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Identification of New mRNA Targets of Puf Protein-Mediated Decay in Yeast

Randi J. Ulbricht
University of Missouri-St. Louis, rjbgwc@studentmail.umsl.edu

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by

Randi J. Ulbricht
B.S. Cell and Molecular Biology, Southwest Missouri State University

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Advisory Committee
Wendy M. Olivas, Ph.D.
Chairperson

Shirley Bissen, Ph.D.

Marc Spingola, Ph.D.

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ABSTRACT

Precise regulation of gene expression is accomplished at many levels. Puf proteins are a widely conserved family of RNA binding proteins that regulate gene expression by influencing the stability of their target mRNA transcripts. Puf family members have been characterized as transcript-specific repressors in *Drosophila, Dictyostelium*, mouse, *C. elegans,* and *Xenopus*. In *S. cerevisiae*, there are 5 conserved Puf family members. Two of them, Puf3p and Puf5p, are known to destabilize their mRNA targets, down-regulating gene expression. The remaining yeast Pufs, Puf1p, Puf2p and Puf4p, are homologous to Puf3p and Puf5p, suggesting that they too have regulatory roles not yet observed. In this work, I attempt to identify new mRNA targets of Puf mediated decay in yeast. In particular, I am interested in targets of the uncharacterized yeast Pufs.

The ability of a Puf protein to regulate its target transcript is dependent on Puf binding to a conserved element in the 3'UTR of the target mRNA. A search for similar 3'UTR elements and previous microarray data helped to identify numerous potential mRNA targets of Puf-mediated decay. In this work, experimental analysis of the candidate mRNAs positively identified three new targets of Puf mediated mRNA decay. Two of the targets, *HXK1* and *TIF1*, are destabilized *in vivo* by Puf1p and Puf5p, respectively. The third target, *YHB1,* is actually stabilized by Puf2p. Interestingly, while

the *TIF1* and *HXK1* 3'UTRs are sufficient for Puf mediated decay as expected, the *YHB1* 3'UTR is not sufficient to confer Puf2p mediated decay onto the *MFA2* coding region. In addition to these functional studies, I also demonstrate Puf interaction with conserved sequence elements in each 3'UTR. Together, my work provides evidence that all yeast Puf proteins selectively regulate mRNA decay, and in some cases, Puf proteins may up-regulate gene expression.

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Chapter 1: INTRODUCTION

The typical eukaryotic mRNA consists of a 5' untranslated region (UTR), a coding region and a 3'UTR. Post-transcriptional modifications required for efficient translation and proper regulation of the mRNA include polyadenylation at the 3'end and placing a protective 7-methylguanosine (m7G) cap structure on the 5' end. There are many proteins performing a variety of different functions that are bound to the mRNA at the 3'UTR, the 5'UTR, the cap, and the poly(A) tail. Cap binding proteins, including the translation initiation complex (eIF4F), are associated with the 5' end of translating mRNA (for review, see Gallie, 1998). At the 3' end of the RNA, Poly(A) binding proteins (Pab1p) are bound to the poly(A) tail. In addition, multiple proteins have been shown to binding 3'UTRs (for review, see Wilusz and Wilusz, 2004). An important aspect to the mRNA layout is that its apparent structure is maintained by interactions between proteins at the extreme ends. For example, Pab1p binds to a cap-associated translation initiation factor, eIF4G, *in vitro* (Tarun and Sachs, 1996). These interactions allow the mRNA to conform to a "circularized" structure with the 3' and 5' ends of the RNA in close proximity. The circular structure has many implications on mRNA regulation. Namely, the mRNA structure promotes efficient translation as well as helps to stabilize the mRNA (Schwartz and Parker, 1999).

Eukaryotic mRNA Decay

The stability of a transcript is an important post-transcriptional property that influences gene expression. Eukaryotic mRNA decay, including that of *Saccharomyces cerevisiae*

(budding yeast), occurs by a very well studied mechanism. mRNA decay begins with deadenylation. In yeast, this occurs primarily by the Ccr4p/Pop2p complex. After deadenylation, the transcript is decapped in both humans and yeast by a complex containing Dcp1p/Dcp2p. After decapping, the mRNA will be quickly degraded by the 5' to 3' exonuclease Xrn1p (for reviews, see Long and McNally, 2003; Parker and Song, 2004).

Transcript-specific regulation of mRNA decay occurs at the steps of deadenylation and decapping and most often requires unique *cis* elements within the transcript. These elements are most commonly found in the 3'UTR region but may also be found in the 5'UTR and coding region, depending on the transcript. The yeast *MFA2* 3'UTR contains sequences required for rapid deadenylation and decapping of *MFA2* (LaGrandeur and Parker, 1999). Another yeast transcript, *PGK1,* requires the context of the start codon for its inherent stability (LaGrandeur and Parker, 1999). Regulatory *cis* elements often serve as binding sites for RNA binding proteins that induce changes in mRNA stability. The most well-known examples are AREs (AU Rich Elements). AREs are instability elements found in many eukaryotic 3'UTRs. ARE binding proteins bind to AREs and recruit the exosome or other decay enzymes to facilitate rapid degradation of the mRNA (for review, see Wilusz and Wilusz, 2004). Not all trans-acting factors simply bind to a single site on the RNA to regulate the RNA. *C. reinherdtii psb*A mRNA contains a 3'UTR element required for regulation of its decay. However, the 5'UTR of *psb*A mRNA is also required for efficient binding of the regulatory protein complex to the transcript (Katz and Danon, 2002).

RNA binding proteins that regulate mRNA decay may act by many possible mechanisms. The ARE binding proteins represent one possible mechanism to destabilize a transcript. In this situation, protein binding recruits the decay machinery to the mRNA (Wilusz and Wilusz, 2004). Other RNA binding proteins may influence a transcript's stability by affecting the interactions between the 3' and 5' ends of the mRNA. The ability of the poly (A) tail to inhibit decapping and promote translational efficiency is thought to be due in large part to binding of Pab1p to the poly (A) tail and Pab1p's concurrent association with the 5' cap complex (for review, see Parker and Song, 2004). This type of stabilizing mechanism implies the presence of a link between the regulation of mRNA decay and translation initiation. Such a link has been investigated in many different ways. First, translation initiation has an inverse relationship with mRNA decay rates. This relationship was illustrated by a study in which mutations in translation initiation factors that down-regulate translation initiation resulted in increased rates of both decapping and deadenylation (Schwartz and Parker, 1999). Evidence of this relationship between translation and stability can also be seen in the stable *PGK1* transcript. In this example, the sequences surrounding the start codon are responsible for both RNA stabilization and increasing the translational efficiency of *PGK1* (LaGrandeur and Parker, 1999).

While mRNA decay regulation is often dependent on the mRNA sequence and regulatory proteins, it can also depend on other cellular factors. An exciting recent discovery in the field of eukaryotic mRNA decay was the discovery of processing bodies (p-bodies) in the yeast cytoplasm. These punctate spots were visualized by GFP (Green Fluorescent Protein) tagging of various decay factors, particularly decapping machinery

and the exonuclease Xrn1p (Sheth and Parker, 2003). The presence of the p-bodies is dependent on the presence of RNA and is influenced by blocking decay steps, further suggesting that these are sites of decay. More recently, additional investigations have revealed that p-bodies are absent in mid-log phase yeast cells, but as the cells enter late-log phase and get further into stationary phase, the p-bodies appear and get larger. Other results further suggest that the presence of p-bodies is influenced by cellular stresses such as diauxic shift, ultra-violet irradiation, and glucose starvation (Teixeira et al. 2005). It seems that as translation is inhibited by these conditions, the state of mRNA decay in the cell changes and p-bodies are formed.

The Puf Family

Members of a particular family of 3'UTR binding proteins, called Puf proteins, are known as translational repressors that influence mRNA decay of their target transcripts. The well studied Puf family members are listed in Table 1.1. Members of the Puf family are characterized by the presence of a highly conserved repeat domain (RD). The RD contains eight imperfect repeats of about 36 amino acids each, which together comprise the RNA binding domain of the protein (Wang et al. 2002). The RD is not only sufficient for RNA binding activity, but is also sufficient for its regulatory activities (Zamore et al. 1997; Wharton et al. 1998; Jackson et al. 2004). Crystal structures of a human Puf protein, HsPUM1, bound to *Drosophila hunchback* mRNA revealed that the repeat domain has a curved structure (Wang et al. 2002). The concave surface interacts with the mRNA, making specific hydrogen bonds and stacking amino acid aromatic rings between the mRNA bases. The convex outer surface is modeled as a potential binding site for

additional protein factors that participate in regulatory activities (Edwards et al. 2001; Wang et al. 2001).

The ability of Puf proteins to interact with protein partners is an important contribution to Puf function. In higher eukaryotes, one of these partners is Nanos. Nanos homologs in *C. elegans*, *Drosophila* and *Xenopus* have been found to interact with their respective Pufs (Wharton et al. 1998; Sonoda et al. 1999; Nakahata et al. 2001). It has been proven, in the case of Pumilio, that Nanos is required for *hunchback* regulation (Wharton et al. 1998; Sonoda et al. 1999). Another known contributor to *hunchback* regulation is Brat. Brat is recruited to the RNA-Pumilio-Nanos complex and thought to bind to Pumilio with the help of Nanos (Sonoda and Wharton 2001; Edwards et al 2003). There are no homologs to these known protein partners in yeast.

All Puf family members studied have been shown to bind to UGU sequence elements in the target mRNA's 3' UTR (see Table 1.1). The sequences flanking the UGU element may be important for the specificity of the protein for its target (for review see, Wickens et al. 2002). Studies have shown that yeast Puf3p specifically requires a UGUA sequence in the 3' UTR of *COX17* mRNA for binding and regulation. The consensus Puf3p binding site on *COX17* has been experimentally expanded to UGUANAUAU (Jackson et al. 2004), which is strikingly similar to the human PUM1, murine PUM2, *Drosophila* Pum and *Xenopus* Pum binding sequences of UGUANAUA (Zamore et al. 1997; Wang et al. 2002; White et al. 2001; Nakahata et al. 2001; Murata and Wharton, 1995). Yeast Puf5p has been shown to bind the UGU containing sequence, UUGUAUGUA, in the 3'UTR of *HO* mRNA (Tadauchi et al. 2001) A microarray study identified apparent consensus binding sequences for three yeast Pufs (Puf3p, Puf4p and

Puf5p). Each of these consensus sequences contains a UGUA core sequence followed by a UA within 6 nucleotides downstream (Gerber et al. 2004).

Although the mRNA binding sequences for the different Puf proteins are similar, the Puf proteins show specificity for their own preferred target binding site. Puf3p, and not Puf5p, can bind *COX17* mRNA (Jackson et al. 2004). However, modifying the *COX17* mRNA UGU sequence to more closely resemble the Puf5p native binding site from *HO* mRNA allows Puf5p to bind this modified 3'UTR (unpublished observation, John Jackson Jr.). Therefore, the sequence specific mRNA/Puf protein interaction is limited to sequences including and flanking the UGU sequence element.

More than one site of Puf binding has been observed in some Puf target mRNAs including, *COX17* and *hunchback*. *Drosophila* Pum (DmPum) binds two UGU regions on *hunchback* mRNA. Each of these regions seems to bind DmPum equally and independently (Zamore et al. 1999). *In vivo*, both regions are necessary for full regulation of *hunchback,* however one region contributes more strongly to mRNA regulation than the other (Wharton and Struhl, 1991; Curtis et al. 1997). The Puf3p target, *COX17,* also contains two sites for Puf binding (Jackson et al. 2004). *In vitro* binding affinities of Puf3p for these two regions of *COX17* differ significantly, however *in vivo*, they are equally important for Puf3p regulation. Moreover, the presence of one site allows only partial regulation, and both sites are required for full regulation of *COX17* (Jackson et al. 2004). Foot printing assays have determined that Puf protein binding protects a 30 nucleotide region, suggesting that Puf proteins require a total sequence of about 30 nucleotides for binding (Wharton et al. 1998). In *COX17* and *hunchback,* the two binding sites lie about 40-45 nucleotides apart on the mRNA. The

spatial relationship of the two sites in each case allows for simultaneous binding of two Puf proteins to the target 3'UTR. Furthermore, the functional assays suggest that concurrent binding occurs *in vivo* (Jackson et al. 2004).

In addition to the sequence selectivity of Pufs for target binding, Puf proteins also show a degree of differentiation in regards to regulation. Although only Puf5p is capable of regulating the decay of *HO* RNA (Tadauchi et al. 2001), we know that both Puf5p and Puf3p can bind to the *HO* target sequence *in vitro* (Houshmandi and Olivas, 2005). From this information, we are assured that the ability of a Puf to regulate decay is more complicated than its protein binding ability.

Organism	Puf	Binding Sequence	Target	Reference
Human	HsPUM1	UUGUANAUA	?	Zamore et al. 1997
Drosophila	DmPum	UUGUANAUA	*hunchback,* cyclin B	Wharton and Struhl, 1991; Asaoka-Taguchi et al. 1999
Dictyostelium	PufA	UGU	*pkaC*	Souza et al. 1999
C. elegans	FBF	UUCUUGUGU	*Fem-3*	Zhang et al. 1997
Murine	PUM2	UGUANAUA	?	White et al. 2001
Xenopus	X-Pum	UGUA	*cyclin B1*	Nakahata et al. 2001
Yeast	Puf3p	UGUANAUA	*COX17*	Jackson et al. 2004
Yeast	Puf5p	UUGUAUGUA	*HO*	Tadauchi et al. 2001
Yeast	Puf6p	UUGU	*ASH1*	Gu et al. 2004

Table 1.1. Puf Proteins and Their Targets. Well studied Puf proteins and the organism from which they were identified are listed. The mRNA sequence that each binds is also listed under "Binding Sequence". For some Puf family members, their endogenous targets have not been identified, but the binding sequence has been determined by either binding to the *Drosophila hunchback* mRNA or by *in vitro* selection experiments (i.e. SELEX). The core UGU sequence element in each binding sequence is underlined.

A Closer Look at Yeast Puf Proteins

There are six members of the Puf family of RNA binding proteins in *Saccharomyces cerevisiae*: Puf1p, Puf2p, Puf3p, Puf4p, Puf5p, and Puf6p (Figure 1). Puf3p binding promotes rapid deadenylation and decay of the *COX17* mRNA (Olivas and Parker, 2000). Puf5p stimulates decay of the *HO* transcript (Tandauchi et al. 2001). The 3'UTRs of these Puf targets are sufficient for Puf mediate RNA decay. This conclusion was made based on the fact that *HO* 3'UTR fused to the *ADE2* coding region is sufficient to destabilize the *ADE2* transcript (Tandauchi et al. 2001) and the *COX17* 3'UTR similarly destabilizes the *MFA2* transcript (Jackson et al. 2004). Puf6p is a divergent member of the Puf family and therefore was not considered in most studies. However, recently Puf6p was characterized as a translational repressor of *ASH1* mRNA (Gu et al. 2004).

Outside the RD of the yeast Puf proteins, there seems to be very little similarity between the proteins (Figure 1.1). Yet the similarity between the RDs is striking. A comparison of the amino acid sequence similarity (using BLAST programs) among the RDs indicates that Puf1pRD and Puf2pRD show the most similarity to each other at 79% similarity. Puf1pRD and Puf2pRD are each about 45% similar to Puf3p. Puf3pRD, Puf4pRD and Puf5pRD are all fairly similar to each other (56-58%). While Puf6pRD shares 42% similarity with Puf3pRD, no other PufRD showed significant alignment with Puf6pRD, demonstrating the divergence of this RD from the other yeast Pufs.

In Figure 1.2, I have aligned regions of amino acid sequences from five yeast Puf proteins and DmPum that are spatially located on the RNA binding surface of the RD. Comparing the sequences shown, it is obvious that Puf3pRD is most similar to DmPum-RD. Amino acids of DmPum-RD and yeast PufRDs that are predicted to participate in

hydrogen bonding or stacking interactions with the mRNA are highlighted (Wang et al. 2002). In accordance with the overall similarities between the yeast RDs mentioned earlier, these amino acids that are likely involved in direct RNA interactions are well conserved in Puf3pRD, Puf4pRD and Puf5pRD, however they often differ in Puf1pRD and Puf2pRD. The importance of the similarities and differences in these regions is unknown. Mutational analyses of Puf3pRD have indicated that while the direct interactions are important, the surface architecture of the RD might be a large contributor to the target binding specificity of a PufRD (Houshmandi and Olivas, 2005). However, the divergence of Puf1p and Puf2p from the others might suggest a slight difference in target preference. In line with this hypothesis, the microarray study that identified an apparent consensus RNA binding sequence for Puf3p through Puf5p was unable to identify a consensus sequence for Puf1p and Puf2p (Gerber et al. 2004).

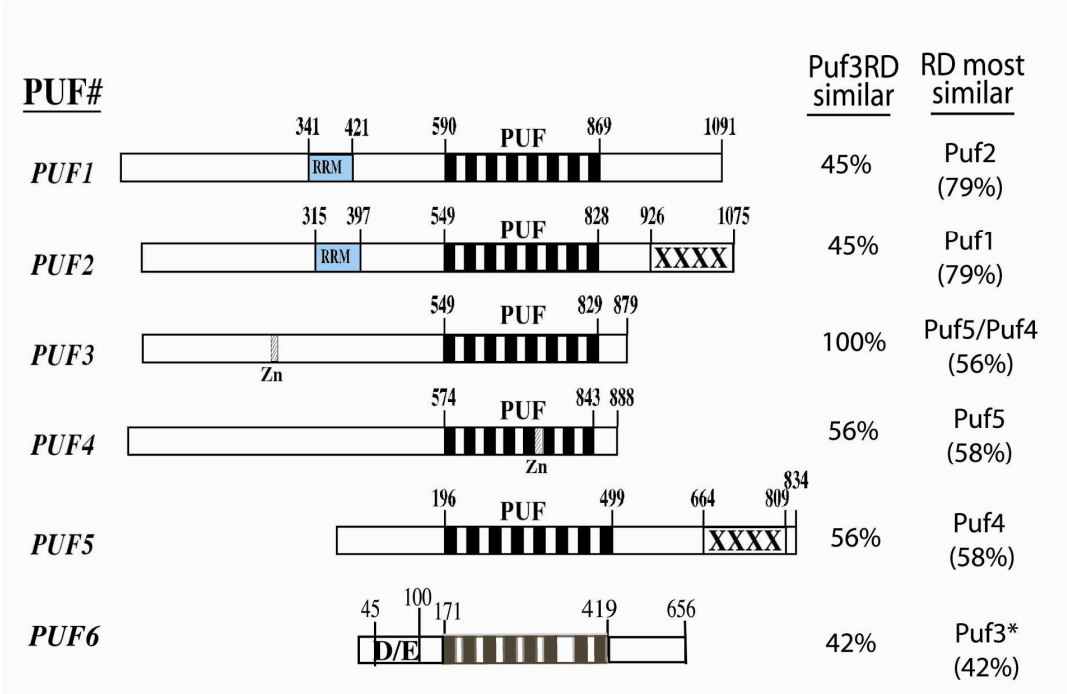


Figure 1.1. Alignment and of the Yeast PUFs. The yeast Puf protein sequence elements are drawn to scale. Each yeast *PUF* contains a conserved repeat domain (RD) with 7-8 repeats. Each repeat is represented with black rectangles. *PUF1* and *PUF2* also contain an RNA Recognition Motif (RRM). *PUF3* and *PUF4* contain a putative zinc finger domain. *PUF2* and *PUF5* contain regions of slight homology represented by XXXXXX. *PUF6* contains a glutamic and aspartic acid-rich region (D/E). The amino acid similarity of each Puf protein RD to Puf3pRD is listed under "Puf3RD similar". The RD sharing the most similarity is listed under "RD Most similar" and the percent similarity to this PufRD is listed in parentheses. **PUF6* RD only shows similarity within acceptable P values to Puf3pRD.

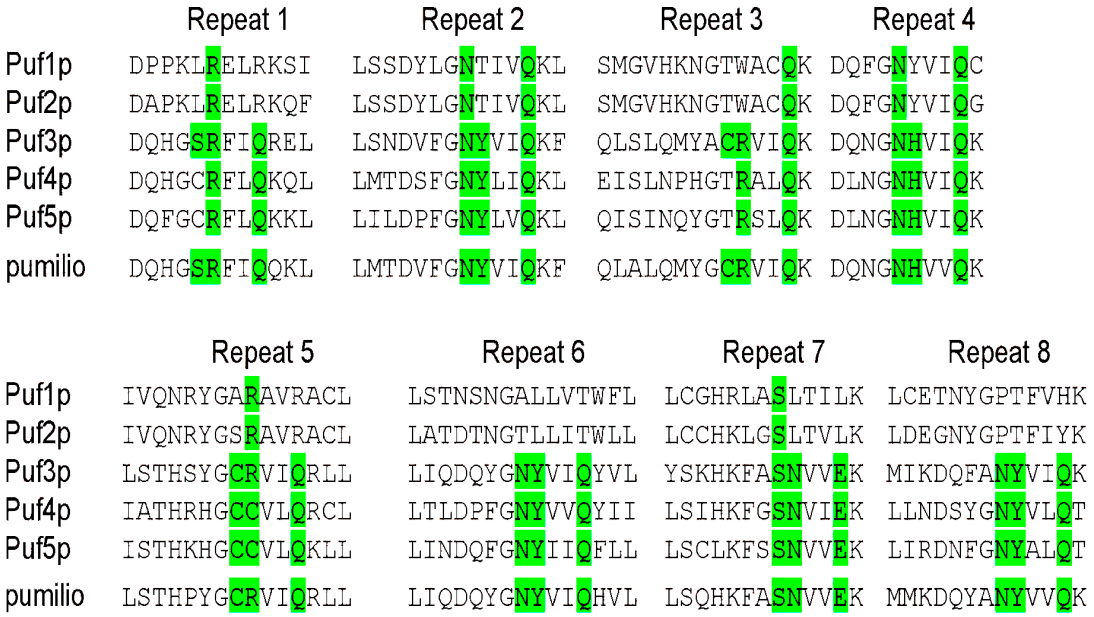


Figure 1.2. Alignment of Yeast Puf and Pumilio Repeat Domains. Amino acids that lie on the inner RNA binding surface of the RD of Puf1-5 and *Drosophila* Pumilio are shown above. Amino acids shown by Wang et al (2001) in Pumilio RD to directly interact with the mRNA are highlighted. Amino acids in the yeast Pufs sharing identity with these Pumilio amino acids are also highlighted. The amino acids are grouped according to the repeat to which they belong. The repeat number is indicated over each group.

Research Goals

Of the six yeast Puf proteins, there is only direct evidence available that Puf5p and Puf3p regulate mRNA decay. However, considering their homology, it is likely that all Puf proteins have the ability to regulate the decay of their own target mRNAs. Thus far, the only identified mRNA targets of *S. cerevisiae* Puf proteins are *HO*, *COX17* and *ASH1* mRNA (Tandauchi et al. 2001; Olivas and Parker, 2000; Gu et al. 2004). Broad studies on the yeast Puf proteins have implied that there are many other uncharacterized Puf protein mRNA targets. Olivas and Parker (2000) identified via microarray analysis over 150 RNAs differentially expressed in yeast deleted of *PUF1* through *PUF5* genes. Another microarray study identified more than 700 RNAs bound to Puf1p through Puf5p *in vivo* (Gerber et al., 2004). Thus, the goal of my thesis work was to identify and characterize new mRNA targets whose decay is regulated by yeast Puf proteins. Using available microarray data and computer BLAST programs, I was able to formulate a large pool of likely candidate Puf mRNA targets. From this pool, I have successfully identified new mRNA targets of Puf1p, Puf2p and Puf5p. Furthermore, I have shown that the stability of each of these mRNAs is dependent on Puf proteins, further confirming that each of the yeast Puf proteins posses the ability to regulate such processes. In addition, I have identified binding sites within the 3'UTRs of the mRNA targets required for Puf binding and regulation.

Chapter 2: RESULTS

The work within my thesis includes identification of potential mRNA targets of the yeast Puf proteins, and the experimental determination of targets that are directly regulated by the Puf proteins at the level of decay. Using a variety of molecular biology techniques, I have identified at least three positive targets of Puf-mediated mRNA decay in yeast.

Identification of Candidate mRNA Targets of Puf Proteins

Previous studies have identified hundreds of mRNAs potentially regulated by Puf proteins. I exploited the information from two such studies to pool my own list of potential targets of Puf1p through Puf5p. (Because *PUF6* is less conserved compared to the rest of the yeast *PUF*s, it was not considered in my or the previous studies.) The first study was a microarray experiment in which poly(A)+ mRNA levels were compared between wild-type (WT) *S. cerevisiae* and a strain with all five *PUF*s deleted (Olivas and Parker, 2000). A significant difference in mRNA levels implied that an mRNA is potentially regulated by at least one Puf protein. This study identified 168 mRNAs as differentially expressed and therefore as potential Puf targets (Olivas and Parker, 2000). The second study was published in 2004 by Gerber *et. al*. and aimed to identify RNAs physically associated with the yeast Puf proteins (Puf1p-Puf5P). Tagged Puf proteins were immuno-precipitated, then the RNAs that co-precipitated with each Puf were isolated and identified via microarray. This study yielded hundreds of RNAs bound to each Puf protein, with some RNAs bound to multiple Puf proteins.

I also employed one final resource to aid in development of my list of potential Puf targets. A BLAST program called Yeast Genome Pattern Matching, or PatMatch (<http://seq.yeastgenome.org/cgi-bin/SGD/PATMATCH/nph-patmatch>), available on the *Saccharomyces* Genome Database enables the user to locate short sequences in specific regions of the *S. cerevisiae* genome. I used this program to identify mRNAs with potential Puf binding sites within the 3'UTR. Since the binding sites seem to be relatively conserved between the known Puf proteins and their targets (see Table 1.1), I searched for sequences in 3'UTR regions common to Puf binding sites. First, I searched only for 3'UTR sequences containing UGUA (the essential core Puf3p binding sequence; Jackson et al. 2004). This generated a list of hundreds of mRNA's. Since both the Gerber microarray and studies in the Olivas lab found downstream AU regions to be significant (Jackson et al. 2004), I searched for a UGUA sequence followed by an AU-rich element to narrow my pattern search results.

Since the accumulated list of potential targets acquired from each of these resources contained more mRNAs than I could possibly investigate in a timely manner, I developed a short list of mRNAs considered most likely to be Puf protein targets. Specifically, the candidates on this short list have been identified by more than one of the three methods discussed. mRNAs from this narrower pool were then the subjects of further investigation. I have investigated 28 of these potential target mRNAs. 18 of them do not appear to have Puf-regulated mRNA decay (determined by methods discussed later). While I will not dwell on these negative results, it is significant that a large number of potential targets identified by microarray experiments are not Puf targets for regulated decay. It illustrates the necessity for work such as mine that goes beyond these

microarray-based experiments. I will focus for the remainder of my thesis on three positive targets of mRNA decay; *HXK1*, *TIF1* and *YHB1*. A schematic diagram of each of the 3'UTRs of these mRNAs is seen in Figure 2.1. The locations of each 3'UTR UGUA sequence element relative to the stop codon are indicated. In the following sections, I will provide evidence that these three mRNAs are targets of Puf-mediated mRNA decay.

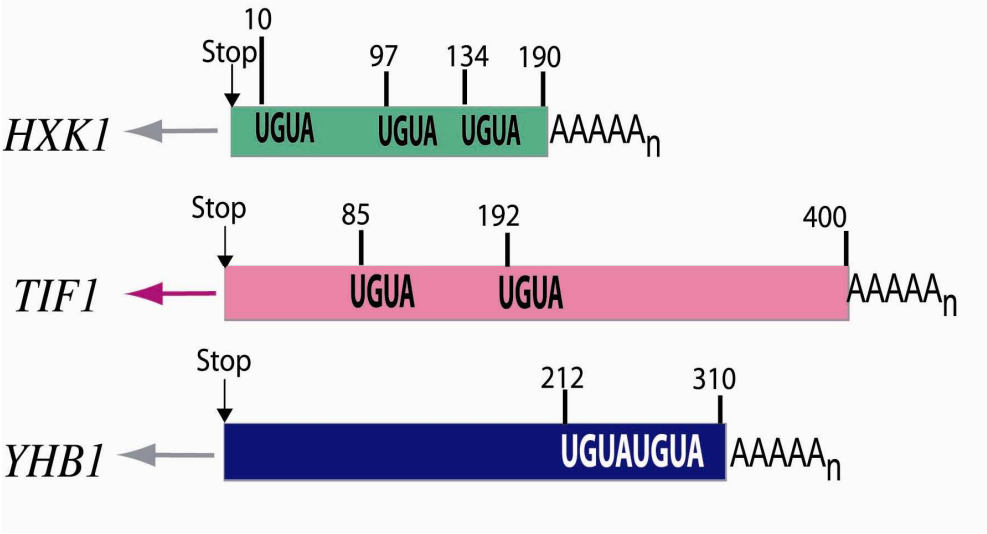


Figure 2.1. Schematic Diagram of Potential Puf Target mRNA 3'UTRs. Stop codons are indicated by black arrows above each diagram. The nucleotide positions of UGUA regions relative to the stop are indicated. The length in nucleotides of each 3'UTR is also indicated. Arrows to the left indicate the coding region which is not depicted here.

Confirming Potential Targets

To identify true Puf targets from the pooled short-list of potential candidates, I subjected candidates to studies to determine if Puf proteins influenced their steady-state mRNA abundance and if the stability of the mRNA is affected by a *puf* deletion (*puf*Δ**)**. The results for three of these candidates *HXK1, TIF1* and *YHB1* are presented in the following sections.

Detecting Differential Steady-State RNA Abundance

Comparing the abundance of mRNAs in strains with individual *PUF*s deleted versus WT yeast will help determine if any particular *PUF* influences mRNA levels of the candidate transcripts. The steady-state abundance of an mRNA remains constant unless there is a change in either the transcription rate or decay rate of the transcript. Therefore, if a Puf protein influences the stability of a transcript, I would expect to see a difference in its relative abundance between yeast with and without that *PUF*. In the following section, I measured the steady-state abundance of the candidate mRNA targets in WT and *puf*Δ yeast and used this information to determine if the mRNA might be a true target and which of the five Puf proteins potentially regulates that mRNA.

To determine the level of an mRNA species, I first isolated total RNA from WT and individual *puf*Δ yeast grown under similar conditions. A number of the candidate mRNAs are preferentially transcribed or translated during oxidative stress or stationary phases of growth. Therefore, to decrease the likelihood of overlooking important information, I harvested total RNA from cell cultures in mid-log phase of growth (Optical Density (OD) $_{600}$ of 0.4) and entering stationary phase (OD $_{600}$ of 1.0). Northern blots

from these steady-state populations were then probed with a γ^{32}P end-labeled DNA oligonucleotide complementary to the selected mRNA target, and RNA loading was normalized to 7S RNA, a constitutively expressed RNA Polymerase III transcript. A difference in mRNA abundance between WT and a *puf*Δ suggests that somehow that Puf protein is influencing the level of that mRNA. Optimally, at least a two-fold difference in mRNA levels between any one deletion strain and WT was considered to be significant.

Differential mRNA Abundance Detected in Three Target Transcripts

From the steady-state mRNA analysis, I discovered significant differential abundance in three potential Puf protein mRNA targets. Two of the transcripts, *TIF1* and *YHB1,* are significantly over-expressed in the absence of Puf2p, with 2 and 2.8 times the amount of mRNA in *puf2*Δ than in WT, respectively (Figure 2.2B). This inducing effect is only seen from cultures entering stationary phase and not from those in mid-log phase. Because it is apparent that this growth condition is reproducibly important to Puf regulation, all subsequent *in vivo* investigations with these transcripts were performed in yeast entering stationary phase. Results for the third candidate, *HXK1,* from mid-log phase cultures are also seen in Figure 2.2A. It appears that levels of *HXK1* decrease in the absence of Puf2p but increase more than 2-fold in the absence of Puf3p or Puf1p.

It is noteworthy that the results shown in Figure 2.2 were only seen for these mRNAs. The same Northern blots were subjected to probing for numerous other potential mRNA candidates with no differences in mRNA levels found (results not shown). Thus, the expression patterns seen in Figure 2.2 are transcript-specific and not a global change in mRNA abundance.

The data seen in Figure 2.2 show only that the amount of mRNA present in a steady-state cell population has changed as a result of the *puf*Δ. Indirect regulation, transcriptional changes or stability changes may be responsible for the abundance changes observed. The following studies will focus on determining if differential mRNA levels are due to direct effects by the Puf proteins on mRNA stability**.**

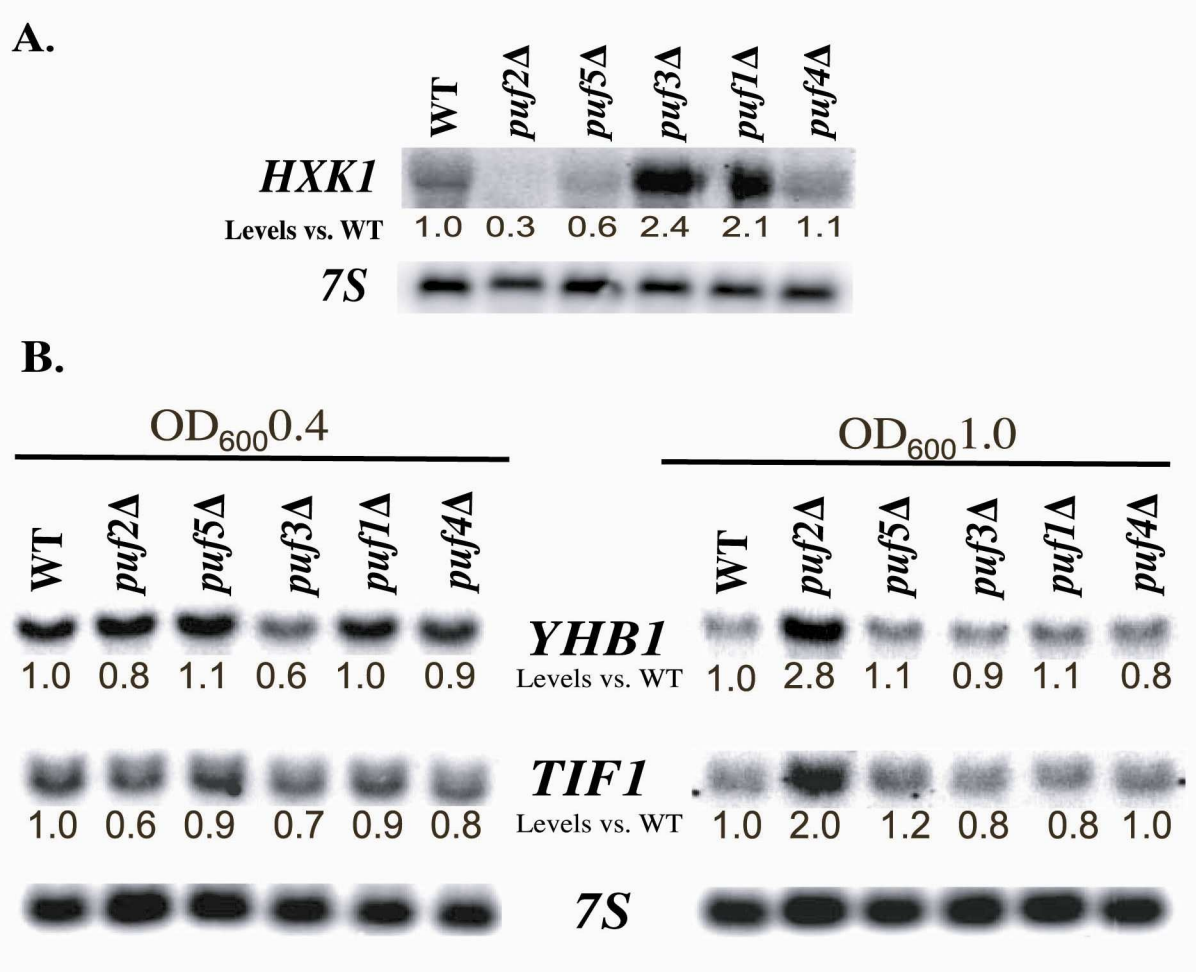


Figure 2.2. Steady-State Levels of *HXK1, TIF1* and *YHB1* mRNAs in Budding Yeast. Total RNA was isolated from steady-state populations of WT and individual *puf* deletion (Δ) yeast strains while in mid-log phase (OD_{600}0.4) and entering stationary phase (OD_{600}1.0). Northern blots were probed for each mRNA of interest as well as 7S RNA. The fraction of RNA detected compared to WT after normalization to the loading control 7S RNA (bottom panels) is indicated as "Levels vs. WT". **A**. Top panel. Autoradiogram of radioactively labeled *HXK1* probe hybridized to a Northern blot containing RNA harvested from yeast grown to an OD_{600} of 0.4. Bottom panel. Same Northern as in top panel probed for constitutively expressed 7S RNA. **B**. RNA for these Northern blots was isolated from cultures grown to ODs indicated above each panel. Top panels. Northern blots probed for *YHB1*. Middle panel. Same Northerns (as in top panel) probed for *TIF1*. Bottom panel. Same Northerns as above probed for 7S.

Puf Protein Effect on Target mRNA Decay Rates

Conclusive evidence that the differential mRNA levels observed in Figure 2.2 are due to direct changes in mRNA stability can be obtained by monitoring the decay rates of the potential targets in WT and *puf***Δ** strains. I used transcriptional shut-off experiments to determine the half-life and therefore the stability of potential targets. This assay is performed in yeast strains with a temperature-sensitive mutation to RNA Polymerase II (*rpb1-1*). First, *rpb1-1* containing yeast are grown at 24°C to an OD_{600} of 0.4 or 1.0, then the cells are switched to media at 37°C. The temperature shift inactivates the mutant RNA Polymerase II, preventing transcription of new mRNAs. Cell populations are isolated over a time course after shift to 37°C. The half-life of the selected mRNA species is determined on a Northern blot by analyzing the time at which half of the initial pool of mRNA has decayed. A significant change in half-life of the potential target mRNA in the absence of a Puf protein compared to WT will confirm that mRNA stability is regulated by that protein.

Determining the stability of some candidate transcripts can be an exceptional challenge. Complex regulation of the gene can interfere with steady-state Northern results as well as the shut-off assays. Transcriptional regulation of some genes is very sensitive to factors such as sugar levels, sugar type, growth conditions, temperature and oxidative stress (for review, see Gasch and Werner-Washburne, 2002). Variation in transcription can be deceiving in steady-state mRNA analysis and also cause difficulties in obtaining consistent results from transcription shut-off experiments. To bypass these complications, the 3'UTR of the mRNA of interest was fused downstream of the *MFA2* coding region in a yeast expression plasmid. Previous studies have confirmed that *MFA2*

is not Puf-regulated under normal mid-log phase conditions (unpublished observations, Wendy M. Olivas) and that the 3'UTRs of known yeast Puf targets are sufficient for Puf regulation (Jackson et al. 2004; Tadauchi et al. 2001). Transcription of the *MFA2* coding region in this plasmid is under the control of the inducible GAL promoter. Therefore, the new chimera, *MFA2/ X 3'UTR*, can be induced with the addition of galactose to the media and repressed with the addition of glucose. The influence of the candidate mRNA's 3'UTR on the stability of the *MFA2* transcript can be measured by transcriptional shut-off assays.

Influence of Puf Proteins on 3'UTR-Mediated Decay Rates

Results of the described transcriptional shut-off experiments are presented in Figure 2.3. Figure 2.3A depicts the RNA decay rates for the *MFA2/HXK1 3'UTR* construct determined at mid-log phase in *puf1*Δ (purple), *puf3*Δ (green), *puf4*Δ (orange) and WT (red) *rpb1-1* yeast in a graphical representation. It is clear from these results that while deletion of *PUF3* and *PUF4* had no effect on decay, deleting *PUF1* stabilized the transcript. The half-life increased from 4 (+/- 1) minutes in WT to 8.5 (+/- 0.5) minutes in the *puf1*Δ. The results indicate that Puf1p destabilizes the *HXK1* transcript and that the 3'UTR of *HXK1* is sufficient to mediate this decay

Decay rates of the *MFA2/TIF1 3'UTR* construct derived from *rpb1-1* transcriptional shut-off experiments performed at the OD_{600} of 1.0 are depicted in Figure 2.3B. The half-life of this construct in WT (red) and *puf2*Δ (blue) yeast is 6.5 (+/- 1.3) and 5.5 (+/- 1.5) minutes, respectively. This does not appear to be a significant difference in stability. However, in the *puf5*Δ (black**)** the half-life increases to 12 (+/- 0.3) minutes. Hence it appears that while Puf2p does not affect stability of the RNA,

Puf5p destabilizes *MFA2/TIF1 3'UTR* about 2-fold. As expected, there were no detectible differences in the stability of *MFA2/TIF1 3'UTR* in mid-log phase cells (results not shown).

It was quite unexpected to discover that Puf5p destabilizes the *MFA2/TIF1 3'UTR*. The steady-state results in Figure 2.2 gave no indication of Puf5p regulation. However, it has been noted that in yeast, a change in steady-state levels of mRNA does not necessarily reflect a similar change in decay. For example, *COX17* mRNA shows a 2-fold increase in *puf3*Δ steady-state populations compared to WT, however, Puf3p destabilizes the transcript about 6-fold as measured by half-life analysis (Olivas and Parker, 2000). So, regardless of the steady-state findings, the decay assays have revealed that *TIF1* is regulated by Puf5p *in vivo* and that the 3'UTR of *TIF1* is sufficient to mediate this decay.

Finally, I tested the stability of the *MFA2/YHB1 3'UTR* construct in the various *PUF* deletion yeast at OD_{600}1.0. The data from this set of experiments were also unexpected. The decay rate of *MFA2/YHB1* 3'UTR does not significantly differ from WT in *puf2*Δ or *puf5*Δ, remaining 6-8 minutes in each strain (Figure 2.3C). As expected, there are also no differences in stability between the *puf*Δ strains and WT at an OD_{600} of 0.4 (results not shown).

Figure 2.3D depicts the control experiments of *MFA2* decay with its native 3'UTR performed at an OD_{600} of 1.0. Half-lives of *MFA2* in WT (red), *puf2*Δ (blue) or *puf5*Δ (black) remain at about 4 minutes. Thus, *MFA2* itself is not regulated by Puf2p or Puf5p at this OD, and the results seen in 2.3B and 2.3C are due strictly to the effects of *TIF1* and *YHB1* 3'UTRs.

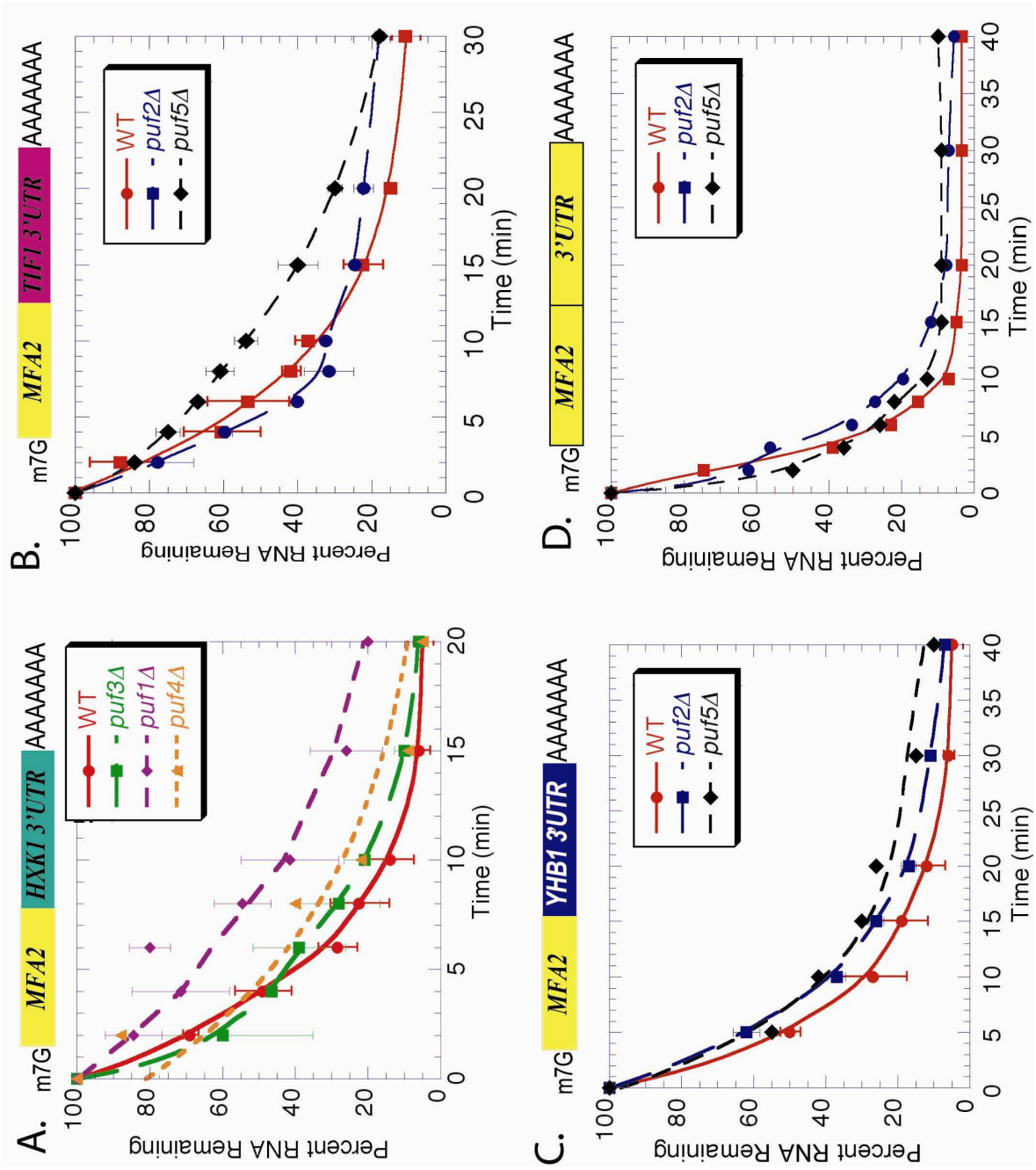

A.
m7G
MFA2
HXK1 3'UTR
AAAAAAA
WT
puf3Δ
puf1Δ
puf4Δ
Percent RNA Remaining
Time (min)
B.
m7G
MFA2
TIF1 3'UTR
AAAAAAA
WT
puf2Δ
puf5Δ
Percent RNA Remaining
Time (min)
C.
m7G
MFA2
YHB1 3'UTR
AAAAAAA
WT
puf2Δ
puf5Δ
Percent RNA Remaining
Time (min)
D.
m7G
MFA2
3'UTR
AAAAAAA
WT
puf2Δ
puf5Δ
Percent RNA Remaining
Time (min)

Figure 2.3. Decay Rates of *MFA2/3'UTR* Chimera mRNAs. Average results of transcription shut-off experiments are presented in graphical form. Experiments were performed in the various *puf*Δ, *rpb1-1* yeast strains. Each line represents the average of two to six experiments. WT (red), *puf* 1Δ (purple), *puf* 2Δ (blue), *puf* 3Δ (green), *puf* 4Δ (orange) and *puf* 5Δ (black). The x-axis represents time (in minutes) after transcription shut-off. Percent RNA remaining from point of transcription shut-off (0 minute) is on the y-axis. All percentages were calculated from phosophoimage analysis of Northern blots probed for a region in the 3'UTR of the selected mRNA after normalizing to 7S RNA, an RNA Polymerase III transcript. **A**. Decay rates of the *MFA2/ HXK1 3'UTR* fusion RNA were calculated by transcriptional shut-off experiments in mid-log phase cells (OD_{600} 0.4). **B.** Decay rates of *MFA2/TIF1 3'UTR* fusion RNA were calculated by transcriptional shut-off experiments in cells entering stationary phase (OD_{600} 1.0). **C**. Decay rates of *MFA2/YHB1 3'UTR* fusion RNA were calculated by transcriptional shut-off experiments in cells entering stationary phase (OD_{600} 1.0). **D.** Control transcriptional shut-off experiments of *MFA2* with its native 3'UTR on the same expression plasmids used in A, B, and C in OD_{600} 1.0 cells.

Puf-Mediated Decay of Endogenous Target mRNA

I expected, based on the previous steady-state levels, that *YHB1* and *TIF1* mRNAs would be subject to Puf2p regulation. I also expected, based on my own results as well as other studies, that the 3'UTR of Puf targets would be sufficient for Puf-regulated decay (Jackson et al. 2004). However, the results above suggest that either the 3'UTRs of *YHB1* and *TIF1* are not sufficient for Puf2p-mediated RNA decay or that the stability of these mRNAs is not regulated by Puf2p.

To investigate the possibility that more than just the 3'UTRs of *YHB1* and *TIF1* are required for Puf2p regulation, I performed transcriptional shut-off experiments on endogenously encoded transcripts from cultures grown to an OD_{600} of 1.0. The results of the *YHB1* mRNA endogenous shut-offs are depicted in Figure 2.4. The *YHB1* endogenous transcript has a half-life of 12 minutes in WT yeast, while in the *puf2*Δ, *YHB1* has a half-life only of 4 minutes. Thus, Puf2p stabilizes endogenous *YHB1*.

It seems contradictory that there was an increase in steady-state *YHB1* mRNA in the *puf2*Δ, yet the half-life assays indicate that Puf2p stabilizes the transcript. I hypothesize that these seemingly conflicting results can be explained by the presence of some sort of feedback loop in which the down-regulation of stability somehow sends a signal for transcriptional up-regulation. Thus, the steady-state results in Figure 2.2 may be a balance of the transcriptional up-regulation and the stability down-regulation.

I also performed transcriptional shut-off experiments to detect decay rates of the endogenous *TIF1* decay in the various *puf*Δ strains (results not shown). However, this transcript has an extremely long half-life in these assays (>30 minutes), making it

difficult to detect differences in decay rates. Therefore, I have no evidence to determine if endogenous *TIF1* is a target of Puf2p mediated decay *in vivo*.

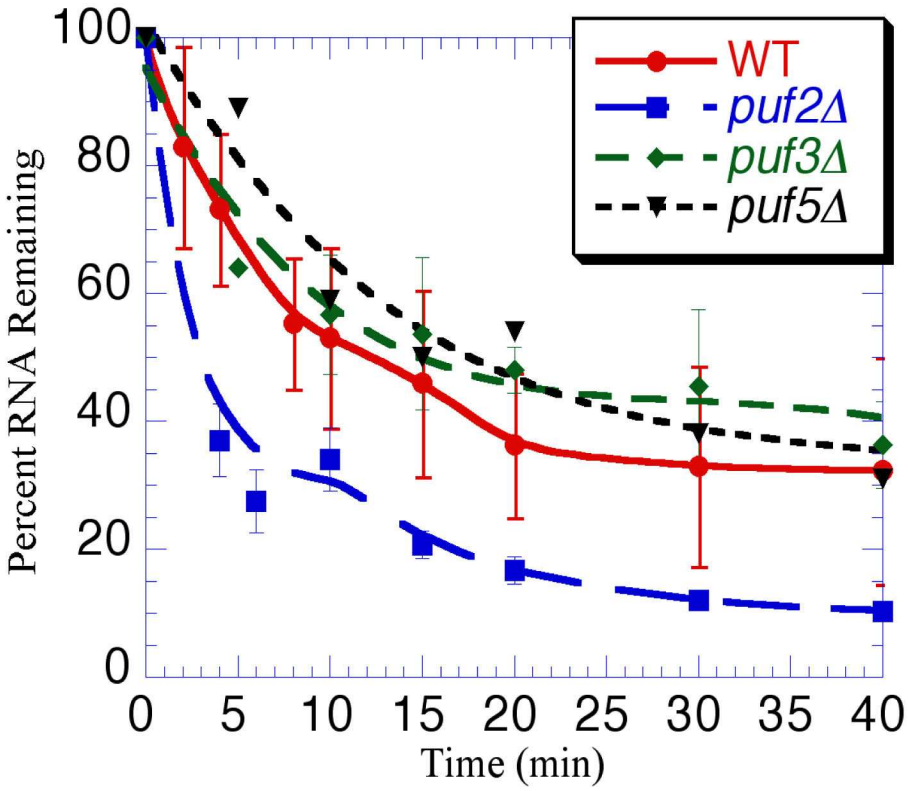


Figure 2.4. Decay of Endogenous *YHB1* mRNA. Transcription shut-offs were performed as described on *rpb1-1* WT (red), *puf2Δ* (blue), *puf3Δ* (green) and *puf5Δ* (black) yeast cultures entering stationary phase. Northern blots were probed for *YHB1* mRNA and 7S RNA, which was used to normalize for proper RNA loading. The X-axis represents time after transcription shut-off and the Y-axis represents percent *YHB1* remaining after shut-off (time 0). The averages of 2-5 experiments are graphed.

Characterization of Puf Target Binding

Previous results indicate that Puf binding to 3'UTR sequences is required for Puf-mediated decay. Similarly, if these new targets are directly regulated by Puf proteins, decay should depend on Puf protein binding to the transcript's 3'UTR in a sequence-dependent manner. To detect this, interactions between the repeat domain (RD) of each protein and sequences from the 3'UTR of each mRNA target were studied.

Based on previous results and the similarity of the Puf protein binding domains, I predicted that Puf binding to target mRNAs would require the core UGUA element. While all eukaryotic Puf binding targets contain a UGU element, previous studies with Puf3p suggest that yeast Pufs require a 3'UTR UGUA sequence followed by AU-rich region (Gerber et al. 2004, Jackson et al. 2004). Thus, potential Puf binding sites on target 3'UTRs were located by selecting UGUA containing regions within a reasonable distance downstream of the stop codon (less than 400 bases downstream of stop, see Figure 2.1). This selection criterion ensured that each contained at least one UGUA followed by an AU-rich region in the 3'UTR. To ensure that no potential binding sites were missed, any UGUA sequence in the selected 3'UTR, with or without an AU-rich region, was tested for its ability to interact with Puf proteins.

In the following sections, I will demonstrate *in vitro* and *in vivo* interactions between yeast PufRDs and target 3'UTR UGUA regions. This will confirm that Puf proteins can bind the mRNA target, confirm that binding is UGUA dependent, and provide additional information as to the sequence preference for different Puf proteins.

***In Vitro* Binding Assays**

I used gel mobility shift experiments to determine if Puf proteins can bind the UGUA regions *in vitro* as predicted. Purified Glutathione S-Transferase (GST) tagged PufRD (GST-PufRD) was incubated with short radiolabeled RNA substrates about 30 nucleotides long. Previous studies have shown that the RD portion of the Puf protein is sufficient for binding (Zamore et al. 1997; Wharton et al. 1998; Jackson et al. 2004). As mentioned in the introduction, the length of the substrates is sufficient for Puf binding (Wharton et al. 1998). The sequence of the RNA substrate is identical to that of the predicted 3'UTR regions, each containing the UGUA core element centered. The sequences of the radiolabeled RNA UGUA regions are listed in Figures 2.5, 2.6, 2.7, and 2.8. The complexes produced in the GST-PufRD plus target RNA co-incubations were resolved from free RNA by non-denaturing polyacrylamide gel electrophoresis.

Target mRNA 3'UTR UGUA regions were tested for binding to multiple PufRDs for several reasons. First, positive binding results, regardless of the target bound, will confirm activity of the newly-purified GST-PufRDs. Purified GST-Puf3pRD and GST-Puf5pRD have been used in previous studies (Jackson et al. 2004; Houshmandi and Olivas, 2005) and are known active binding proteins. However, GST-Puf1pRD and GST-Puf2pRD were newly purified for this study, thus their binding activity had to be determined. Puf4pRD could not be tested because it could not be purified. Second, since we have no target binding sequence data for Puf1p and Puf2p, and the RDs of these proteins differ in positions modeled to be required for RNA binding (Figure 1.2), it is of interest to determine whether these RDs bind to RNA sequences that are similar to other PufRD binding sites. Third, the binding studies will help further characterize the

preferred binding sequence of each tested PufRD. For example, will all PufRD bind to the Puf3pRD consensus sequence UGUANAUAU?

***YHB1 In Vitro* Protein Binding Assays.** Figure 2.5 presents the results of *in vitro* binding assays for GST-PufRD binding to the *YHB1* UGUA Region, as well as to the known Puf3pRD binding site of *COX17* mRNA (Jackson et al. 2004). Sequences of each of the UGUA regions made as *in vitro* transcription products are listed in Figure 2.5A. Notice that both target RNAs contain a core UGUA element (underlined) followed by an AU-rich region. However, while the *COX17* binding site contains the sequence UGUA followed by UAUA, the *YHB1* contains two UGUA elements in tandem followed by UUUA. Phosphoimager analysis of the native gel shifts are seen in Figure 2.5B and 2.5C. Binding of GST-PufRD to the radioactive target is indicated by a mobility shift (arrow), whereas unbound RNA migrates more quickly (free RNA). All the GST-PufRDs tested, including GST-Puf2pRD, are capable of binding to the *YHB1* UGUA Region (Figure 2.5B, left panel). It is important to note that while all four tested GST-PufRDs were able to bind *YHB1*, albeit to different degrees, only GST-Puf3pRD was able to bind *COX17* (Figure 2.5B, right panel). This suggests that GST-PufRDs are not just sticky, but rather selective in regards to binding RNA. In fact, competition experiments in which excess, non-radioactive competitor RNA was added to the radiolabeled *YHB1* UGUA reaction, indicate that the binding is specific (Figure 2.5C). Excess unlabeled *YHB1* (specific competitor, lanes 2, 5, 8, and 11) but not mutant *COX17* (non-specific competitor, lanes 3, 6, 9, and 12; Jackson et al. 2004) competed for GST-PufRD binding and therefore eliminated the shift.

These results also indicate that GST-Puf2pRD and GST-Puf1pRD are active RNA binding proteins. Even though equal amounts of RNA and protein were added to each binding reaction, the intensity of the Puf1pRD shift is considerably less than that of the other PufRDs. This decreased intensity could be due to a lesser affinity of the Puf1pRD for the target or deficient overall activity of the purified GST-Puf1pRD. Previously, the specific activity of similarly purified PufRDs was shown to be about 10% (Jackson et al. 2004). The activity of this particular prep is unknown.

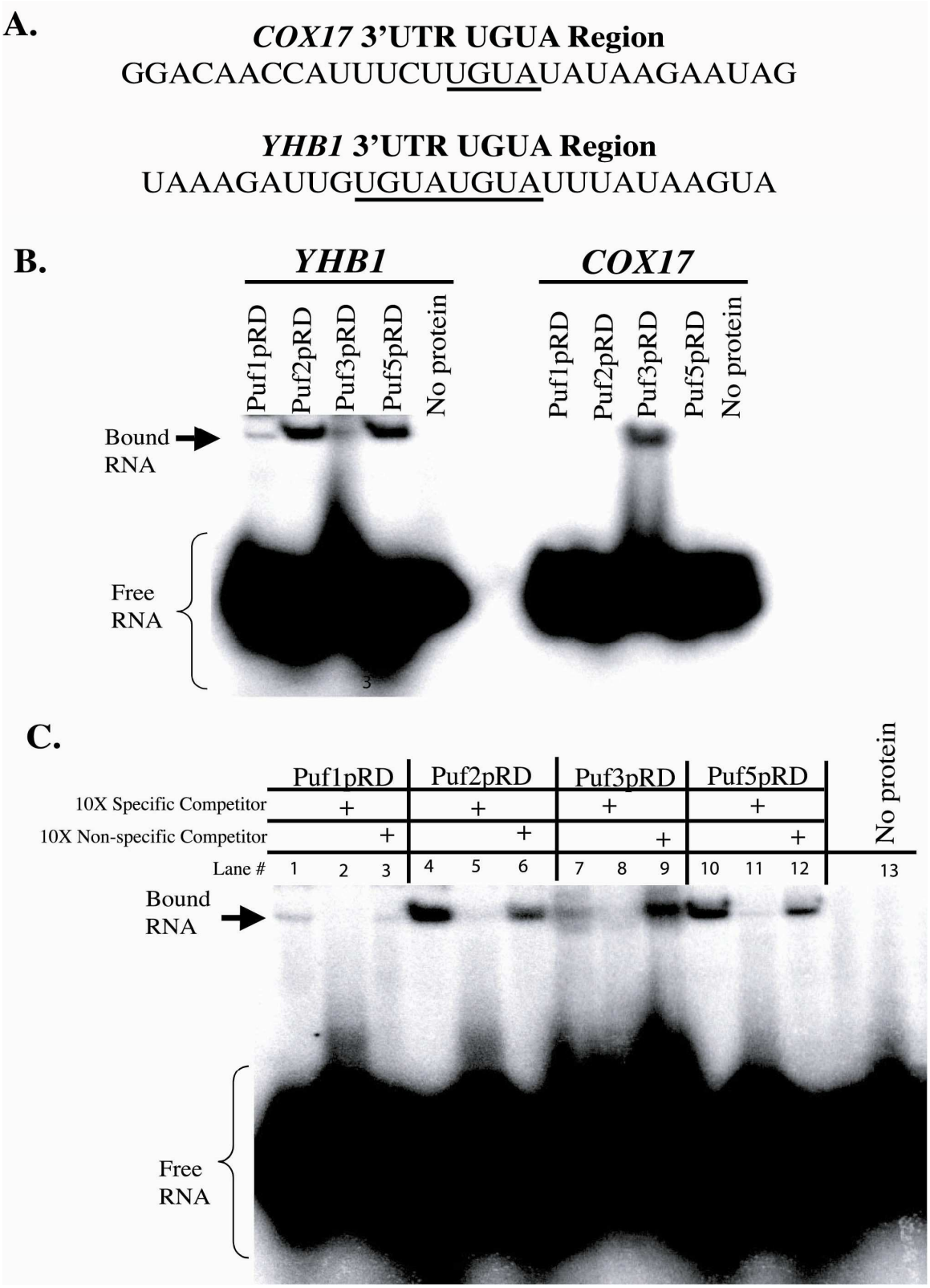

A.
COX17 3'UTR UGUA Region
GGACAACCAUUUCUUGUAUAUAAGAAUAG
YHB1 3'UTR UGUA Region
UAAAGAUUGUGUAUGUAUUUAUAAGUA
B.
YHB1
COX17
Puf1pRD
Puf2pRD
Puf3pRD
Puf5pRD
No protein
Bound RNA
Free RNA
C.
Puf1pRD
Puf2pRD
Puf3pRD
Puf5pRD
No protein
10X Specific Competitor
10X Non-specific Competitor
Lane # 1 2 3 4 5 6 7 8 9 10 11 12 13
Bound RNA
Free RNA

Figure 2.5. *In Vitro* Binding of GST-PufRD to UGUA Regions of *YHB1* and *COX17* 3'UTRs. A. Sequence of *in vitro* transcribed RNAs of *YHB1* and *COX17* 3'UTR. The core UGUA sequences are underlined. **B**. *In vitro* binding reaction to radiolabeled RNA (*YHB1*, left panel; *COX17*, right panel) in the presence or absence of purified GST-Puf1pRD, GST-Puf2pRD, GST-Puf3pRD and GST-Puf5pRD. **C**. *In vitro* binding reactions to radiolabeled *YHB1* in the presence or absence of purified GST-Puf1pRD, GST-Puf2pRD, GST-Puf3pRD and GST-Puf5pRD. Excess unlabeled *YHB1* UGUA Region and mutant *COX17* were used as specific and non-specific competitors, respectively. All *in vitro* binding reactions pictured were separated on a native polyacrylamide gel. Positions of un-bound radiolabeled RNA (Free RNA) and RNA bound by PufRD (Bound RNA) are indicated.

Puf3p and Puf5p each require the core UGUA sequence for binding to their mRNA targets (Jackson et al. 2004; Nakahata et al. 2001). The *YHB1* 3'UTR UGUA region contains the sequence UGUAUGUA, containing two such elements in tandem. In order to determine if this sequence is essential for Puf binding, I mutated UGUAUGUA to ACACACAC and tested the ability of GST-PufRDs to bind this mutant. The mutation completely eliminated binding of GST-Puf1pRD (Figure 2.6B, lanes 2-4) and GST-Puf3pRD (not shown). The mutation also eliminated specific binding of GST-Puf2pRD (lanes 5-7) and GST-Puf5pRD (lanes 8-10), as indicated by lanes 6, 7, 9 and 10, where it is obvious that both unlabeled non-specific and specific competitors were able to compete for binding of the labeled mutant transcript. Thus, the UGUAUGUA sequence is required for specific PufRD binding.

Next, I wanted to determine if only one of the *YHB1* tandem UGUAs is essential for binding. So I mutated the UGUAUGUA sequence to UGUAACAC (Mutant A) or ACACUGUA (Mutant B). In Figure 2.6C and D, *in vitro* binding indicates that each of the UGUAs is essential for specific binding of the GST-PufRDs. No GST-Puf1pRD (lane 1) or GST-Puf3pRD (lane 2) binding was detected to Mutant A. Also, only non-specific binding of GST-Puf2pRD (lanes 4-6) and GST-Puf5pRD (lanes 7-9) occurred with this mutant, as indicated by the ability of specific and non-specific competitors to successfully compete for labeled Mutant A binding (Figure 2.6C, lanes 5, 6 and 8, 9). Binding to Mutant B (Figure 2.6D) by GST-Puf1pRD (lanes 2-4), GST-Puf2pRD (lanes 5-7) and GST-Puf5pRD (lanes 11-13) was also competed off by both nonspecific and specific competitor RNAs. GST-Puf3pRD binding, however, could not be competed off with either competitor (lanes 8-10). Thus, all binding detected to *YHB1* Mutant B is due

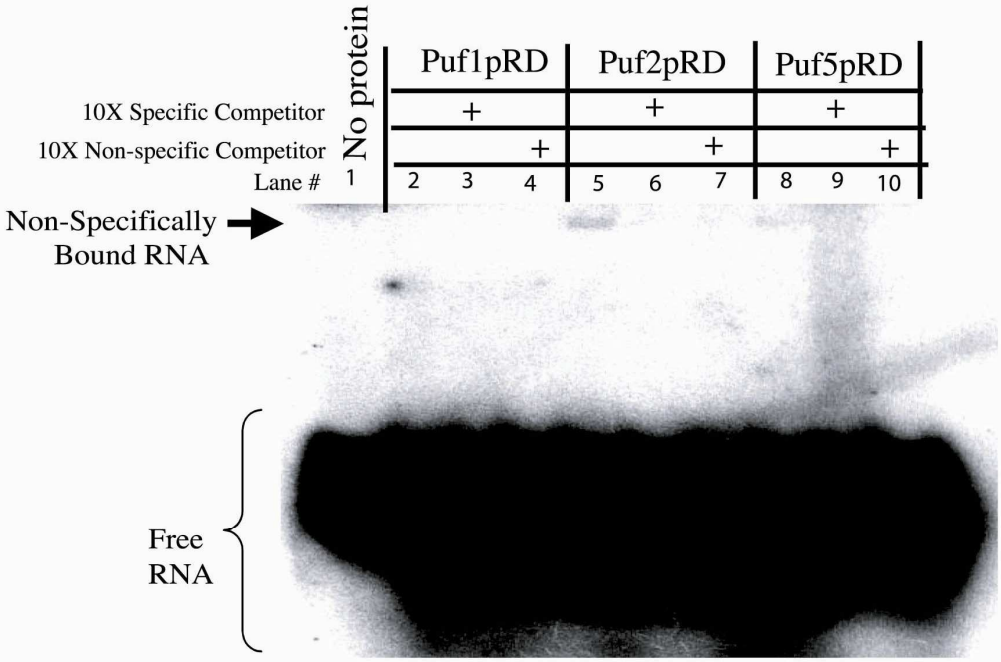
to non-specific interactions. Furthermore, each UGUA in the *YHB1* 3'UTR is required for specific recognition of *YHB1*.

To summarize the *YHB1* binding data, multiple PufRDs have the ability to bind specifically to the 3'UTR of *YHB1,* and this binding is dependent on the core UGUAUGUA binding element. Since I have shown that Puf2p regulates decay of *YHB1 in vivo*, for the purposes of this study I am most interested in the interaction detected between Puf2pRD and *YHB1*.

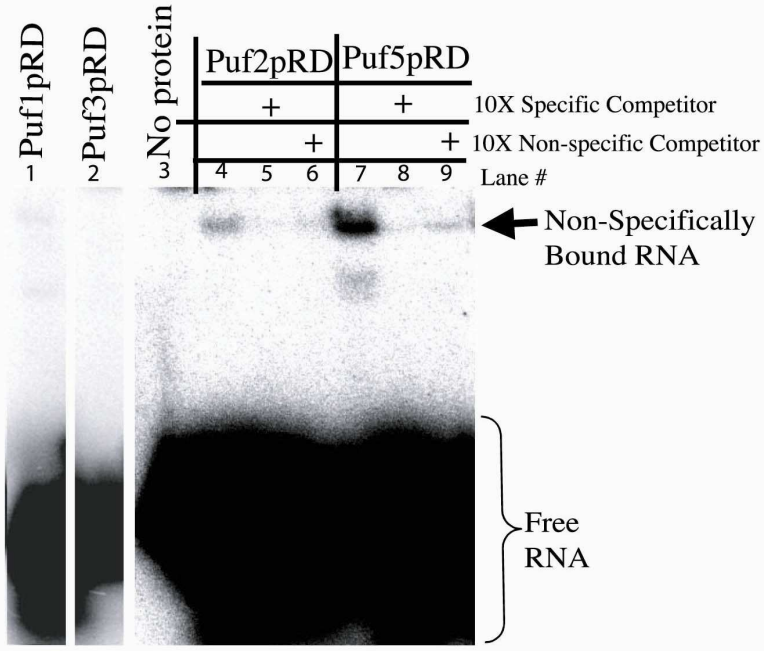
A.

YHB1 Double Mutant	**UAAAGAUUGACACACACUUUAUAAGUA**
YHB1 Mutant A	**UAAAGAUUGUGUAACACUUUAUAAGUA**
YHB1 Mutant B	**UAAAGAUUGACACUGUAUUUAUAAGUA**

B. Double Mutant



C. Mutant A



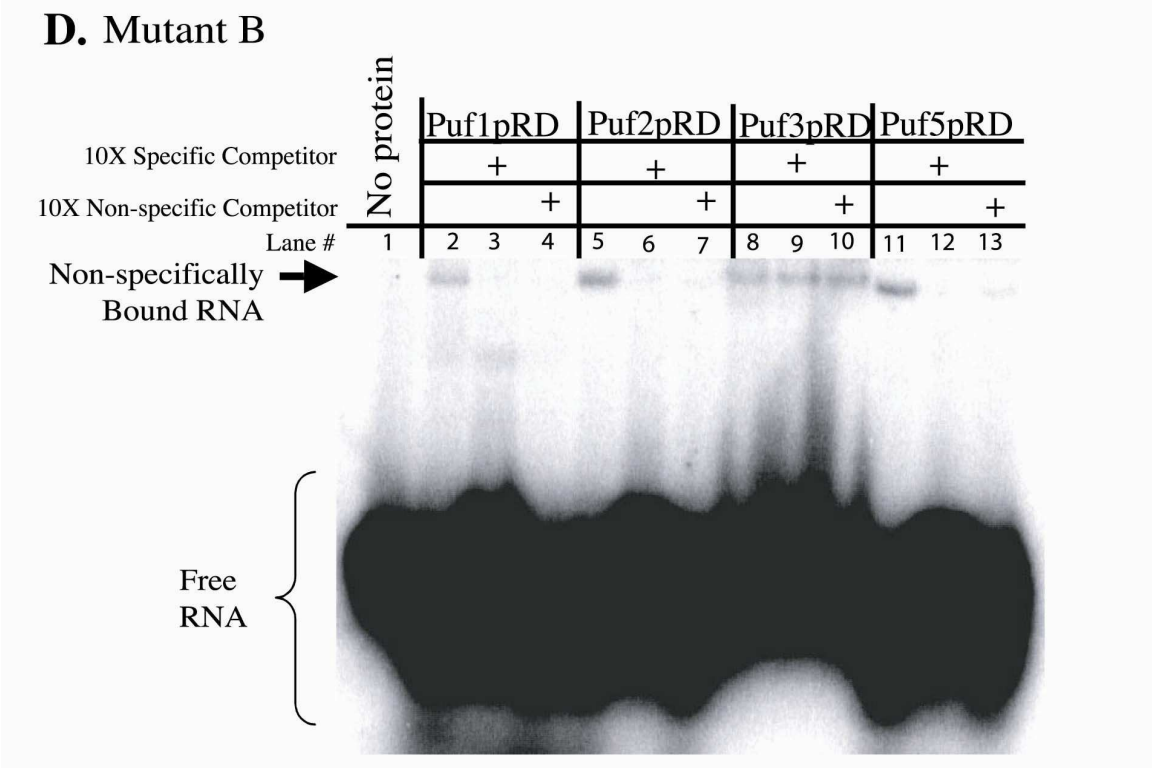


Figure 2.6. ***In Vitro*** **Binding of GST-PufRD to** ***YHB1*** **Mutant UGUA Regions. A.** RNA sequences of mutant *in vitro* transcribed *YHB1* UGUA Regions. UGUA core sequences are underlined. Mutant transcripts contain ACAC (underlined and boxed) in the place of UGUA. **B**. *In vitro* binding reactions of radiolabeled RNA (*YHB1* Double Mutant) in the presence or absence of GST-Puf1pRD (lanes 2-4), GST-Puf2pRD (lanes 5-7) or GST-Puf5pRD (lanes 8-10). **C**. *In vitro* binding reactions of radiolabeled RNA (*YHB1* Mutant A) in the presence or absence of GST-Puf1pRD (lane 1), GST-Puf2pRD (lane 2), GST-Puf3pRD (lanes 4-6) and GST-Puf5pRD (lanes 7-9). **D**. *In vitro* binding reactions of radiolabeled RNA (*YHB1* Mutant B) in the presence or absence of GST-Puf1pRD (lanes 2-4), GST-Puf2pRD (lanes 5-7), GST-Puf3pRD (lanes 8-10) and GST-Puf5pRD (lanes 11-13). All *in vitro* binding reactions in B, C, and D were separated on a native polyacrylamide gel. Excess unlabeled *YHB*1 WT UGUA Region and *COX17* mutant were used as specific and non-specific competitors, respectively. Positions of un-bound radiolabeled RNA (free RNA) and RNA bound to PufRD (Non-Specifically Bound RNA) are indicated.

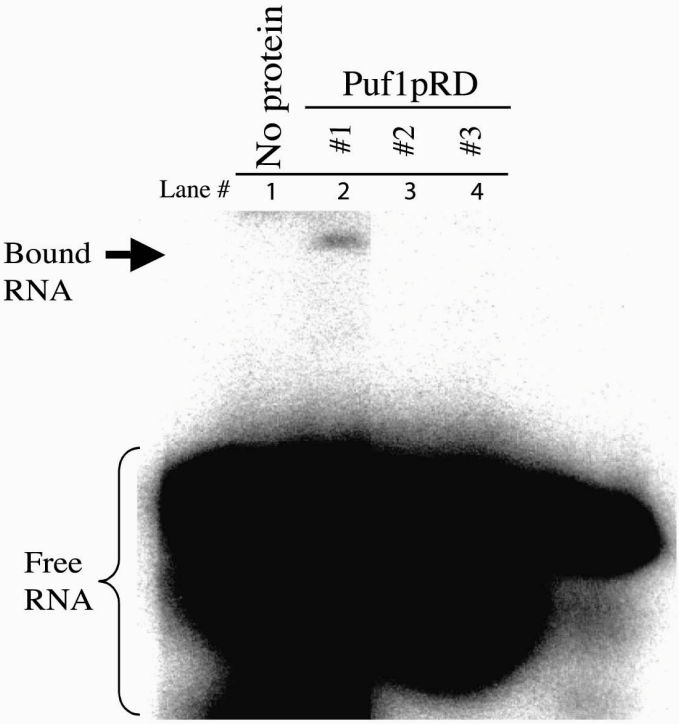
***In Vitro* Binding to *HXK1 3'UTR* UGUA Regions.** Similar *in vitro* binding studies were performed with all three of the UGUA regions of the *HXK1* 3'UTR. The sequences of each of these regions are listed in Figure 2.7A. The core UGUA elements of each are underlined. Since Puf1p has a destabilizing effect on *HXK1* 3'UTR, I was interested in the ability Puf1p to bind *HXK1*. Therefore each of the radiolabeled UGUA regions of *HXK1* was tested for its ability to bind to GST-Puf1pRD. In Figure 2.7B, the results show GST-Puf1RD binding to Region #1 (lane 2), but not to Regions #2 and #3 (lanes 3 and 4). Thus, UGUA Region #1 is the likely site of Puf1p interaction.

Specificity of the Puf1pRD interaction was tested by the addition of excess unlabeled competitors (Figure 2.7B). The addition of unlabeled UGUA Region #1 (specific competitor) led to a shift of less intensity than without competitors (compare lane 1 with lane 2). With the addition of unlabeled non-specific competitor, the intensity of the shift is also somewhat decreased, however a shift remains (lane 3). Deficient activity of purified GST-Puf1pRD could help explain these questionable results. Previous results with *YHB1* binding suggest that GST-Puf1pRD may be less active than the other purified GST-PufRDs. In addition, it remains possible that more than the RD of Puf1p is required for efficient and/or specific binding of the *HXK1* target UGUA region. Still another possible explanation of these results is that the RNA is too short for efficient binding of Puf1pRD and more sequence may be required.

A. *HXK1* 3'UTR UGUA Regions

Region #1 UUAAUGAAAAAAAUGUAAUGAAAUAUAAAU
Region #2 GCGGUCUAGUAUAUGUAAAUAUAGACACAC
Region #3 UAUAUAUUUAUGUGUGUAUAAUCCUAGCUAA

B.



C.

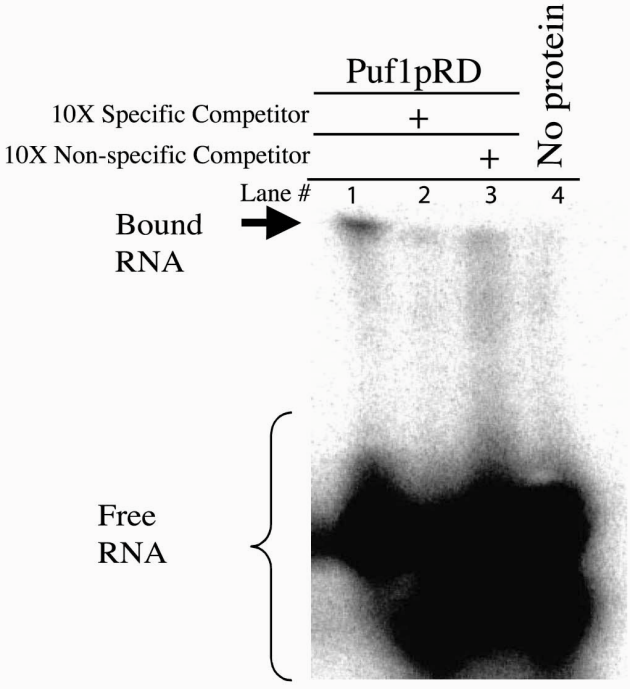


Figure 2.7. *In Vitro* Binding to *HXK1* 3'UTR UGUA Regions. A. Sequences of each of the three transcripts of *HXK1* 3'UTR UGUA regions. The core UGUA sequences are underlined. **B**. *In vitro* binding reactions of the radiolabeled RNAs (UGUA Region #1, lane 2; UGUA Region #2, lane 3; UGUA Region #3, lane 4) in the presence or absence of GST-Puf1pRD were separated on a native polyacrylamide gel. **C.** *In vitro* binding reactions of radiolabeled *HXK1* UGUA Region #1 in the presence or absence of GST-Puf1pRD. Excess unlabeled *HXK1* UGUA Region #1 (lane 2) and *COX17* RNA (lane 3*)* were used as specific and non-specific competitors. Positions of unbound radiolabeled RNA (Free RNA) and RNA bound to Puf1pRD (Bound RNA) are indicated.

***TIF1 In Vitro* Binding Assays.** Finally, I completed *in vitro* binding assays with GST-PufRDs and *in vitro* transcribed *TIF1* UGUA regions. There are two potential binding regions in the *TIF1* 3'UTR. The *in vitro* binding results for UGUA Region #1 are seen in Figure 2.8B. No binding was detected to GST-Puf1pRD (lane 1). Binding was detected, however, with GST-Puf2pRD (lane 2), GST-Puf3pRD (lane 5) and GST-Puf5pRD (lane 8). No shift is seen in lanes 3 and 9 where excess unlabeled *TIF1* RNA (specific competitor) was added to the binding reactions with Puf2pRD and Puf5pRD, yet binding was still detected to GST-Puf3pRD despite the presence of this specific competitor (lane 6). Non-specific competitors did not disrupt the *TIF1*-PufRD interaction in any case (lanes 4, 7 and 10). Thus, Puf2pRD and Puf5pRD bind *TIF1* UGUA Region #1 specifically *in vitro*. However, Puf3pRD binds non-specifically to this region.

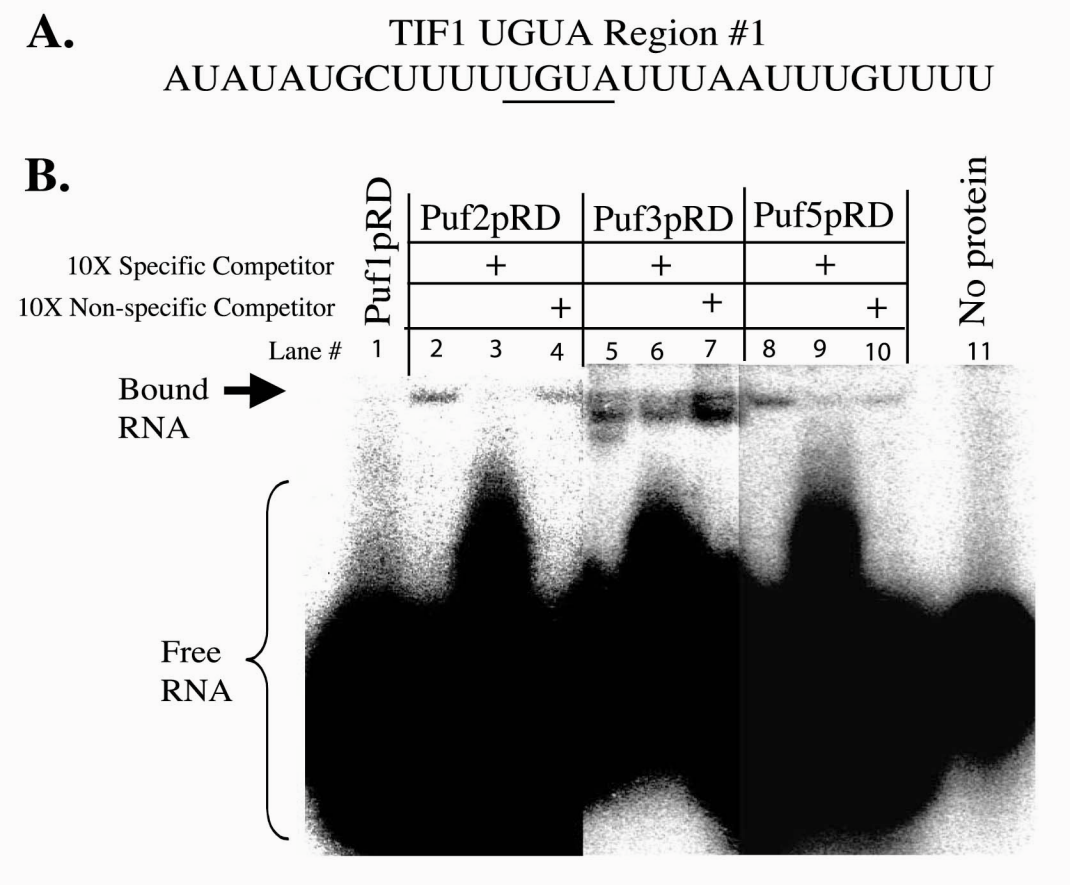


Figure 2.8. ***In Vitro*** **Binding to** ***TIF1*** **3'UTR UGUA Region #1.** **A**. The sequence of *TIF1* UGUA Region #1 *in vitro* transcribed RNA. The UGUA core sequence is underlined. **B**. I*n vitro* binding reactions of radiolabeled RNA in the absence or presence of GST-Puf1pRD (lane 1), GST-Puf2pRD (lanes 2-4), GST-Puf3pRD (lanes 5-7) or GST-Puf5pRD (8-10) were separated on a native polyacrylamide gel. Excess unlabeled *TIF1* UGUA Region #1 and mutant *COX17* were used as specific and non-specific competitors, respectively. Positions of unbound radiolabeled RNA (Free RNA) and RNA bound to PufRDs (bound RNA) are indicated.

***In Vivo* Roles of *TIF1 3'UTR* UGUA Regions.**

My results indicate that Puf5p regulates *TIF1 in vivo,* and that its 3'UTR is sufficient for this regulation. Puf2pRD and Puf5pRD bound to *TIF1* UGUA Region #1 *in vitro*, but *in vitro* binding studies for Region #2 were not definitive (results not shown). While *in vitro* studies are useful in many cases, they are often not representative of what happens *in vivo*. For example, *in vitro* studies showed that Puf3p binds the second UGUA region in *COX17* with much less affinity, however *in vivo*, each site is equally important for full regulation of *COX17* RNA (Jackson et al. 2004). Thus I proceeded with *in vivo* studies to better understand the importance of each of the *TIF1* UGUA regions in Puf regulation within the cell.

For these studies, I first utilized a PCR induced mutation in the 3'UTR of *TIF1* in the *MFA2* fusion construct. I have termed this mutant construct *MFA2*/*tif1*^{1} (Figure 2.9A). The mutation is a UGUA to CGUA substitution in the first UGUA region of *TIF1* 3'UTR (UGUA Region #1). A similar mutation in the *COX17* 3'UTR binding site eliminated Puf3p's ability to bind to the site (Jackson et al. 2004). In addition, similar mutations in the *hunchback* NRE also eliminated DmPum binding and regulation (Wharton et al. 1998). Thus, this *tif1*^{1} mutation is also predicted to be detrimental to Puf binding, and any Puf binding to Region #1 that may occur *in vivo* should be eliminated by the mutation.

In addition to the *tif1*^{1} construct, I also obtained a second mutant construct in which both UGUA regions of the *TIF1* 3'UTR were mutated (*MFA2*/*tif1*^{2x}, Figure 2.9A). To create this double mutant, I used *in vitro* site-directed mutagenesis to mutate the UGUA of the second binding region to ACAC in the *MFA2*/*tif1*^{1} expression plasmid. As

seen in the *in vitro* binding assays (Figure 2.6) and in similar *COX17 in vivo* analyses (Jackson et al. 2004), the UGUA to ACAC mutation will completely eliminate Puf binding to this region. Therefore, with this double mutation, all prospective Puf binding sites should be eliminated.

With these mutant constructs, I could determine if Puf proteins bind these regions *in vivo*. If the first UGUA region of the *TIF1* 3'UTR is bound by a Puf protein *in vivo*, the half-life of the *MFA2*/ *tif1*^{1} mutant compared to *MFA2/TIF1* will reflect the inability of the Puf to destabilize the mutant. By mutating both prospective binding regions, all possibilities of Puf binding and regulation are eliminated, and the decay of the *MFA2*/*tif1*^{2x} mutant should no longer be Puf-regulated *in vivo*.

In Vivo Effect of *TIF1* 3'UTR Mutations on Decay Rates

When I tested the decay rate of both mutants in *rpb1-1* yeast with transcriptional shut-off experiments at the OD_{600} of 1.0, the results indicate a role for both UGUA sites. First, in WT yeast the half-life of the *MFA2*/*tif1*^{1} mutant was 9 (+/- 2.3) minutes (Figure 2.9B, red line, and 2.9C, second red bar). When I tested the same mutant in *puf2*Δ yeast, the half-life was 7.5 (+/- 1.7) minutes, which is not significantly different from WT yeast (Figure 2.9B, blue line, 2.9C, second blue bar). However, in *puf5*Δ yeast *MFA2*/*tif1*^{1} had a half-life of 18 (+/- 2.5) minutes (Figure 2.9B, black line, 2.9C second gray bar). Thus, despite the mutation in UGUA Region #1, *MFA2*/*tif1*^{1} is still destabilized by Puf5p *in vivo,* while Puf2p appears to have no significant effect on the construct.

The construct with both Puf UGUA regions mutated had a half-life of 25 (+/- 6.4) minutes (Figure 2.9B, pink line, 2.9C, last red bar), showing that *MFA2*/*tif1*^{2x} decays similar to that of the *MFA2*/*tif1*^{1}in the *puf5*Δ strain. Thus, with the second UGUA region

eliminated, Puf5p can no longer regulate decay of *TIF1*, indicating that UGUA Region #2 in the *TIF1* 3'UTR is required for regulation by Puf5p *in vivo*.

In Figure 2.9C, I compare the half-lives of the *MFA2/TIF1* WT construct with that of the two mutants, *MFA2*/*tif1*^{1} and *MFA2*/*tif1*^{2x}. Comparing *MFA2/TIF1* with *MFA2*/*tif1*^{1}, the mutation to UGUA Region #1 appears to influence the decay of the construct. In all three yeast strains tested, the mutation causes an increase in the half-life of the construct. This suggests that UGUA Region #1 is involved in stabilizing *TIF1* mRNA. However, because the *MFA2*/*tif1*^{1} mutant maintains the ability to be regulated by Puf5p, Puf5p cannot be the only contributor to *TIF1* regulation. There must be some other factor thar requires the presence of UGUA Region #1 to properly destabilize *TIF1* mRNA.

A.

WT *TIF1* 3'UTR

UAA....73n....AUAUAUGCUUUU**UGUA**UUUAAUUUGUUUUUCUUUU.....78n.....GC
UUUGUCUUUU**UGUA**UAUAUCCGAACGU

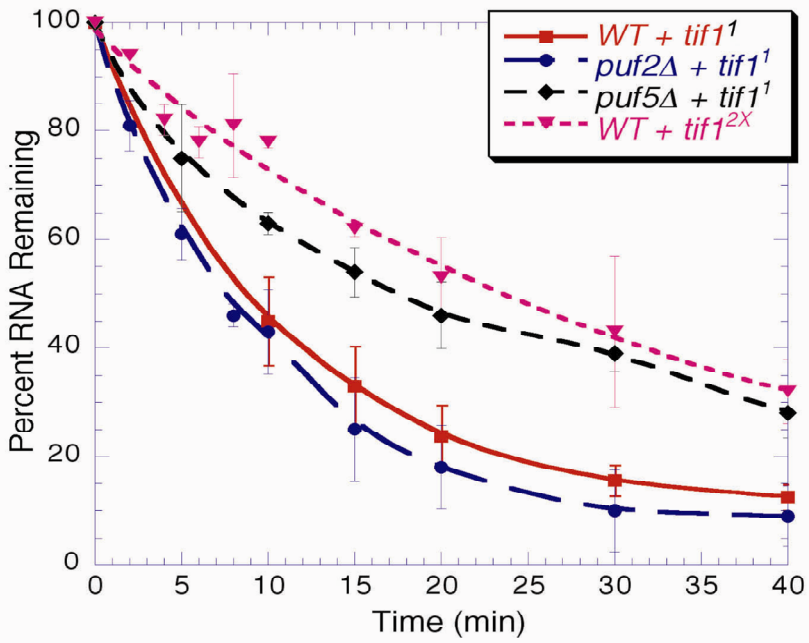
tif1 UGUA Region #1 Mutant (tif1^{1})

UAA....73n....UAUAUGCUUUU**C**GUAUUUAAUUUGUUUUUCUUUU.....78n.....GCU
UUGUCUUUU**UGUA**UAUAUCCGAACGU

tif1 Double Mutant (tif1^{2x})

UAA....73n....UAUAUGCUUUU**C**GUAUUUAAUUUGUUUUUCUUUU.....78n.....GCU
UUGUCUUUU**ACAC**UAUAUCCGAACGU

B.



C.

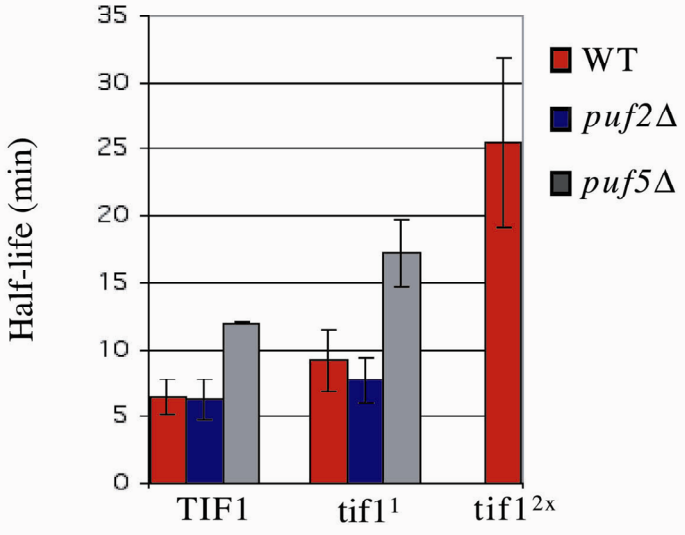


Figure 2.9. Effect of Mutations to *TIF1* 3'UTR on Decay Rates *In Vivo*. A. Sequences of WT (*TIF1*) and mutant (*tif1*^{1} and *tif1*^{2x}) 3'UTRs. The distance from the stop codon (UAA) to the UGUA Region #1 is 73 nucleotides (73n). The distance between this and UGUA Region #2 is 78 nucleotides (78n). UGUA core sequences are bold. Mutations are bold and boxed. **B**. Average decay rates of *MFA2/ tif1*^{1} and *MFA2/tif1*^{2x} in *rpb1-1* yeast strains. *MFA2/ tif1*^{1} in WT yeast (red squares), *MFA2/ tif1*^{1} in *puf5*Δ yeast (black diamonds), *MFA2/ tif1*^{1} in *puf2*Δ yeast (blue circles) and *MFA2/ tif1*^{2x} in WT yeast (pink triangles). The X-axis represents time after transcription shut-off. The y-axis values are the percent RNA remaining after transcription shut-off. Rates of decay were calculated from at least two transcription shut-off assays as described in earlier sections. **C**. Comparison of average half-lives of *MFA2/TIF1, MFA2/ tif1*^{1} and *MFA2/tif1*^{2x}. Average half-lives are presented from *rpb1-1* WT (red), *puf2*Δ (blue), and *puf5*Δ (grey) yeast.

Mode of Regulation

Next I was interested in exploring what aspects of decay are influenced by each Puf protein. Previous studies have determined that *COX17* is regulated by Puf3p in a deadenylation-dependent manner (Olivas and Parker, 2000) However, results presented above suggest that at least Puf2p may regulate decay in a different manner than Puf3p. Thus, different Puf proteins potentially regulate different aspects of decay. I analyzed poly(A) tail distributions of steady-state mRNA from WT and *puf∆* yeast to obtain general information as to the mode of Puf regulation.

Poly(A) Tail Distributions

There are many possible post-transcriptional events that could be influenced by Puf proteins including: initiation of deadenylation, processivity of deadenylation, terminal deadenylation, decapping, or any combination of the above. Observing the relative distribution of poly(A) tail lengths of mRNAs in WT versus *puf*Δ can inform us if the kinetics of individual decay steps are altered relative to the other decay steps. This dependence can in turn give limited information as to the mode of decay regulated by the individual Puf proteins.

The distribution of poly(A) tails in a steady-state mRNA population is visualized by first isolating total RNA from steady-state yeast cultures (WT and *puf*Δ). Then, to create a fragment of the selected mRNA short enough to resolve individual nucleotide differences on a denaturing polyacrylamide gel, the mRNA is cleaved toward the 3' end with a complementary DNA oligonucleotide and RNaseH (see Figure 2.10B). Relative lengths of poly(A) tails in *puf*Δ strains versus WT can be seen with a radiolabeled probe for a sequence in the 3'UTR, downstream of the site of RNaseH cleavage.

Results of Poly(A) Tail Distribution Analysis

I have determined the poly(A) tail length distributions for *YHB1, TIF1*, and *HXK1* in the various *puf* deletion strains. The results can be seen in Figure 2.10. For *YHB1*, there appears to be no change in distribution at either mid-log phase (results not shown) or entering stationary phase (Figure 2.10B), only a greater overall amount in the stationary phase *puf2Δ*. From earlier results, we know that Puf2p stabilizes the *YHB1* transcript. The poly(A) tail analysis suggests a general retardation of all steps of decay rather than impairment of any single step intermediate step, therefore causing no change in the steady-state distribution of mRNA poly(A) tails.

The poly(A) tail distributions of *TIF1* mRNA isolated from cells entering stationary phase (Figure 2.10) are relatively short in WT, *puf3*Δ, *puf1*Δ, and *puf4*Δ strains. Conversely, poly(A) distributions of *TIF1* from *puf2*Δ and *puf5*Δ strains are slightly longer (Figure 2.10C). Thus the poly(A) tail length of *TIF1* is dependent on Puf5p and Puf2p. These data suggest that both Puf2p and Puf5p may alter the kinetics of some step of decay.

The distributions of poly(A) tails for *HXK1* are shown in Figure 2.10D. It is possible that slightly shorter tails accumulate in *puf 1*Δ than in WT, suggesting that Puf1p affects the kinetics of some step of mRNA decay.

While this type of assay indicates alteration of decay kinetics, there are further types of analyses that better detect what specific aspects of decay are regulated by Puf proteins. This is an area of interest that will continue in the Olivas lab after the completion of this thesis.

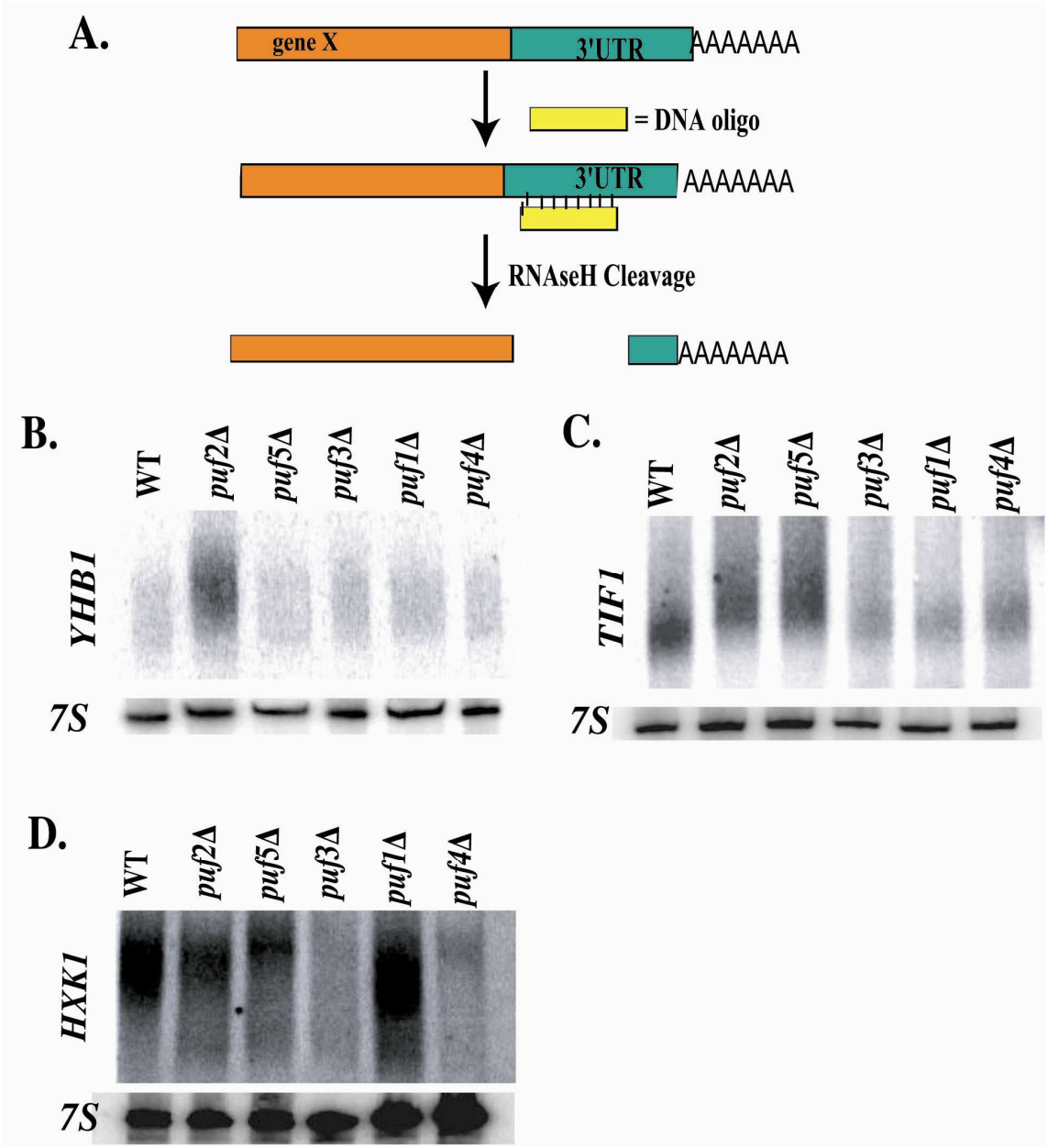


Figure 2.10. Poly(A) Tail Distributions of Steady-State mRNA Targets. **A**. Steady-state poly(A) tail distributions were determined by first annealing a DNA oligo a region within the 3'UTR then cutting the mRNA with RNaseH. The products were resolved on a denaturing polyacrylamide gel. The small 3'UTR fragment and the poly(A) tail was visualized with an end-labeled DNA oligo complimentary to this region of the 3'UTR. **B**. Poly(A) tail distributions of steady-state *YHB1* mRNA from OD_{600}1.0 cells. **C**. Poly(A) tail distributions of *TIF1* steady-state mRNA from OD_{600}1.0 cells. **D.** Poly(A) tail distributions of steady-state *HXK1* from OD_{600}0.4 yeast cells. The 7S RNA loading control is shown below each results panel.

Chapter 3: DISCUSSION

There are many interesting conclusions that may be drawn form this work as well as numerous questions that have been raised. This work has identified three new targets of Puf-mediated mRNA decay in *S. cerevisiae*; one target of Puf1p, one target of Puf2p and one new target of Puf5p. While some of these targets are destabilized by Puf proteins, I have identified a new stabilizing role for Puf proteins. In addition, I have observed that the activity of Puf proteins can be condition-specific. With multiple Puf targets, I can now compare and contrast the preferred binding sequences for the yeast Puf proteins.

HXK1 mRNA Destabilized by Puf1p

Both the steady-state levels of *HXK1* and steady-state poly(A) tail distributions suggest an involvement of Puf1p in regulation of *HXK1*. *In vivo* decay analysis showed that Puf1p destabilizes *HXK1* mRNA, and the *HXK1* 3'UTR is sufficient to mediate this Puf1p-regulated decay. I also showed that Puf1pRD binds to UGUA Region #1 *in vitro*. The binding data supports the *in vivo* decay results (Figure 2.3A), implying that the Puf1p-mediated decay is a direct effect of Puf1p binding to *HXK1* 3'UTR as opposed to indirect regulation. Thus, Puf1p most likely regulates *HXK1* stability by binding to UGUA Region #1 in the 3'UTR of *HXK1* mRNA. The type of regulation between Puf1p and *HXK1* is typical of Puf proteins, where binding promotes decay of the mRNA. This appears to be the same type of Puf-mediated regulation seen previously in *Drosophila, C. elegans, Xenopus* and yeast. These Puf proteins may promote decay of their target

transcripts including by recruiting decay machinery to the mRNA or altering the mRNP structure as to allow for more efficient degradation of the mRNA.

Future work is required to verify that Puf1p directly affects the decay rate of *HXK1* mRNA in a deadenylation-dependent manner. Because of the weak nature of the *in vitro* interaction between the *HXK1* 3'UTR UGUA region and Puf1pRD, an *in vivo* demonstration of this interaction is required to verify that Puf1p destabilizes *HXK1* by direct interaction with this region *in vivo*. Such a study would involve mutating the UGUA element in the *HXK1* 3'UTR of the *MFA2/HXK1 3'UTR* expression vector. I hypothesize that this mutation will eliminate specific interaction with Puf1pRD *in vivo* and therefore eliminate the ability of the fusion to be regulated by Puf1p.

YHB1 is Stabilized by Puf2p

Puf proteins are known as translational repressors that stimulate mRNA decay. However, transcriptional shut-offs in this work have shown that Puf2p stabilizes endogenous *YHB1* mRNA. This is the first instance in which a Puf protein stabilizes a transcript rather than destabilizes the target. Whether this is a novel function for Puf2p on *YHB1* mRNA, a novel function of Puf2p in general, or if other Pufs are also capable of such regulation remains to be seen.

In vitro binding data showed that Puf2pRD has the ability to bind specifically to the 3'UTR of *YHB1* and that this binding is dependent on the core UGUAUGUA element. These results support my hypothesis that Puf2p regulates *YHB1* by binding in a UGUA-dependent fashion to this region of its 3'UTR *in vivo*.

The UGUA region is obviously sufficient for *in vitro* binding of Puf2p, however, as shown by transcriptional shut-offs with the *MFA2/YHB1 3'UTR* chimera, the 3'UTR

of *YHB1* is not sufficient for Puf2p-mediated stabilization. Thus, unlike the characteristics shown for Puf3p and Puf5p, Puf2p regulation does not solely depend on the presence of the target 3'UTR.

A Model for Puf2p Regulated Stabilization

In Figure 3.1, I present a model of the regulatory mechanism of Puf2p on *YHB1* mRNA that incorporates all the observations for Puf2p in this study. In this model, Puf2p binds to the 3'UTR, as we have seen *in vitro*. However, to incorporate observations that the 3'UTR is not sufficient for Puf2p mediated decay, the model includes the hypothesis that Puf2p requires interactions with regions in or near the 5'UTR of *YHB1* (suggested by the double ended arrows) for proper target regulation. Known interactions between 3' and 5' binding proteins (i.e. Pab1p and eIF4E) result in a close spatial relationship between the polar ends of the RNA (Tarun and Sachs, 1996; Schwartz and Parker, 1999). This close proximity would also allow Puf2p to interact with both ends of the RNA, making the 5'UTR rather than the coding region of the RNA the most likely region of Puf2p interaction. *C. reinherdtii psb*A mRNA is regulated by a similar mechanism in which the regulatory protein complex associates with sequences in both UTRs (Katz and Danon, 2002). However, I cannot rule out the possibility that all or a portion of the coding region may be required for Puf2p regulation.

This model does not speculate the nature of the interaction between Puf2p and the 5' end of the RNA. While one possibility is that Puf2p binds directly to the RNA sequence in the 5' region, I do not believe this is a likely occurrence. It is more likely that Puf2p requires protein partners to accomplish this interaction. Previous studies have indicated that the outer surface of the conserved PufRD serves as a site of protein-protein

interaction (Edwards et al. 2001; Wang et al. 2001). This is where Nanos and Brat interact to help DmPum perform its function (Sonoda and Wharton 2001; Edwards et al. 2003). Without known yeast homologs to these proteins, it is difficult to hypothesize what proteins may bind to this region in Puf2p. Candidates for these interacting partners include translation initiation factors, other cap binding proteins known to be associated with the 5'end of the RNA or perhaps some novel protein yet to be characterized. A complex of proteins, as is the case with *psb*A (Katz and Danon, 2002), may also be responsible for maintaining the 3' to 5' interaction.

This model also takes into account Puf2p's unique stabilizing effect on *YHB1* mRNA. The interactions between Pab1p and translation initiation factors promote the looped mRNA structure and help to stabilize mRNA (Tarun and Sachs, 1996). Puf2p interaction with both ends of the RNA may strengthen the 5' and 3' interaction, further stabilizing the target mRNA. Thus, while Pufs (such as Puf1p, Puf3p and Puf5p) that destabilize their mRNA targets do so via the 3'UTR, Pufs (such as Puf2p) that stabilize their mRNA targets require sequences outside the 3'UTR.

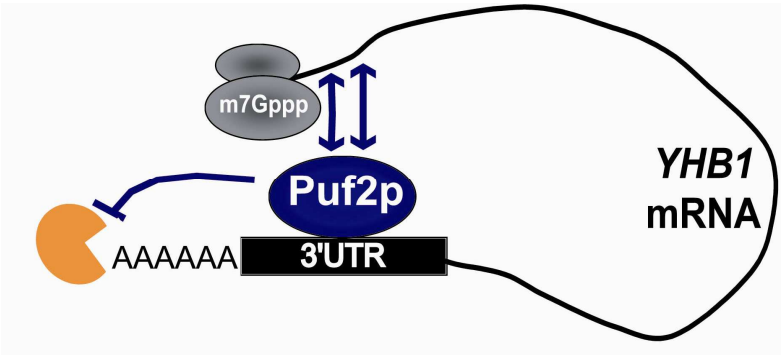


Figure 3.1. Puf2p Model. Puf2pRD binds to the 3'UTR of *YHB1* (or any other target mRNA). It may also interact, indirectly or directly, with RNA sequences in the 5' end of the mRNA (indicated by double-ended arrows). Both of these interactions may be required to properly inhibit decay of the RNA.

Future Directions for Puf2p and *YHB1* mRNA Research

The novelty of Puf2p has raised some very interesting possibilities as well as created a wealth of future goals. To support the model I have presented here, future work should involve first determining the minimal regions of *YHB1* required to permit Puf2p regulation. According to the model, adding the *YHB1* 5'UTR to the *MFA2*/*YHB1* 3'UTR construct would allow stabilization by Puf2p. In addition, mutational studies will be helpful in determining that the UGUA region in the 3'UTR is truly required for Puf2p sensitivity *in vivo*. The more long-range goal is to determine the protein partners required for Puf2p to regulate the mRNA.

On a larger scale, this work implies the presence of a Puf protein function not yet discovered in other organisms. This work is the first to characterize a Puf protein with a role in gene expression up-regulation by stabilizing the mRNA rather than down-regulation by destabilizing the mRNA. I hypothesize, based on the conserved nature of the PufRDs and the fact that at least 38 eukaryotic Puf proteins are still uncharacterized, that this type of regulation may also be present in other organisms.

TIF1 mRNA: A Target of Multiple Puf Proteins?

This study has positively identified *TIF1* as a target of Puf5p. *In vivo* decay experiments indicate that Puf5p destabilizes *TIF1* mRNA and that sequences outside the 3'UTR are not required for this regulation. The poly(A) tail distributions of *TIF1* also support a role for Puf5p in regulation of decay. While Puf5p can bind UGUA Region #1 in the *TIF1 3'UTR in vitro*, *in vivo* mutational studies showed that Region #1 is dispensable for Puf5p–mediated decay. However, Region #2 is required for proper regulation by Puf5p

in vivo, suggesting that Puf5p binds in a UGUA dependent fashion to this region and stimulates decay of *TIF1* mRNA.

While Region #1 is not required for Puf5p mediated decay, the mutational studies suggest that the UGUA Region #1 is a regulatory element. Because the single mutation to UGUA Region #1 stabilized the RNA, while the double mutant stabilized the target even further, each region must be partially involved in decay regulation. Furthermore, because the mutation to UGUA Region #1 still allowed regulation by Puf5p, this region must be involved in regulation by some decay process other than Puf5p-mediated decay. I hypothesized earlier that this region is a binding site for some unknown protein. Because this region contains a UGUA element, this other regulatory protein may be a Puf protein. While evidence suggests that it is probably not Puf2p or Puf5p, any one of the other Pufs not yet tested may bind to this region *in vivo* to destabilize *TIF1*. Thus, it remains a future goal to test the decay of *MFA2*/*TIF1 3'UTR* mutants in all *puf*Δ yeast in hopes of discovering that one of the other Puf proteins is responsible for binding to UGUA Region #1 and destabilizing *TIF1* mRNA.

The steady-state mRNA levels of *TIF1* did not predict Puf5p's now proven involvement in regulation of the transcript, nor did they predict the involvement of any other Puf protein in *TIF1* decay. Thus the results seen in Figure 2.2 may be the result of the balance of multiple levels of decay regulation occurring *in vivo*. I have shown evidence that Puf5p as well as some other factor each destabilize and down-regulate *TIF1*. It also remains possible that Puf2p stabilizes *TIF1 in vivo*, as it does with *YHB1*. The presence of two UGUA regions, each confirmed to have potential Puf interaction capabilities, would allow for binding of multiple Pufs concurrently. Concurrent binding

of two Puf proteins to a single 3'UTR has been shown for both *COX17* and *hunchback* mRNA (Jackson et al. 2004; Wharton et al. 1998). It may seem contrary for the cell to have two opposing forces working on the same RNA. However, transcriptional regulatory processes often have both inducers and inhibitors. The opposing forces in these cases allow for more precise regulation of gene expression. In the same manner, precise regulation in the case of transcription and mRNA decay may be enhanced because each regulatory protein is subject to condition-specific regulation itself, altering the balance of expression under different conditions.

Steady-state levels of *TIF1* mRNA and its poly(A) tail distributions suggest a role for Puf2p in regulating *TIF1* mRNA. Furthermore, specific Puf2pRD binding was detected with at least one of the UGUA containing regions in *TIF1 3'UTR in vitro*. However, no apparent differences in decay rates of a construct containing the *TIF1* 3'UTR were detected with or without *PUF2*. At the least, these results suggest that the 3'UTR of *TIF1* is not sufficient to mediate regulation by Puf2p. Because my results with *YHB1* mRNA suggest that Puf2p regulation requires sequences outside the 3'UTR, based on this data alone I cannot rule out *TIF1* mRNA as a target of Puf2p mediated decay. I have attempted to obtain half-life estimations of the endogenous full-length *TIF1* transcript in the *puf2Δ* strain. These attempts have been fruitless due to the abnormally long half-life of this transcript under the high cell density conditions. Therefore, I have little data to determine if sequences outside of the *TIF1* 3'UTR would allow Puf2p-mediated decay of *TIF1* mRNA. I hypothesize that if *TIF1* is regulated by Puf2p, it is regulated in a similar manner as *YHB1,* whereas Puf2p probably interacts with regions in the 5'UTR to stabilize the mRNA. To tesr this hypothesis, I propose adding the 5'UTR

to the *MFA2/TIF1* 3'UTR construct and then determining if the 5'UTR sequence will allow for Puf2p sensitivity *in vivo*.

Translation and Stability

As I mentioned, the stability of *TIF1* has made detecting Puf-mediated decay difficult. I hypothesize that the stable *TIF1* transcript is efficiently translated, which is known to stabilize a transcript. By placing the *TIF1* 3'UTR onto a transcript like *MFA2* that is degraded efficiently and not stabilized by translation, I was able to eliminate the stabilization of *TIF1* transcript caused by translation and detect only the influence of the Puf proteins on decay (Figure 2.3B). Similarly, I hypothesize that *YHB1* is also translated more efficiently than the *MFA2/YHB1 3'UTR* construct, explaining why *YHB1* endogenous mRNA has a much longer half-life than the 3'UTR fusion.

Condition Specific Regulation by Yeast Puf Proteins

Previous studies with the yeast Puf proteins, including the microarray experiments, were performed on yeast cultures grown to mid-log phase (~OD_{600}0.4) (Olivas and Parker, 2001; Jackson et al. 2004; Gerber et al. 2004). As I have confirmed, this condition is optimal for many of the experiments commonly utilized to study RNA decay. However, regulation of two of the mRNA targets for Puf-mediated mRNA decay only occurs at a higher cell density (OD_{600}1.0), not at the OD_{600} of 0.4. Many conditions within the cell and in the media change as the cultures grow in density and undergo diauxic shift, including cytoplasmic mRNA decay processes. P-bodies, proposed sites of RNA processing, become apparent only under these conditions that inhibit translation initiation (Teixeira et al. 2005). From my studies, Puf regulation is also condition specific in some

cases. Perhaps, Puf function is also linked to the translation state of the RNA. In the future, it will be interesting to see if conditions that promote p-body formation and alter the translation state of mRNAs within the cell also allow Puf regulation of these condition specific targets. Similarly, it would be interesting to see if conditions that do not allow p-body formation do not allow Puf-regulated decay of the target mRNA.

Binding Preferences for the Yeast Puf Proteins

Consistent with previous observations, my studies suggest that each of the yeast Puf proteins requires a UGUA element for efficient binding both *in vitro* and *in vivo*. In the case of *YHB1*, I showed that multiple Puf proteins are capable of binding to the UGUA region and this binding is dependent on the sequence UGUAUGUA. So the binding preferences of Puf1pRD and Puf2pRD are similar to that of the other PufRDs, suggesting that the amino acid differences in these RDs (Figure 1.2) do not have a significant influence on the sequence preferences of these proteins. Qualitative observations with *in vitro* binding assays of *YHB1* and *TIF1* determined that despite the incredible similarity between the two proteins (78% similar), Puf2p can bind sequences that Puf1p cannot bind. This suggests that these two very similar proteins have distinct binding preferences.

While I showed that multiple Puf proteins can bind *YHB1* 3'UTR *in vitro*, only Puf2p has the ability to regulate this RNA *in vivo*. This observation is consistent with previous observations suggesting that the ability of a PufRD protein to bind an mRNA is not necessarily indicative of the ability of that Puf to regulate the mRNA *in vivo*. Taking this information into account, it is not surprising that Puf5pRD bound *TIF1* UGUA

Region #1 *in vitro*, but Puf5pRD was shown to have no *in vivo* role with this region of *TIF1*.

In Table 3.1, I have aligned each of the target mRNA's UGUA regions positive for Puf binding in this study, as well as the UGUA regions of *HO* and *COX17* known to be bound by Puf5p and Puf3p. In addition to the common UGUA element, each target UGUA region also contains a downstream Adenine (underlined) as well as an AU-rich region downstream of the UGUA, as predicted. In fact, Puf2p, Puf3p and Puf5p seem to share the binding preference UGUAUNUA. This sequence may be universal to yeast Puf binding sites and therefore useful in future searches for Puf mRNA targets.

It is difficult to discern from the primary sequences how Puf proteins are able to selectively recognize their own target mRNAs for regulation *in vivo* upon examination of the RNA binding sequences collectively. There are no apparent sequences unique to the Puf2p target versus targets bound but not regulated by Puf2p *in vivo*. Neither are there apparent RNA sequences unique in the UGUA regions of Puf5p regulated RNAs. On the other hand, only targets bound and regulated by Puf3p contain the sequence CNUGUA. The C upstream from the UGUA was predicted by the Gerber et al. (2004) microarray to be part of the Puf3p consensus binding sequence. Thus, this C may important for Puf3p recognition *in vivo*. The Puf1p target appears to be fairly unique compared to the other RNA sequences and therefore may be distinguishable *in vivo*. Identification of additional targets of all of the yeast Pufs will be helpful in determining and/or confirming the consensus sequences for each of the Puf proteins.

RNA	Binding Sequence	Binding Proteins				*In Vivo*
		Puf1p	Puf2p	Puf3p	Puf5p	Regulation
HO	AAGUUGUAUGUAAUAA	nd	+	-	+	Puf5p
YHB1	AUUGUGUAUGUAUUUA	+	+	+	+	Puf2p
TIF1(1)	UUUUUGUAUUUAAUUU	-	+	-	+	?
TIF1(2)	UUUUUGUAUAUAUCCG	nd	nd	nd	+	Puf5p
COX17(1)	UUCUUGUAUAUAUAAG	-	-	+	-	Puf3p
COX17(2)	UACCUGUAAAUAUGUG	nd	nd	+	-	Puf3p
HXK1(1)	AAAAUGUAAUGAAAUA	+	nd	nd	nd	Puf1p

Table 3.1. Alignment of Puf Protein Target 3'UTR UGUA Regions. Sequences of each Puf target UGUA region identified in this study and by other studies (Tadauchi et al. 2001; Jackson et al. 2004) are listed. In some cases, there is more than one UGUA region in the target 3'UTR. The number of the UGUA region listed is placed in parentheses next to the name of the RNA. Elements common to each UGUA region are underlined within the sequence. Puf proteins shown to bind either *in vitro* or *in vivo* are indicated [(+) = interaction detected, (-) = no interaction detected, (nd) = no data available]. The Puf protein shown to regulate decay *in vivo* of each transcript is indicated (*in vivo* regulation).

Summary

In review, the results of this study have positively identified three new targets of Puf-mediated mRNA decay in budding yeast. Puf1p and Puf2p have been verified as active target-specific regulators of mRNA decay, further suggesting that each of the yeast Puf proteins are regulators of mRNA decay *in vivo*. Each of the yeast Puf proteins rely on the presence of 3'UTR UGUA containing sequences for target binding and regulation. While Puf1p, Puf3p and Puf5p, as well all other studied eukaryotic Pufs destabilize their target mRNAs, Puf2p is unique in that it stabilizes its target. Puf2p is also unique in that sequences outside of the 3'UTR are required for its regulatory abilities.

CHAPTER 4: MATERIALS AND METHODS

Table 4.1. Strains Used in This Study.

STRAIN	GENOTYPE	SOURCE/REFERENCE
yWO3	MATa, *his4-539*, *leu2-3, lys2-201, trp1-1, ura3-52*	yRP683 Hatfield et al. 1996
yWO5	MATa, *leu2-3, lys2-201, trp1-1, ura3-52, cup1::LEU2/PM*	yRP840; Hatfield et al. 1996
yWO7	MATα, *leu2-3, ura3-52, rpb1-1*	yRP693; Caponigro et al. 1993
yWO14	MATa, *his4-539*, *leu2-3, trp1-1, ura3-52, cup1::LEU2/PM*, *puf2::URA3*	yRP1237; Olivas & Parker, 2000
yWO17	MATa, *his4-539*, *leu2-3, trp1-1, ura3-52, cup1::LEU2/PM*, *puf5::TRP1*	yRP1240; Olivas & Parker, 2000
yWO18	MATa, *his4-539*, *leu2-3, trp1-1, ura3-52, cup1*::*LEU2/PM*, *puf3::NEO*	yRP1241; Olivas & Parker, 2000
yWO20	MATa, *his4-539*, *leu2-3, trp1-1, ura3-52, cup1::LEU2/PM*, *puf1::NEO*	yRP1243; Olivas & Parker, 2000
yWO22	MATa, *leu2-3, lys2-201, trp1-1, ura3-52, cup1::LEU2/PM, puf4::LYS2*	yRP1245; Olivas & Parker, 2000
yWO43	MATα, *his4-539*, *leu2-3, trp1-1, ura3-52, rpb1-1, cup1::LEU2/PM*, *puf3::NEO*	yRP1360; Olivas & Parker, 2000
yWO48	MATα, *his4-539*, *leu2-3, ura3-52, rpb1-1, puf2::URA3*	Wendy Olivas
yWO49	MATα, *leu2-3, trp1-1, ura3-52, rpb1-1, puf5::URA3*	Wendy Olivas
yWO102	MATa, *leu2-3, trp1-1, ura3-52, cup1::LEU2/PM*, *puf1::URA3*	Randi Ulbricht
yWO104	MATa. *his4-539, leu2-3, lys2-201, ura3-52, rpb1-1*	Randi Ulbricht
yWO105	MATα, *his4-539*, *lys2-201, ura3-52, rpb1-1, puf4::LYS2*	Randi Ulbricht

Table 4.2. Plasmids Used in This Study.

PLASMID	DESCRIPTION	MARKER(S)	SOURCE/REF.
pWO4	GAL vector	*URA3, AMP*	pRP22; Caponigro et al. 1993
pWO12	pGEX-PUF3RD	*AMP*	Jackson et al. 2004
pWO20	pGEX-PUF5RD	*AMP*	Jackson et al, 2004
pWO21	pBS-PUF2RD	*AMP*	John J. Jackson
pWO22	pGEX-PUF2RD	*AMP*	John J. Jackson
pWO24	GAL-MFA2pG	*URA3, AMP*	pRP485; Decker & Parker, 1993
pWO27	GAL- MFA2/HXK1 3'UTR	*URA3, AMP*	Randi Ulbricht
pWO48	pBS-PUF1RD	*AMP*	Randi Ulbricht
pWO49	pGEX-PUF1RD	*AMP*	Randi Ulbricht
pWO53	GAL -MFA2/*tif1*^{1} 3'UTR	*URA3, AMP*	Randi Ulbricht
pWO54	GAL -MFA2/*tif1*^{1} 3'UTR	*LEU2, AMP*	Randi Ulbricht
pWO55	GAL-MFA2/YHB1 3'UTR	*URA3, AMP*	Randi Ulbricht
pWO56	GAL-MFA2/YHB1 3'UTR	*LEU2, AMP*	Randi Ulbricht
pWO57	GAL- MFA2/HXK1 3'UTR	*LEU2, AMP*	Randi Ulbricht
pWO58	LEU, CEN Vector	*LEU2, AMP*	pRS415; Brachmann et al. 1998
pWO61	GAL-MFA2pG	*LEU2, AMP*	Randi Ulbricht
pWO70	GAL -MFA2/TIF1 3'UTR (WT)	*URA3, AMP*	Randi Ulbricht
pWO71	GAL -MFA2/TIF1 3'UTR (WT)	*LEU2, AMP*	Randi Ulbricht
pWO72	GAL -MFA2/*tif1*^{2x} 3'UTR	*URA3, AMP*	Randi Ulbricht
pWO73	GAL -MFA2/*tif1*^{2x} 3'UTR	*LEU2, AMP*	Randi Ulbricht

Table 4.3. Oligonucleotides Used in This Study

OLIGO	DESCRIPTION	SEQUENCE
oWO9	T7 Promoter	taatacgactcactatag
oWO10	*COX17* Binding Region #1	ctattcttatatatacaagaaatggttgtccctatagtgagtcgtatta
oWO11	*COX17* Mutant	ctattcttatatagtgtagaaatggttgtccctatagtgagtcgtatta
oWO138	*HXK1* Binding Region #1	atttatatttcattacatttttttcattaactatagtgagtcgtatta
oWO139	*HXK1* Binding Region #2	gtgtgtctatatttacatatactagaccgcctatagtgagtcgtatta
oWO209	*YHB1* Binding Region	atcttataaatacatacacaatctttactatagtgagtcgtatta
oWO210	*TIF1* Binding Region #1	aaaacaaattaaatacaaaaagcatatatctatagtgagtcgtatta
oWO211	*TIF1* Binding Region #2	acgttcggatatatacaaaaagacaaagcctatagtgagtcgtatta
oWO222	*HXK1* Mutant #2	gtgtgtctatattgtgttatactagaccgcctatagtgagtcgtatta
oWO266	*HXK1* Binding Region #3	ttagctaggattatacacataaatatatactatagtgagtcgtatta
oWO268	*YHB1* Mutant A	atcttataaagtgttacacaatctttactatagtgagtcgtatta
oWO269	*YHB1* Mutant B	atcttataaatacagtgtcaatctttactatagtgagtcgtatta
oWO272	*YHB1* Double Mutant	atcttataaagtgtgtgtcaatctttactatagtgagtcgtatta
oWO270	*TIF1* Mutant #1	aaaacaaattaaagtgtaaaagcatatatctatagtgagtcgtatta
oWO280	*TIF1* Mutant #2	acgttcggatatagtgtaaaagacaaagcctatagtgagtcgtatta
oWO21	7S Probe	gtctagccgcgaggaagg
oWO105	*HXK1* probe	cataagggcatcactcataag
oWO125	*HXK1* cutter	gccaatgataccaagagacttac
oWO136	*PUF2RD* Up Primer	cgcggatcccctccaccatcattatcggatagt
oWO137	*PUF3RD* Down Primer	tctgcccgggaaacagaaacgcctctggc
oWO144	*PUF1RD* Up Primer	cccggatccgaattcgcaaattccgatgaataccaaatcaattcg

oWO145	*PUF1RD* Down Primer	cccccgccggcgcagctgcgaaatgctgctgttatgatgctgc
oWO153	*HXK1* 3'UTR Down primer	ccgaagcttccgagctatcctacgactttc
oWO159	*YHB1* Probe	cgcctaaacttgcacggttgac
oWO160	*HXK1* 3'UTR UP Primer	gccagatctcttggtatcattggcgcttaatg
oWO176	*YHB1* Cutter	gatgaccaatcatagcttgc
oWO182	*TIF1* Probe	gtagcgatgtcggatggc
oWO183	*TIF1* Cutter	catagcgccaacgtcttcg
oWO231	*TIF1 3'UTR* Down Primer	ccgaagcttctctatacaaggcagaggg
oWO238	*MFA2* Probe	atattgattagatcaggaattcc
oWO239	*TIF1* 3'UTR Up Primer	ccgaagcttctctatacaaggcagaggg
oWO249	*TIF1* 3'UTR Probe	caaccttcgtgccgagagtc
oWO262	*YHB1* 3'UTR Up Primer	ggcagatctgtcaaccgtgcaagtttag
oWO263	*YHB1* 3'UTR Down Primer	ccgaagcttgcttccatgacaggttccg
oWO265	*YHB1* 3'UTR Probe	ctggcgttaacgtgaagtg
oWO310	*TIF1* SDM Primer #1	ggttgaaataccctatactaattgtttgctttctcttttacactatatccg aacgtatctatctgaaatttttc
oWO311	*TIF1* SDM Primer #2	gaaaaatttcagatagatacgttcggatatagtgtaaaagacaaag caaacaattagtatagggtatttcaacc

Yeast Strains

The genotypes of the *S. cerevisiae* strains used are listed in Table 4.1.

The *S. cerevisiae* strains yWO102, yWO104 and yWO105 were obtained by mating. The parent haploid strains were crossed and the diploids sporulated. The resulting tetrads were dissected and each spore was genotyped. yWO102 was obtained by crossing yWO7 and yWO20. yWO3 and yWO7 were crossed to make yWO104, which was crossed to yWO22 to obtain yWO105.

Radioactive Labeling of Probes

All Northern probes were 5' end-labeled using γ ^{32}P-ATP and T4 Polynucleotide Kinase (NEB). 200ng of each indicated DNA oligonucleotide were radiolabeled according to manufacturer's recommendations.

Northern Blot Preparation

Total RNA was isolated from yeast as previously described (Caponigro et al. 1993). 40μg of RNA was separated on a 1% formaldehyde-agarose gel and blotted to NytranSupercharge membrane (Schleicher and Schuell). All Northern blots were probed using γ ^{32}P end-labeled oligonucleotides. Corrections for loading were made by stripping blots and re-probing for 7S RNA, a constitutively expressed RNA Polymerase III transcript (Felici et. al.,1989). All quantification of RNA was accomplished using ImageQuant software (Molecular Dynamics).

In Vivo Steady-State mRNA Levels

Yeast strains yWO5 (wild-type), yWO14 (*puf2*Δ), yWO17 (*puf5*Δ), yWO18 (*puf3*Δ), yWO20 (*puf1*Δ) and yWO22 (*puf4*Δ) were grown in synthetic media with 2% dextrose to OD_{600} of 0.4 or 1.0 and harvested. RNA was isolated from frozen cell pellets as described. Northern blots were probed with the following end-labeled oligonucleotides; oWO105 (*HXK1)*, oWO159 (*YHB1)*, oWO182 (*TIF1)* and oWO21 (*7S)*.

Site-Directed Mutagenesis

In vitro site-directed mutagenesis was performed to mutate *TIF1* 3'UTR binding region #2 (UGUA to ACAC) with the QuickChange XL Site-directed Mutagenesis Kit (Stratagene). The primers oWO310 and oWO311 were used in this PCR based mutagenesis reaction of pWO53 as recommended by the manufacturer (Stratagene). Resulting mutants (pWO72, *MFA2/tif1 3'UTR* mutant #2) were confirmed by sequencing.

In Vivo Decay Analysis

Steady state transcriptional shut-off experiments were performed essentially as described (Caponigro et al. 1993). Decay of steady-state mRNA was monitored in strains containing the temperature sensitive *rpb1-1* RNA Polymerase II allele, in which transcription is rapidly repressed following a shift from 24°C to 37°C. All yeast transformations were accomplished by LiAc high efficiency transformation (Gietz and Schiestl, 1996).

Transcriptional shut-off experiments of the *MFA2/HXK1 3'UTR* transcript were performed in yeast strains transformed with pWO27. This plasmid will express a fusion RNA containing the coding region of *MFA2* and the 3'UTR of *HXK1*. It is derived from pWO24, where the transcription of *MFA2* is under the control of the *GAL UAS* (upstream activating sequence). The *MFA2* 3'UTR was replaced by the 3'UTR of *HXK1* by inserting the PCR amplified 3'UTR of *HXK1* (531 nucleotides*)* into *BamH*I and *Hind*III sites of pWO24. *HXK1 3'UTR* was amplified from genomic DNA with the primers oWO160 and oWO153. pWO27 was transformed into yWO7 (WT), yWO43 (*puf3*Δ), yWO102 (*puf1*Δ), and yWO105 (*puf4*Δ).

Transcriptional shut-offs of the *MFA2/ TIF1 3'UTR* were performed in yeast strains containing pWO70 or pWO71. These plasmids will express a fusion RNA containing the *MFA2* coding region and *TIF1 3'UTR* with transcription regulated by the *GAL UAS*. pWO70 was made by PCR amplification of the *TIF1 3'UTR* from genomic DNA with the primers oWO231 and oWO239. The PCR product was ligated into pWO24 between *Bgl*II and *Hind*III sites, replacing the 3'UTR of *MFA2* with that of *TIF1*. Similarly, the *Bgl*II/*Hind*III fragment was ligated into pWO54 (see below) to make pWO71. pWO70 (*URA* marker) was transformed into yWO7 and yWO43, while pWO71 (*LEU* marker) was transformed into yWO48 (*puf2*Δ) and yWO49 (*puf5*Δ).

Transcriptional shut-off assays of the *MFA2/tif1 3'UTR* mutants were performed similar to that of *MFA2/TIF1 3'UTR*. Creation of the *MFA2/tif1*^{1} 3'UTR mutant (pWO53) occurred via a spontaneous error in the PCR amplification of the *TIF1* 3'UTR as described above. Sequencing this PCR product ligated into pWO24 revealed a U to C mutation 84 nucleotides down-stream from the stop codon. To make pWO54, pWO53

was cut with *Pvu*II, and the fragment containing *GAL-MFA2/tif1*^{1} *3'UTR* was ligated into pWO58, which contains the *LEU2* marker. The *MFA2/tif1*^{2x} expression plasmid pWO72 was made by site-directed mutagenesis (described previously) of pWO53. pWO72 was then transformed into yWO7.

MFA2/YHB1 3'UTR transcriptional shutoffs were performed in yeast strains containing the plasmids pWO55 or pWO56. The 3'UTR of *YHB1* was amplified from genomic DNA using the primers oWO262 and oWO263. The PCR product was inserted between the *Bgl*II and *Hind*III sites of pWO24 to yield pWO55. To make pWO56, pWO55 was digested with *Pvu*II and the *GAL-MFA2/YHB1 3'UTR* fragment was inserted into pWO58. pWO55 (*URA* marker) was transformed into yWO7. pWO56 (*LEU* marker) was transformed into yWO48 and yWO49.

Control shut-off experiments of the native *MFA2* mRNA were performed using either pWO24 or pWO61. pWO61 was created by digesting pWO24 with *Pvu*II and ligating the product containing *GAL- MFA2* into pWO58. pWO61 was transformed into yWO48 and yWO49, while pWO24 was transformed into yWO7.

Transcriptional shut-off experiments were performed by growing 200ml yeast cultures containing the appropriate expression plasmid in synthetic media with 2% galactose at 24°C to an OD_{600} of 1.0 (entering stationary phase) or an OD_{600} of 0.4 (mid-log phase). Shut-offs of *MFA2/ YHB1 3'UTR*, *MFA2/TIF1 3'UTR*, *MFA2/tif1 3'UTR* mutants and *MFA2* control transcripts were all performed on yeast entering stationary phase. 100ml of each culture was harvested by centrifugation and cells were resuspended in 20ml of 37C media containing 4% dextrose, then 2ml aliquots of the culture were harvested at various time points following temperature shut-off. Mid-log phase shut-offs

(*MFA2/HXK1 3'UTR*) were performed as decribed above, but with harvesting the entire 200ml of the yeast culture. Northern blots of RNA prepared from each time point were probed with the following ^{32}P end-labeled oligonucleotides complementary to 3'UTR sequences: oWO238 (*MFA2*), oWO249 (*TIF1, tif1*), oWO265 (*YHB1*) and oWO105 (*HXK1*).

Steady-state transcriptional shut-off experiments were also performed to monitor decay of endogenous *YHB1* using the following modifications. yWO7, yWO43, yWO48 and yWO49 yeast strains were grown to an OD_{600} of 1.0 in 200ml rich media cultures with 2% Dextrose. 100ml of each culture was harvested and cells resuspended in the same media at 37C for shut-off. Northern blots were probed with P^{32} end-labeled oWO159.

Protein Purification

The *GST-PUF1RD* fusion construct was created by PCR-amplification of an 1140 nucleotide region of genomic *PUF1* (amino acids 551-934) using the primers oWO144 and oWO145. The PCR product was inserted into pBluescript (Stratagene) between *Bam*HI and *Not*1 to yield pWO48. pWO48 was digested with *BamH*I and *Pvu*II then cloned into pGEX-6P-3 (Amersham Biosciences) between *BamH*I and *Sma*I to create pWO49, the GST-Puf1pRD expression vector. To create the *GST-PUF2RD* fusion construct, nucleotides 1453-2712 were amplified from genomic *PUF2* (encoding amino acids 485-904) with the primers oWO136 and oWO137. This product was inserted into pBluescript between the *BamH*I and *Xma*I sites, creating pWO21. The *BamH*I-*Xma*I digestion product of pWO21 was then ligated into pGEX-6P-3 to yield the GST-Puf2pRD expression vector pWO22. Each construct was verified by sequencing. The

GST fusion constructs were transformed into BL-21 protease deficient *E. coli* and were purified as recommended by Amersham Biosciences with modifications as described in Jackson et al. Eluates were dialyzed in 50mM Tris-HCL pH 8.0 and verified by western analysis with anti-GST antibodies.

In Vitro Binding Assays

Short RNAs containing potential 3'UTR Puf-binding sites were transcribed from single stranded oligonucleotide templates that contained the T7 RNA polymerase promoter (oWO10-11, oWO138-139, oWO209-211, oWO222, oWO266, oWO268-270, oWO272, oWO280; see Table 4.3). The T7 RNA polymerase primer (oWO9) was annealed to its promoter. Transcription was performed using the T7-MEGAshortscript kit (Ambion) as described in Jackson et al. in the presence or absence of α^{32}P-UTP. Each reaction was treated with DNaseI. Short radiolabeled transcripts were separated on a 10% denaturing polyacrylamide gel, eluted from gel slice and ethanol precipitated. Non-radiolabeled transcripts were purified using a Nucleotide Removal Kit (Qiagen).

The short radiolabeled target transcripts were incubated with 1X binding buffer (10mM Hepes at pH7.5, 50mMKCl, 1mM EDTA, 2mM DTT, 200u/ml RNasin, 0.1mg/ml bovine serum albumin, 0.01% Tween, 0.1mg/ml poly(rU), 10µg/ml yeast tRNA), in the presence or absence of GST-Puf2pRD (0.25µM), GST-Puf5pRD (0.2µM), GST-Puf1pRD (0.1µM), GST-Puf3pRD (1.0µM) for 30 minutes at room temperature. Each reaction was treated with 5µg heparin for an additional 10 minutes at room temperature. Products were separated on 8% native polyacrylamide gel at 4ºC.

For the competition assays, a 10-fold excess of unlabeled short competitor transcript was added to the binding reaction above prior to protein incubation. The *COX17* mutant binding site with a UGUA to ACAC mutation (oWO11) was used as non-specific competitor.

Poly (A) Tail Analysis

Steady-state RNA was harvested from yeast strains yWO5, yWO14, yWO17, yWO18, yWO20 and yWO22 grown in synthetic media with 2% dextrose to OD_{600} 0.4 or 1.0. RNA was isolated as described above. RNaseH reactions were performed as described (Muhlrad and Parker, 1992). In brief, oWO83, oWO176 and oWO125 were annealed to *TIF1, YHB1* and *HXK1* mRNAs respectively, then mRNAs were digested with RNaseH (Promega) as recommended. Following phenol extraction and ethanol precipitation, the products were separated on a 6% denaturing polyacrylamide gel. Each gel was electroblotted to Nylon memebrane. The resulting blot was probed with P^{32} end-labeled oWO182 (*TIF1*), oWO159 (*YHB1*), or oWO105 (*HXK1*).

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