involved in axial polarity; its mRNA is repressed and destabilized by Puf5p (Seay et al. 2006). Dhh1p is an RNA helicase that associates with the Ccr4/Not complex (Maillet and Collart 2002); *DHH1* mRNA interacts with Puf5p (Seay et al. 2006).

In vitro systems have been instrumental in deciphering mechanisms of translational control (Gebauer and Hentze

2007; Mathews et al. 2007 and references therein; Wu et al. 2007). To analyze PUF-mediated repression in *S. cerevisiae*, we developed an assay in which recombinant, canonical PUF proteins repressed translation in a yeast cell extract. Using that assay, we analyzed key features of the mRNA that are required for repression and uncovered differences in repression of different target mRNAs.

RESULTS

PUF repression recapitulated in vitro

To better understand the function of PUF proteins, we used a cell-free system that supports PUF-mediated repression. Purified recombinant PUF proteins were combined with reporter mRNAs and a crude yeast extract that supports translation in vitro (Fig. 1A; Iizuka and Sarnow 1997; Wu et al. 2007). The purity and integrity of all purified proteins were verified by SDS-PAGE gel electrophoresis (Supplemental Fig. 1). To test Puf5p-specific repression, we used a luciferase reporter with the firefly luciferase open reading frame (ORF) and the 3'UTR from *HO* mRNA. A *Renilla* luciferase reporter lacking PUF binding sites was included in every reaction as an internal control (Fig. 1A). Recombinant Puf5p repressed the *HO* reporter 10-fold, but had no effect on the control reporter (Fig. 1B). Repression was specific by two criteria: A mutant reporter that lacked PUF binding sites, *HO* mut, was unaffected by Puf5p; and a mutant Puf5p incapable of binding RNA was unable to repress (Fig. 1B). The GST tag used to purify the proteins did not affect protein activity as 6xHis-tagged Puf5p behaved identically (data not shown).

The concentration of Puf5p needed for repression correlated well with the K_d of the protein for its binding site in the *HO* 3'UTR. Half-maximal repression required 45 nM protein, while the apparent K_d of the protein for its binding site in *HO* is ~100 nM (Fig. 1C; Hook et al. 2007). The *HO* mut reporter was unaffected by levels of Puf5p threefold in excess of the K_d (Fig. 1C).

Kinetics of repression

To examine the kinetics of repression, we quantitated firefly luciferase at 10-min time points throughout a 1-h incubation. In the absence of Puf5p, firefly luciferase activity increased throughout the hour and accumulated linearly from 10 to 30 min (Fig. 2A). In the presence of Puf5p, firefly luciferase levels were reduced at every time point. Thus, repression was continuous throughout the time course (Fig. 2A,B).

To examine the stability of the mRNA in the translation extract, we re-isolated RNA from the extract at 10-min time points and performed a Northern blot to detect firefly luciferase mRNA (Fig. 2C). Before the RNA was re-isolated, each sample was combined with a known amount of a control

FIGURE 1. Puf5p represses translation *in vitro*. (A) The *in vitro* translation assay. Yeast cytoplasmic extracts and two luciferase reporter RNAs were combined with reaction buffers and allowed to translate for 1 h at 30°C. Each reaction contained two luciferase reporters: The *HO* reporter consisted of a short 5'UTR, the firefly luciferase ORF, and the *HO* 3'UTR. The boxes in the diagram labeled 4 and 5 represent the binding sites of Puf4p and Puf5p, respectively. The control reporter was composed of the 5'UTR of *CAN1*, the *Renilla* luciferase ORF, and the 3'UTR from *PGK1*. All reporters carried a m7GpppG cap structure and a 50-nt poly(A) tail. After incubation, levels of firefly and *Renilla* luciferase were determined using a luminescence-based detection system. (B) Puf5p specifically represses the *HO* reporter. The first graph shows the firefly (FF) luciferase response, the second graph shows the control *Renilla* (Ren) luciferase response, and the last graph shows normalized responses (FF/Ren). Normalization consists of dividing the firefly by the *Renilla* response and setting a control reaction (containing only buffer) to 1.0 for each reporter RNA. (C) Titration of Puf5p. Increasing concentrations of Puf5p were added to translation reactions containing either the *HO* or *HO* mut reporter.

RNA to normalize for variations in the re-isolation process. Puf5p had no significant effect on mRNA stability during the translation assay (Fig. 2C). In the same experiments, we measured luciferase levels at the final time point and verified that Puf5p had been active (Fig. 2D). Thus, the decrease in luciferase activity was due to an effect on translation rather than on decay.

In vivo, *HO* is a target of both Puf4p and Puf5p (Goldstrohm et al. 2006; Hook et al. 2007). Puf4p was also active *in vitro*: purified recombinant Puf4p repressed the *HO* reporter to a comparable extent as Puf5p in a *puf4Δ* extract (Fig. 3). The effects of Puf4p and Puf5p were not additive (data not shown).

Conservation

A PUF protein from *C. elegans*, FBF-2, also showed specific repression activity in yeast extracts. To test its activity, we used reporters bearing 0 to 3 binding sites for FBF, termed FBEs, in an unrelated 3'UTR. Reporters containing FBEs were repressed in the presence of FBF-2, and repression increased linearly as the number of FBEs increased (Fig. 4A). A control reporter that lacks FBEs, 0 FBE, was unaffected by FBF-2, and a mutant FBF-2 that cannot bind RNA had no effect on any reporter (Fig. 4A). FBF-2 repression was effective, requiring as little as 66 nM protein to effect significant repression of the 3 FBE reporter (Fig. 4B). Although the worm protein appeared to be less active than the yeast proteins, repression was specific. We conclude that molecular interactions required for PUF function are conserved from yeast to *C. elegans*.

Differential regulation

Puf5p regulates multiple mRNAs via their 3'UTRs. To compare the behavior of different targets *in vitro*, we constructed luciferase reporter mRNAs in which the 3'UTR was derived from a Puf5p target. We tested the 3'UTRs of *CIN8*, *DHH1*, and *RAX2* mRNAs (see Introduction). As a control, we chose the *PGK1* 3'UTR because it contains no putative Puf5p binding sites and was negative in tests for Puf5p targets.

All three additional reporters were specifically repressed by Puf5p, while the control reporter was unaffected (Fig. 5). The extent of repression differed among mRNAs: The *HO* reporter was repressed

more efficiently than the other three reporters. At the concentration of protein used (330 nM), *HO* was repressed by 80%, while *CIN8*, *DHH1*, and *RAX2* were repressed by 30%–40%. We conclude that the *HO* 3'UTR mediated particularly efficient repression.

An element in the *HO* 3'UTR

We next sought to understand the basis of the enhanced repression of *HO* versus other mRNAs. The *HO* 3'UTR contains two PUF binding elements that recruit Puf4p and Puf5p. Puf5p can bind to the Puf4p site in the *HO* 3'UTR, albeit with reduced affinity (Hook et al. 2007). In principle,

FIGURE 2. Puf5p represses continuously. (A) Kinetics of translation and repression. Firefly and *Renilla* luciferase levels were measured every 10 min during a 1-h incubation at 30°C. (B) Kinetics as a proportion of total luciferase activity. The data in panel A are expressed as the level of firefly luciferase from the *HO* reporter over time in the presence or absence of Puf5p. (C) RNA stability. Total RNA was re-isolated from translation reactions at various time points during a translation reaction, and firefly luciferase mRNA was detected via Northern blot. The control (cont.) RNA is a 500-nt fragment of firefly luciferase added after the translation reaction ends to control for variation in RNA re-isolation. To verify that Puf5p was active in this experiment, luciferase levels were measured at the 60-min time point of the same incubations (D). (Right) The graph quantifies the mRNA levels. To determine the percent RNA remaining, background readings were subtracted from the *HO* signal and then divided by the signal of the control RNA, where the RNA detected at 1 min is assumed to be equal to 100%. (D) Puf5p was active in the assays analyzing kinetics (see panel C).

the Puf4p binding site might be responsible for enhanced repression of the *HO* reporter. To test this idea, we mutated each PUF site in the *HO* 3'UTR and assayed repression by Puf5p. The loss of the Puf5p binding site almost completely abrogated Puf5p repression, while the loss of the Puf4 binding site had only a marginal effect (Fig. 6). Therefore, the Puf5p site is sufficient to promote enhanced repression of *HO*.

We next tested whether the identity of the Puf5p binding site was critical for repression. The *CIN8* Puf5p binding site promotes intermediate repression in the context of the *CIN8* 3'UTR (Fig. 5). To determine whether the *HO* Puf5p binding site was responsible for the enhanced repression, we replaced it with the Puf5p binding site from *CIN8* (Fig. 7A). The *CIN8* Puf5p binding site was able to promote maximal repression from within the *HO* 3'UTR, indicating that the identity of the Puf5p binding site does not determine the level of repression for the *HO* reporter. All of the putative Puf5p sites in the four reporters have been tested previously using electrophoretic mobility shift assays (EMSAs) to determine their relative affinity for Puf5p. In these assays, the Puf5p site in *HO* bound Puf5p twofold less tightly than did the binding sites in the *RAX2* and *CIN8* 3'UTRs (Seay et al. 2006; Hook et al. 2007). Thus, repression and affinity were not well correlated. We therefore hypothesized that features outside the canonical binding site were responsible for enhanced repression activity of the *HO* 3'UTR.

Role of additional sequences

To determine whether enhanced repression of *HO* was due to a discrete sequence outside of the canonical PUF binding sites, we created chimeric reporters using the *HO* and *CIN8* 3'UTRs. Sequences from the *HO* 3'UTR were placed in the *CIN8* 3'UTR while maintaining the same spacing relative to the Puf5p binding site (Fig. 7B). Two sequences from the *HO* 3'UTR enhanced repression of the *CIN8* reporter: segment A, containing the Puf4p site, and segment B. The contributions of segments A and B were not additive; when both A and B were combined in mutant *CIN8-AB*, the level of repression was equivalent to either mutation alone (ANOVA, $P \geq 0.9$, Fig. 7B). None of the chimeric reporters were repressed as well as the wild-type *HO* reporter. Since *CIN8-B* was significantly more repressed than *CIN8* alone, the sequence between the two PUF sites in the *HO* 3'UTR may be important for regulation.

An element in the *HO* 3'UTR

To identify sequences important for regulation in the *HO* 3'UTR, we analyzed transversion mutations covering 8–10-nucleotide (nt) blocks across the *HO* 3'UTR, excluding the two PUF sites. Segment E, ~10 nt downstream from the Puf5p binding site, showed significant de-repression (Fig. 8). We further narrowed down the critical region by subdividing segment E into 3-nt blocks, labeled E1–E3 (Fig. 8). The first 3 nt in segment E were identified as the most important for enhanced Puf5p repression.

To rule out the possibility that the loss of repression seen with mut E was due to an effect on Puf5p binding, we assayed the relative affinity of Puf5p for *HO* or *HO* mut E

FIGURE 3. Puf4p represses the *HO* reporter. Puf4p was added to translation reactions with either the *HO* or *HO* mut reporter. The final concentrations of Puf4p were 0, 0.5, or 2.5 μ M.

FIGURE 4. *C. elegans* FBF-2 is active in yeast extracts. (A) Repression by FBF-2 protein. Purified FBF-2 or an FBF-2 mutant was added to translation reactions with reporters containing 0–3 FBF binding elements (FBEs). FBF-2 specifically repressed only those reporters containing FBEs, and repression was directly correlated with the number of FBEs. The FBF-2 mutant protein contains alanine substitutions in the RNA-contacting residues of repeat 6 that greatly reduce its affinity for RNA. (B) Titration of FBF-2. Translation reactions containing the 3 FBE reporter were incubated with increasing concentrations of FBF-2.

using EMSAs (Fig. 9). A set of 5'-radiolabeled RNAs containing the Puf5p binding site with or without mut E was used in the EMSAs (Fig. 9A). Binding of Puf5p to the two RNAs was indistinguishable, demonstrating that segment E did not reduce repression activity by preventing the interaction with Puf5p (Fig. 9B–D). In contrast, other mutations in segments B and D (shown in Fig. 7) disturbed Puf5p binding (data not shown), and therefore were not pursued further. We conclude that segment E's role in repression is independent of Puf5p binding.

Proximity to the ORF is important

The *HO* 3'UTR is four times longer than that of *CIN8* mRNA (253 vs. 67 nt). We considered the possibility that a longer 3'UTR might decrease the efficacy of repression. To test this idea, we used the *HO* 3'UTR as a scaffold, and introduced extra sequence on either the 5' or 3' end of the *HO* 3'UTR; the extra sequence was derived from vector sequence or "CA" repeats. Insertion of up to 70 nt between the PUF site and poly(A) tail did not affect repression (Fig. 10A). In contrast, insertion of 66 or more nucleotides between the ORF and the *HO* 3'UTR reduced repression significantly ($P \leq 0.001$): expression of the reporter mRNAs doubled (Fig. 10B). The effect on repression reached a plateau at 66 nt from the ORF and was never

abolished completely; thus, the distance effect only accounts for part of the enhanced repression mechanism.

Since the reduction in repression closely matched the change in repression between *HO* and *HO* mut E, we tested whether mut E and an elongated 3'UTR might block the same interaction. If so, a mutant reporter with both a longer 3'UTR and mut E would behave similarly to either single mutant alone. An RNA carrying the longest 5' spacer mutation and mut E1 was not repressed more efficiently than were RNAs carrying the individual mutations ($P \leq 0.001$, Fig. 10B). We infer that both mutations perturb the same event.

DISCUSSION

The cell-free translation assay described here recapitulates PUF repression in vitro and demonstrates specific regulation for all three PUF proteins tested: yeast Puf4p and Puf5p, and *C. elegans* FBF-2. The specificity of the assay was validated by three criteria: only 3'UTRs from mRNA targets shown to be regulated in vivo were affected, the binding site and the PUF protein's binding activity were both required, and the amount of protein required for repression was comparable to its apparent K_d for the RNA. The non-canonical yeast protein Puf6p required substantially more protein to repress in analogous assays (Deng et al. 2008). Using the cell-free translation assay, we verified that Puf5p regulates three targets in addition to *HO*: *DHH1*, *RAX2*, and *CIN8*. Indeed, validation of targets may be a useful adjunct of the system. Differences in mechanisms of regulation among target mRNAs may be more easily discerned in the extract than in vivo.

FBF-2, a *C. elegans* PUF, was active in the yeast extract. Therefore, FBF-2 must be able to productively interact with yeast proteins to enact repression. Although FBF-2 appeared to be less active than the two yeast PUF proteins, the binding elements were suboptimal. Regardless, the ability of a PUF protein to function in the context of a different species demonstrates that PUF protein interactions, and likely function, are conserved.

FIGURE 5. Multiple target 3'UTRs mediate repression, but the *HO* 3'UTR is particularly efficient. Five reporters containing the firefly luciferase ORF and the 3'UTR from the mRNA indicated were tested in vitro. The *PGK1* 3'UTR served as a negative control (it is not a Puf5p target and contains no Puf5p binding elements). For detailed description of 3'UTRs, see Materials and Methods.

FIGURE 6. The Puf4p site is not required for repression. The *HO* 3'UTR was mutated to determine the contribution of each PUF binding element. For each site, the core “UGU” sequence was mutated to “ACA,” which eliminates PUF binding (Hook et al. 2007). The crossed-out boxes indicate the site that was mutated in each reporter.

The *HO* 3'UTR mediated more efficient repression than the other 3'UTRs tested. Our studies identified at least two contributing factors that underlie this efficiency: specific sequence elements, and distance between the PUF sites and termination codon. Mutational analysis of the *HO* 3'UTR revealed three sequence elements outside of the canonical PUF binding sites required for maximum repression by Puf5p. Two of the three mutations reduced the affinity of Puf5p for the RNA and therefore were unlikely to play a mechanistic role (data not shown). The third mutation uncovered an “AUU” sequence (identified as “mut E1” in Fig. 8) required for maximal repression of the *HO* reporter. In addition to this element, the distance between the termination codon and the *HO* 3'UTR was important: insertion of 66 nt or more reduced repression (Fig. 10). A double-mutant *HO* reporter combining the “AUU” mutation with a longer 3'UTR was repressed to the same extent as either single mutant (Fig. 10). From this finding, we infer that both mutations perturb the same mode of repression. The effects of distance between the ORFs and PUF sites suggest communication with the translating or terminating ribosome. However, we have not measured either elongation or termination directly, and other interpretations are possible. Puf6p, a non-canonical PUF protein, regulates 60S joining via an interaction with eIF5B (Deng et al. 2008). Repression by Puf6p requires its N-terminal region, outside the RNA-binding domain; in contrast, the RNA-binding domains of the PUF proteins tested here are sufficient for repression. This suggests that the mechanisms of repression by Puf5p and Puf6p differ, but further studies will be required to test this notion definitively.

We propose a model in which Puf5p recruits a partner repressor protein via the “AUU” element in the *HO* 3'UTR. We speculate that the resulting complex disrupts translation elongation or termination. Thus, the PUF protein and its segment E partner interfere with translation. The cell-free system we have described should enable tests

of this model, including the basis of the distance effect, identification of the proposed partner protein, and elucidation of the mechanism of repression.

MATERIALS AND METHODS

Yeast strains

All yeast extracts were derived from the *S. cerevisiae* strain MBS (*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* [rho b] L-o, M-o) with the exception of the MBS *puf4Δ* extract (Iizuka and Sarnow 1997; Wu et al. 2007). The MBS *puf4Δ* is a *puf4::HIS3* derivative of MBS constructed for this study (Longtine et al. 1998).

Protein expression constructs and purification

The recombinant Puf4p and Puf5p protein expression constructs used in this study have been described previously (Hook et al. 2007). The RNA-binding defective mutant of Puf5p has four mutations in PUF repeats 7 and 8: S455A, N456A, N520A, and Y521A. The FBF-2 expression construct (amino acids 121–632) has also been described previously (Bernstein et al. 2005). The FBF-2 RNA-binding defective mutant carries three mutations in repeat 6: N294A, Y295A, and Q298A. The resultant GST fusion proteins were isolated from BL-21 gold *E. coli* cells (Invitrogen)

FIGURE 7. Segments of *HO* enhance repression of *CIN8*. (A) *CIN8* versus *HO* binding sites. In the *HO CIN8 bs* reporter, the Puf5p binding site in the *HO* 3'UTR (UGUAUGUAAU) was replaced with the Puf5p binding site from the *CIN8* 3'UTR (UGUAAUAAU). (B) Chimeric 3'UTRs. The *HO* 3'UTR was divided into four segments that were then inserted into the 3'UTR of the *CIN8* reporter while maintaining the same orientation and distance relative to the Puf5p site, as shown.

FIGURE 8. A sequence in the *HO* 3'UTR enhances Puf5p repression. A 9-nt mutation in the *HO* 3' UTR, mut E, reduces repression. The mut E element was further subdivided into three mutants 3 nt long, E1–E3. Each mutant contained a transversion mutation in the bracketed sequence. Another variation of the *HO* reporter (*HO* long 3') included an extra 25 nt of vector sequence that is present in all *HO* mutant reporters for technical reasons (see Materials and Methods). The sequences highlighted in gray indicate the PUF binding sites. The highlighted sequence constitutes the smallest region in mut E that is required for optimal repression of the *HO* 3'UTR.

and purified as described using the following elution buffer: 5 mM HEPES-KOH (pH 7.4), 30 mM potassium acetate, 0.5 mM magnesium acetate, 20% glycerol, 0.5 mM DTT, and 20 mM glutathione (reduced) (pH 8.0) (Bernstein et al. 2005).

Luciferase constructs and synthetic mRNAs

pSP65A, a gift from Allan Jacobson's laboratory (University of Massachusetts Medical School), contains the 5'UTR from the *CAN1* mRNA, the firefly luciferase ORF, and the 3'UTR from *PGK1*. pSP65-Ren was created by replacing the firefly luciferase ORF with the *Renilla* luciferase ORF in pSP65A. The control *Renilla* reporter was transcribed from a PCR product amplified from pSP65-Ren that began at the SP6 promoter and ended at the poly(A) tract. The *HO* luciferase reporter was transcribed from the pYC2-*HO* plasmid. pYC2-*HO* contains sequence from the *HO* locus spanning 2000 nt upstream to 1000 nt downstream from the ORF with the ORF replaced by the firefly luciferase gene. The PCR template used for transcription included 8 nt upstream of the ATG of luciferase and the 67-nt *HO* 3'UTR. A T3 promoter and a 50-nt poly(A) tail were added 5' and 3', respectively, by an additional primer sequence. For the *HO* 3'-UTR mutants, the reverse primer included an additional 25 nt of vector sequence to avoid overlapping with the mutations. The reporters containing 0–3 FBEs were transcribed from the pGL3 promoter v2 plasmid (Promega) with 1, 2, or 3 FBEa sequences (derived from the 3'UTR of the *gld-1* mRNA from *C. elegans*) introduced by oligo ligation into the *Xba*I site (Crittenden et al. 2003).

The *CIN8*, *DHH1*, *PGK1*, and *RAX2* reporters were assembled by ligation of the firefly luciferase ORF and the 3'UTR of interest and PCR selecting for the final product. The PCR product was then cloned into pET-15b using the *Xho*I and *Hind*III sites. The 3'-UTR length was chosen based on data from a tiling array of the yeast transcriptome (David et al. 2006). The 3'-UTR sequences included downstream from the stop codon for each reporter are: *HO*, 67 nt; *CIN8*, 253 nt; *DHH1*, 167 nt; *PGK1*, 76 nt; and *RAX2*,

89 nt. PCR templates were created using a primer specific to the 5' end of firefly luciferase and the vector sequence directly downstream from the *Hind*III site. The reverse primer also coded for a 50-nt poly(A) tail. All PCR products were subsequently prepared for transcription by phenol/chloroform extraction, ethanol precipitation, and resuspended in DEPC-treated water. RNA was transcribed from the PCR products using the MEGAscript kit (Ambion) with 20% of the suggested GTP and 6 mM 7mG cap analog (New England Biolabs).

Preparation of yeast extracts and translation reactions

Yeast extracts were prepared as described, and yeast were lysed by the liquid nitrogen grinding method (Iizuka and Sarnow 1997). Extracts were treated with micrococcal nuclease (Takara) for 10 min at room temperature, and the reaction was quenched with EGTA. Extracts were then stored in 150- μ L aliquots at -80°C . For each extract, a titration was performed to determine the volume of extract that maximized translation (typically 3–5 μ L of 15–20 mg/mL extract). For analysis of repression by Puf4p (Fig. 4), extract was prepared from a Δ *puf4* strain (see strains).

FIGURE 9. Mutation of segment E does not affect Puf5p binding. (A) Complete sequences of the two RNA oligos used in EMSA analysis. The Puf5p site is underlined, and the mut E mutation is highlighted. (B,C) Representative EMSA autoradiograms. The lower band in each gel is radiolabeled unbound RNA and the upper band is RNA bound to Puf5p. The K_d shown was calculated from four independent experiments. (D) Quantitation. The fraction of RNA bound by Puf5p was plotted versus protein concentration.

FIGURE 10. 3'-UTR length affects repression. (A) The *HO* 3'UTR was expanded by the inclusion of either 25 or 70 extra bases of downstream vector sequence. (B) The 3'UTR was expanded by introducing random sequence between the stop codon and the *HO* 3'-UTR sequence. The number indicates the length of the additional sequence. The *HO* 5'-spacer 185 mut E1 contains both a spacer sequence and mutation E1 from Figure 5.

Translation reactions were assembled using ~60 μ g of yeast extract, 10 ng of firefly reporter RNA, 30 ng of *Renilla* reporter RNA, 2.5 μ L of 6 $\times$ translation buffer, 1 μ L of 4mg/mL creatine kinase, and 0.1 μ L of RNasin (Promega) in a 15- μ L reaction (Iizuka and Sarnow 1997). Reactions were incubated for 60 min at 30°C, and then luciferase levels were measured. Both firefly and *Renilla* luciferase levels were measured using the Dual-Luciferase Reporter Assay System from Promega on a Turner 20/20ⁿ luminometer with a 10-sec integration for both measurements according to the manufacturer's directions.

Northern blots

Translation reactions to assay mRNA stability were performed under the same conditions as described above except the concentration of firefly reporter RNA was increased to 100 ng per 15- μ L reaction. At each time point, 15 μ L of translation reaction was added to 185 μ L of stop buffer and mixed vigorously. Stop buffer: 7 mM tris-HCl (pH 7.5), 33 mM EDTA, 0.5% SDS, 20 mg proteinase K, and 2 ng of control luc fragment RNA was added to control for variations in RNA re-isolation. Luc fragment RNA consisted of nucleotides 1150–1652 of firefly luciferase. After the addition of stop buffer, reactions were incubated for 30 min at 37°C to allow proteinase K digestion, and subsequently extracted

with phenol/chloroform and ethanol precipitated. Ten percent of the purified RNA was loaded onto a 1.0% agarose, 3.7% formaldehyde, 1 $\times$ MOPS gel, and run for 2 h at 100 V. RNA was transferred overnight onto a charged nylon membrane (Millipore) by capillary action using 10 $\times$ SSC. Blots were hybridized in Ultrahyb (Ambion) and washed according to the manufacturer's directions. The probe used to detect firefly luciferase was complementary to nucleotides 1131–1640 of firefly luciferase and was internally labeled with [α -³²P]UTP.

EMSAs

Electrophoretic mobility shift assays were performed and analyzed as previously described (Koh et al. 2009) except the reaction buffer consisted of 10 mM HEPES (pH 7.4), 1 mM EDTA, 50 mM KCl, 2 mM DTT, 0.1 mg/mL BSA, and 0.02% Tween 20 (v/v). The differences between our reported K_{AS} and those previously reported is likely due to variations in protein activity and the lack of competitor RNA in our assays. Our EMSAs were performed only for comparison of the two RNA oligos indicated. The K_d values and standard errors reported were from four independent experiments.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

ACKNOWLEDGMENTS

We thank Shubendu Ghosh and Allan Jacobson at the University of Massachusetts Medical School for providing strains, reagents, and protocols for the cell-free translation experiments. We also thank members of the Wickens laboratory for advice and discussion. We thank Laura Vanderploeg and the Biochemistry Media Laboratory for help with figure preparation. M.W. is supported by grants from the NIH (GM31892 and GM50942). J.J.C. was supported by the NIH Genetics Training Grant (UW-Madison) during this work.

Received December 28, 2009; accepted February 10, 2010.

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RNA 2010 16: 1217-1225 originally published online April 28, 2010

Access the most recent version at doi:[10.1261/rna.2070110](https://doi.org/10.1261/rna.2070110)

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