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**THE FUNCTION OF PAB1 IN TRANSLATION
AND IN
PUF3 DEPENDENT DEADENYLATION**

BY

DARREN J. LEE

B.S., University of California, Davis 2001

Submitted to the University of New Hampshire

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Doctor of Philosophy

in

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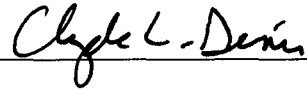
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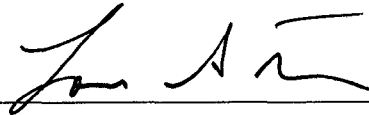
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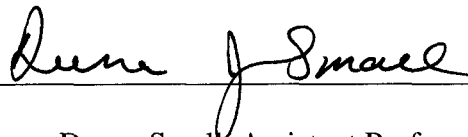
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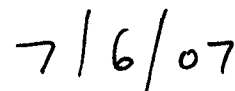
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Date

DEDICATION

I hereby dedicate this work to my loving family. My parents, Irving and Marian Lee have always supported me and stood by me throughout my entire educational career. My brother, Ryan Lee, I am very proud of for all of his academic accomplishments. Without the support of my family I would not have achieved the level of education that I have today.

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TABLE OF CONTENTS

DEDICATION.....	iii
ACKNOWLEDGEMENTS.....	iv
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
ABSTRACT.....	x

CHAPTER	PAGE
GENERAL INTRODUCTION.....	1
I: MEASUREMENT OF <i>IN VIVO</i> TRANSLATION RATES IN STRAINS CONTAINING PAB1 VARIANTS, DEFECTS IN mRNA DEGRADATION COMPONENTS, AND DEFECTS IN TRANSLATION COMPONENTS.....	21
Introduction.....	21
Materials and Methods.....	26
Results.....	30
Discussion.....	48
II: <i>COX17</i> DEADENYLATION RATES IN PAB1 VARIANTS.....	53
Introduction.....	53
Materials and Methods.....	57
Results.....	60
Discussion.....	84
GENERAL DISCUSSION.....	86
REFERENCES.....	94

LIST OF TABLES

NUMBER	PAGE
1 – Yeast Strains Used.....	28
2 – Plasmids Used.....	29
3 – Absolute Values for Figure 4.....	32
4 – Absolute Values for Figure 5.....	34
5 – Absolute Values for Figure 6.....	40
6 – Absolute Values for Figure 7.....	43
7 – Absolute Values for Figure 8.....	47
8 – Sequence of Probes Used.....	57
9 – Summary of <i>COX17</i> Deadenylation Rates.....	82

LIST OF FIGURES

NUMBER	PAGE
1 – Pathways of mRNA Degradation.....	3
2A, 2B, 2C – Various Models of Deadenylation.....	8
3A, 3B, 3C, 3D – Translation Initiation.....	15
4 – <i>In Vivo</i> Translation Rates of Strains Containing Defects in Components Involved in mRNA Degradation or Translation.....	32
5 – <i>In Vivo</i> Translation Rates of Strains Containing PAB1 Variants.....	34
6 – <i>In Vivo</i> Translation Rates of Strains <i>prt1-63</i> and <i>PAB1</i> Variants.....	40
7 – <i>In Vivo</i> Translation Rates of Strains <i>cdc33-1</i> and <i>PAB1</i> Variants.....	43
8 – <i>In Vivo</i> Translation Rates of Strains Containing <i>ccr4Δ</i> or <i>caf1Δ</i> and PAB1 Variants.....	47
9A, 9B, 9C, 9D – Deadenylation Assay of <i>PAB1</i> in <i>PUF3</i> and <i>puf3Δ</i> Background.....	61
10A, 10B, 10C, 10D – Deadenylation Assay of <i>PAB1-ΔRRM1</i> in <i>PUF3</i> and <i>puf3Δ</i> Background.....	63
11 – Summary of <i>GAL1</i> Deadenylation Assay.....	64
12 – Summary of <i>MFA2</i> Deadenylation Assay	65
13A, 13B, 13C, 13D – Deadenylation Assay of <i>PAB1-ΔRRM2</i> in <i>PUF3</i> and <i>puf3Δ</i> Background.....	67
14A, 14B, 14C, 14D – Deadenylation Assay of <i>PAB1-ΔRRM3</i> in <i>PUF3</i> and <i>puf3Δ</i> Background.....	69

15A, 15B, 15C, 15D – Deadenylation Assay of <i>PAB1-ΔRRM4</i> in <i>PUF3</i> and <i>puf3Δ</i> Background.....	71
16A, 16B, 16C, 16D – Deadenylation Assay of <i>PAB1-ΔP</i> in <i>PUF3</i> and <i>puf3Δ</i> Background.....	73
17A, 17B, 17C, 17D – Deadenylation Assay of <i>PAB1-ΔC</i> in <i>PUF3</i> and <i>puf3Δ</i> Background.....	75
18A, 18B, 18C, 18D – Deadenylation Assay of <i>PAB1</i> in <i>PUF3</i> and <i>puf3Δ</i> Background at 37°C.....	80
19A, 19B, 19C, 19D – Deadenylation Assay of <i>cdc33-1</i> in <i>PUF3</i> and <i>puf3Δ</i> Background at 37°C.....	81
20A, 20B, 20C – Models of <i>PUF3</i> Mediated <i>PAB1</i> Self-Association.....	91

ABSTRACT

THE FUNCTION OF PAB1 IN TRANSLATION

AND IN

PUF3 DEPENDENT DEADENYLATION

by

Darren J. Lee

University of New Hampshire, December 2007

The involvement of the poly(A)-binding protein (PAB1) in deadenylation and translation is well known. How PAB1 inhibits deadenylation and promotes translation is not well understood. I have analyzed PAB1 variants, containing entire domain deletions and substitutions of yeast residues with human residues. Chapter I discusses and provides *in vivo* translation rates of strains containing PAB1 variants, defects in mRNA degradation proteins, and defects in translation components. In chapter II, I address the role of PAB1 in regulated deadenylation. For this analysis I studied the effect of PUF3, a member of the PUF family of proteins that bind specific 3' UTR sequences and accelerate deadenylation and/or repress translation of the target transcript.

The analysis of *in vivo* translation rates, showed that while PAB1 did not have a major role in translation, its RRM1 and RRM2 domains were the most important for translation. I found defects in translation initiation factors, eIF4E and eIF3, had different effects on translation in combination with two PAB1 variants in the RRM2 domain that affect different aspects of translation *in vitro*. CAF1 and CCR4, two components of the CCR4-NOT complex responsible for deadenylation, have recently been shown to have separate functions in deadenylation. It has been suggested CAF1 and CCR4 have roles in

translation. I show CAF1 and CCR4 do in fact have roles in translation, but as with deadenylation, those roles in translation are different.

The mechanism for PUF mediated deadenylation has recently been shown to involve recruitment of CCR4 via CAF1, suggesting PUF proteins accelerate deadenylation by increasing the local concentration of deadenylases around the mRNA. Since PUF proteins are involved in repression of translation it has been suggested that they also accelerate deadenylation through perturbation of the mRNP complex. In this work I show that PUF3 requires the PAB1 RRM1 domain for deadenylation of *COX17* mRNA. Additionally, I show that PUF3 bypasses the requirement of the PAB1 P domain for deadenylation, and is required for acceleration of deadenylation through defects in the cap binding protein, eIF4E. This suggests that PUF3 interacts with PAB1 to disturb the mRNP complex to accelerate deadenylation of *COX17* mRNA.

GENERAL INTRODUCTION

Proper and appropriate gene expression is essential to cellular processes ranging from following developmental cues to monitoring metabolic activity. Multiple levels of control are utilized to obtain proper gene expression. These controls include: promoter enhancers and suppressors to regulate transcriptional initiation, chromatin remodeling complexes which positively and negatively regulate transcriptional elongation, mRNA transcript stability controls the amount of protein that can be translated, various translation initiation complexes regulate the translational process, and events such as methylation and phosphorylation occur post-translationally to further regulation protein function. One area of particular interest is mRNA transcript stability, specifically factors contributing to differential transcript stability. Illustrating the wide-range of transcripts stability is the stable mRNA transcript, *PGK1*, which has a 33 minute half-life (Muhlrad et al., 1995), and the unstable mRNA transcript, *MFA2*, which has a 3.5 minute half-life (Muhlrad and Parker, 1992). Since the mRNA half-life is directly related to the amount of protein that is translated from the mRNA it is important to understand the factors contributing to how one mRNA has a longer half-life compared to another mRNA with a shorter half-life. Additionally, translationally competent mRNA is absolutely required for the translation of protein. It is therefore important to understand the factors that control translation and the factors involved in determining mRNA degradation. Furthermore, a link between mRNA degradation and translation initiation has been shown (Schwartz and Parker, 1999), which indicates the need to understand the

mechanisms behind both processes. In this dissertation I have focused on two areas: how the PUF proteins act to deadenylate *COX17* mRNA, and how PAB1 and components involved in both mRNA degradation and translation affect translation.

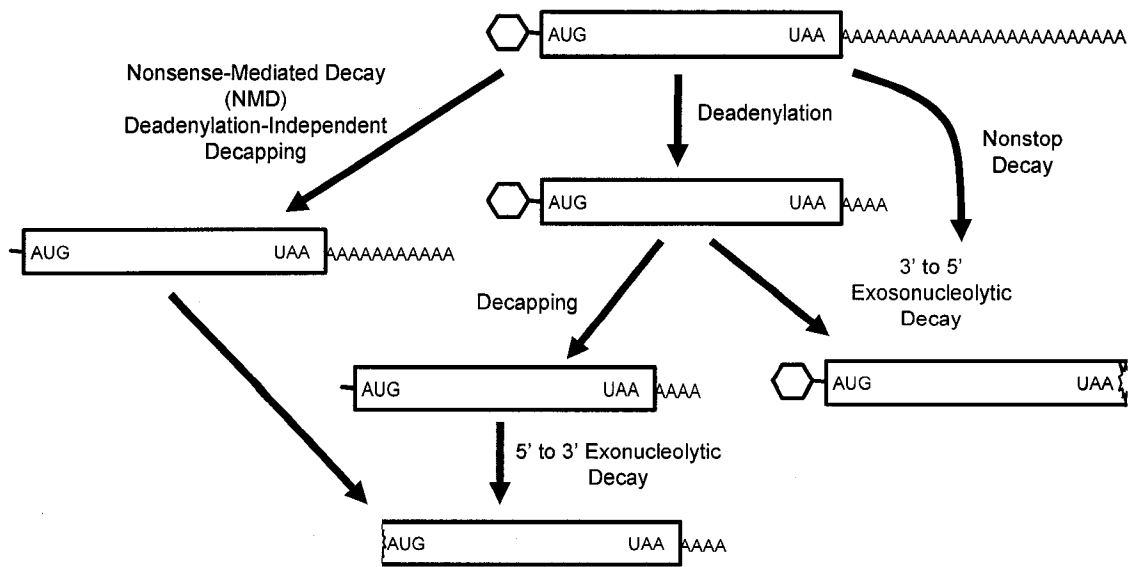


Figure 1 – Pathways of eukaryotic mRNA degradation. From Collier and Parker 2004. Several pathways of mRNA degradation are known. The major degradation pathway requires deadenylation of the poly(A) tail, however, other deadenylation independent pathways exist.

mRNA Degradation

The pathways of mRNA degradation can be grouped into two types, deadenylation dependent and deadenylation independent (Figure 1). The deadenylation dependent pathway is the major mRNA degradation pathway. Degradation of the mRNA body occurs following exonucleolytic digestion of the adenines (deadenylation) at the 3' end of the mRNA and decapping of the 5' 7-meG cap. In yeast, the poly(A) tail is initially trimmed by the PAN2/PAN3 complex from 100-110 A's to 70-90 adenines (A's) (Tucker et al., 2002). The remaining poly(A) tail is digested to approximately 8-12 A's, by the catalytic component of the CCR4-NOT complex, CCR4 (Chen et al., 2002). Next, the DCP1/2 complex removes the 5' cap with the assistance of additional proteins, such as DHH1, EDC1, EDC2, LSM1-7, and PAT1 (Dunckley and Parker, 1999; Bouveret et al., 2000; Schwartz et al., 2003; Bonnerot et al., 2000; Hilleren and Parker, 1999; He and Parker, 1999; Hatfield et al., 1996). Following deadenylation and decapping, XRN1 then digests the mRNA in the 5' to 3' direction (Dahanukar et al., 1999). Also, a multi-component complex, called the exosome, can digest the mRNA in the 3' to 5' direction following deadenylation (Anderson and Parker, 1998; Mitchell et al., 1997). The exosome complex can also digest mRNA, through the nonstop decay pathway, as a result of a transcriptional error in which a stop codon has been skipped (Frischmeyer et al., 2002; Beelman and Parker, 1995). Deadenylation independent mRNA degradation can occur through two mechanisms. First, adenylated mRNA can be digested endonucleolytically (Binder et al., 1994). Second, a nonsense-mediated decay (NMD) pathway degrades mRNA containing a premature stop codon (Muhlrad et al., 1994; Cao and Parker, 2001). NMD is thought to have evolved as a surveillance mechanism that

quickly degrades aberrant mRNA containing premature stop codons in order to reduce the number of truncated transcripts (Cali and Anderson, 1998). The first step of NMD is decapping, followed by XRN1 mediated 5' to 3' exonucleolytic decay (Muhlrad and Parker, 1994).

Several observations suggest that deadenylation is the rate limiting step of mRNA degradation. First, sequences promoting rapid degradation also promote increased deadenylation (Decker and Parker, 1993; LaGrandeur and Parker, 1999). Second, stable transcripts have a much slower deadenylation rate compared to transcripts that degrade rapidly (Decker and Parker, 1993; LaGrandeur and Parker, 1999). Lastly, decapping does not occur until the poly(A) tail is approximately 8-12 A's, which is the minimal length of poly(A) that PAB1 can bind (Decker and Parker, 1993; Sachs et al., 1987). Since deadenylation is the initial and rate limiting step in mRNA degradation, understanding how deadenylation occurs will provide valuable insight into how mRNA degradation occurs. CCR4 is of particular interest because it is responsible for the majority of deadenylation (Tucker et al., 2001). Further characterization of CCR4 will provide valuable information about mRNA degradation. How CCR4 determines which mRNA is to be deadenylated more quickly than others is an important area of study.

CCR4

The *CCR4* gene was initially identified by Clyde L. Denis in 1984 as a block to derepression of the *ADH2* gene (Denis, 1984). Subsequent studies revealed that the CCR4 protein contains an acidic activation domain at the amino terminus, a leucine rich repeat region, and a deadenylase domain (Draper et al., 1994; Malvar et al., 1992). Two activation domains present in the N-terminal region were shown to be able to activate

LacZ expression when expressed from a GAL promoter, this shows that CCR4 has some role in transcription initiation (Draper et al., 1994). However, deletion of upstream *ADH2* activation sequences did not affect CCR4 regulation of *ADH2* expression, indicating that CCR4 is involved in regulating *ADH2* expression through mechanisms other than simple activation (Denis and Malvar, 1990). The leucine rich repeat region was shown to be important in blocking the derepression of *ADH2* (Malvar et al., 1992). However, since CCR4 is part of a multi-protein complex called the CCR4-NOT complex and the LRR was shown to be important in contacting additional proteins in the CCR4-NOT complex (Clark et al., 2004; Draper et al., 1994; Ohn et al., 2007) the function of the LRR in *ADH2* expression may be to simply contact the remainder of the complex. Initially, the major role of CCR4 was thought to be in transcription initiation, whereby the CCR4 activation domain was thought to play a part in transcription initiation and the leucine rich repeat region was utilized to contact additional proteins involved in proper promoter placement (Draper et al., 1994). Until recently, the C-terminal exonuclease domain was largely unexplored, but in 2001 CCR4 was discovered to be responsible for the majority of mRNA deadenylation in the cell (Chen et al., 2002; Tucker et al., 2001).

Additional components of the CCR4-NOT complex are CAF1, NOT1-5, CAF40, and CAF130 (Chen et al., 2001). NOT1 serves as a scaffold for the CCR4-NOT complex; CAF1, CAF40, CAF130 and the remaining NOTs all bind to NOT1 (Chen et al., 2001). CAF1 acts as a bridge between CCR4 isolating it from the rest of the complex (Bai et al., 1999). The NOTs were identified in a screen for genes capable of bypassing a delta insertion in the *HIS3* promoter, which indicates that they have a role in

transcriptional initiation (Collart and Struhl, 1994; Collart and Struhl, 1993; Viswanathan et al., 2004). NOT5 and NOT3 contact TFIID (Collart and Struhl, 1994; Lemaire and Collart, 2000) and NOT4 appears to have ubiquitin ligase activity (Irie et al., 1994; Albert et al., 2002) and a putative RNA binding motif. Genetic evidence also suggests the involvement of the CCR4-NOT complex in transcriptional elongation (Denis et al., 2001). Currently, CAF40 and CAF130 remain largely uncharacterized. What is also not well understood is how the CCR4-NOT complex components work together to affect deadenylation and how the rates of mRNA deadenylation are regulated. In particular, the mechanism whereby CCR4 targets specific mRNA for faster or slower deadenylation remains to be elucidated.

It has been shown that certain mRNA binding proteins, such as the PUF family of proteins, act on specific target mRNA to suppress translation and activate deadenylation (Crittenden et al., 2003; Olivas and Parker, 2000; Edwards et al., 2001; Wang et al., 2002; Wang et al., 2001; Crittenden et al., 2002). Furthermore, it has been shown that PUF3 and PUF5 interact with or require CCR4 to deadenylate mRNA (Olivas and Parker, 2000; Goldstrohm et al., 2007; Goldstrohm et al., 2006; Tucker et al., 2002; unpublished data). How CCR4 works in concert with the PUF proteins is a particular area of interest. CCR4 could work by a simple recruitment model, whereby RNA binding proteins, such as the PUFs, bind specific mRNAs to bring them in contact with the deadenylase (Figure 2A). Another model suggests that factors causing mRNP disruption or rearrangement could result in the stalling of translation initiation which could also allow degradatory proteins access to the mRNA (Figure 2B). These models have been hypothesized to explain how the PUF proteins work to suppress mRNA.

PUF Family of Proteins

The PUF protein family members all share a conserved RNA binding domain capable of recognizing a consensus repeat consisting of four core ribonucleotides, UGUR. The PUF name is derived from the two proteins first characterized; *Pumilio*, in *Drosophila melanogaster* and FBF, found in *Caenorhabditis elegans*. The PUF family of proteins consists of at least four subfamilies (Spasov and Jurecic, 2003). The following PUM family members share 49% sequence identity: *Pumilio* in *Drosophila*, Puf 8 and 9 in *C. elegans*, and PUM1 and PUM2 in mammals. The FBF subfamily shares ~20% sequence identity to the PUM family and its members consist of seven proteins found in *C. elegans* and the PUF5 protein found in *Saccharomyces cerevisiae*. Another subfamily sharing more similarity to PUM than FBF contains the PUF proteins found in plants and PUF3 and PUF4 from yeast. The last subfamily with very distant sequence similarity consists of the yeast proteins PUF1, PUF2, and PUF6.

Crystallography and mutational studies showed that the RNA binding domain of the Pum protein in *Drosophila* forms a crescent in which *hb* mRNA binds to conserved residues on the inner surface, whereas Nos and Brat bind to the outer surface along the eighth repeat (Edwards et al., 2001; Wang et al., 2001; Muhlrud and Parker, 1999). Strikingly, this C-terminal region has 78% and 79% sequence similarity to the mammalian PUM1 and PUM2 genes (Spasov and Jurecic, 2002). This observation suggests that the RNA binding region is the most important for function.

In the eukaryotes that PUF proteins have been identified, the biological roles are very diverse. These roles range from stem cell maintenance, the mating switch in yeast

and worm, embryological development, and a mitochondrial role. Clearly, PUF proteins have various biologically important roles which will be addressed further.

Pum1 and *Pum2* are highly expressed in mouse fetal and adult hematopoietic stem cells (Spasov and Jurecic, 2003) and *PUM2* is expressed in human germline cells (Nakahata et al., 2003). This is suggestive of a mammalian role for PUM in stem cells.

The FBF protein in *C. elegans* is responsible for translational repression of *gld-1* mRNA, which prevents germline cell differentiation allowing for stem cell proliferation (Crittenden et al., 2003; Crittenden et al., 2002). *FBF* is highly expressed in germline stem cells and *fbf-1/fbf-2* mutants do not have germline stem cells (Crittenden et al., 2002). Another mRNA that FBF acts on is *fem-3*, which is involved in the sperm/oocyte switch (Kraemer et al., 1999). The FBF protein in *C. elegans* works with NANOS-3 to accomplish translational repression of *fem-3* mRNA (Kraemer et al., 1999), and represses expression of *gld-1* mRNA (Crittenden et al., 2002).

Drosophila is another system that may have a *Pum* involvement in stem cells. *Pum* is highly expressed in germline stem cells and *pum* ^{-/-} stem cells undergo uncontrolled differentiation (Lin and Spradling, 1997). Pumilio (Pum) plays a role in establishing a *hunchback* (*hb*) protein gradient in the developing *Drosophila* embryo necessary for proper abdominal segmentation (Murata and Wharton, 1995; Wreden et al., 1997). A 32 ribonucleotide sequence called the *Nanos* recognition element (NRE) is recognized by Pum. Translational repression and deadenylation of *hb* occurs when Pum binds the NRE located in the 3' UTR. It is important to note that Pum cannot accomplish this suppression of *hb* without the proteins Nanos (Nos) and Brain Tumor (Brat) (Edwards et al., 2001; Wang et al., 2002; Wang et al., 2001; van Hoof et al., 2000).

Recognition of the *hb* NRE is accomplished independently of Nos and Brat and complementation of a *pum* mutant is possible by *Pum* repeats alone (Wharton et al., 1998).

In *Saccharomyces*, deadenylation of *COX17* mRNA is dependent on PUF3 and the PUF recognition sequence in the 3'UTR is essential for PUF3 mediated deadenylation (Olivas and Parker, 2000; Jackson et al., 2004). Mutation analysis of PUF3 has indicated that the outer surface is important for deadenylation of *COX17* mRNA (Houshmandi and Olivas, 2005) suggesting that contact with additional proteins is important for deadenylation. Another PUF protein in *Saccharomyces*, PUF5, is required for deadenylation of *HO* mRNA (Goldstrohm et al., 2007). The 3'UTR of *HO* has also been shown to confer translational suppression when fused to the *ADE* gene (Tadauchi et al., 2001). These results show that in yeast, both PUF3 and PUF5 suppress expression of their target mRNA transcripts through acceleration of the deadenylation rate, and PUF5 is capable of translation suppression of a chimeric mRNA. This indicates that PUF in yeast is a suitable model for analyzing PUF function and is likely to exploit similar mechanisms of action as those used in higher eukaryotes.

At the molecular level, PUF proteins are involved in proper protein expression at a post-transcriptional level. It has been shown that PUF proteins are capable of suppressing translation of specific mRNAs and promoting the deadenylation of target mRNAs. The precise mechanism explaining how deadenylation is stimulated by the PUF proteins remains to be discovered. Additionally, the mechanism whereby PUF proteins suppress translation remains to be determined. Two models describing how PUF proteins suppress expression are the recruitment model and mRNP disruption model.

As described in the recruitment and mRNP disruption models in the *CCR4* section above, PUF proteins could act by contacting specific mRNA and mRNA degradatory proteins thereby recruiting the degradatory proteins to specific mRNA or PUF could act by contacting components of the mRNP complex thereby causing them to dissociate from the mRNA allowing degradatory proteins access to the mRNA. A third model could be envisioned which combines the recruitment model and mRNP disruption model (Figure 1c). PUF proteins could cause mRNP destabilization or rearrangement and simultaneously bring mRNA degradation proteins to the mRNA. The mRNP disruption model does provide a mechanism for the translational suppression caused by PUF proteins. However, since translation initiation and mRNA deadenylation are linked (Schwartz and Parker, 1999), it is possible that both models are correct. In order to obtain more information for either model the proteins involved in translation need to be further characterized. Information about the translational involvement of proteins involved in mRNA degradation and components of the mRNP complex would shed more light on how translation occurs, and specifically, prove or disprove the mRNP disruption model.

CAF1

CCR4 associated factor (CAF1) was first identified in a screen for overproduction of the mammalian α -amylase driven by a *PGK* promoter (Sakai et al., 1992). This gene was termed *PGK promoter overproduction*, or *POP2*. *POP2* was the first term for this gene, but due to the association with *CCR4* I will refer to it as *CAF1*. Additional phenotypes of the *caf1* allele are: a block in glucose derepression, temperature sensitivity, sporulation defects, and reduced carbohydrate reserves.

In the CCR4-NOT complex, CAF1 is the primary contact between CCR4 and the NOT proteins (Bai et al., 1999). CAF1, being an RNase of the DEDD superfamily, has deadenylase activity (Daugeron et al., 2001) and is required for deadenylase activity *in vivo* (Tucker et al., 2001; Daugeron et al., 2001). However, mutations in the RNaseD domain of CAF1 did not affect *in vivo* deadenylation activity (Viswanathan et al., 2004), and a lesion in CCR4 caused a more severe block in deadenylation (Tucker et al., 2001). This also suggests another role for CAF1, as the CCR4 protein is responsible for the majority of cytoplasmic deadenylase activity (Tucker et al., 2002). Mutation of CAF1 residues important for CCR4 contact were shown to cause deadenylation defects, further indicating CAF1 has a separate role in deadenylation from CCR4 (Ohn et al., 2007). Clearly, the exact role of CAF1 in mRNA deadenylation is unknown and further study of this protein is necessary to determine its precise function.

A translational role for CAF1 has been suggested through two observations. First, a *caf1* deletion displayed a synthetic lethality with *PAB1-ΔRRM2* which can be rescued by over-expression of *STM1* and *DHH1* (Ohn et al., 2007). STM1 has a role in translation and makes contact with the ribosome (Van Dyke et al., 2006; Van Dyke et al., 2004) and DHH1 has role in both decapping and translation (Coller et al., 2001; Lodomery et al., 1997; Nakamura et al., 2001; Navarro et al., 2001). Second, over-expression of *CAF1* rescues the lethality observed in a *cdc33-1* strain, which has defects in the translation initiation factor eIF4E (Ohn et al., 2007). For the purpose of determining if CAF1 has a role in translation and what that role is, I have measured *in vivo* translation rates in a *caf1Δ* strain in combination with the *cdc33-1* allele and *PAB1-RRM2* variants, and over-expressed *CAF1* in a strain carrying the *cdc33-1* allele.

Translation

Translation describes a process resulting in the conversion of mRNA into protein. In all eukaryotes the 80S ribosome is responsible for translation. The 80S ribosome can be divided into the two core components, the 40S and 60S ribosomes. Ribosomes are composed of many proteins and several RNA. In yeast, all ribosomal proteins also have a homolog in mammalian systems (Mager et al., 1997), so translation in yeast is probably representative of translation in mammals. The yeast 60S ribosome is composed of 42 ribosomal proteins and three RNAs: 5S (121 nt), 5.8S (158 nt), and 25S (3392 nt). The smaller 40S ribosome consists of 32 proteins and a 18s RNA (1798 nt). By weight, the majority of the ribosome is RNA, compared to protein. In the 60S ribosome, the RNA to protein ratio is ~61% to 39%, and the 40S subunit has a 54% RNA to 46% protein ratio (Verschoor et al., 1998).

Translation begins with the formation of the 43S complex, which occurs when eIF2-GTP- Met-tRNA_i^{Met} binds the 40S ribosomal subunit. This process is facilitated by the translation initiation factors, eIF3, eIF1, and eIF1A (Figure 3A). Binding of the 43S complex with the mRNP complex through eIF4G occurs in mammals through eIF3, but in yeast it is thought that eIF2 bridges the eIF4G-eIF3 interaction. When the 43S complex binds the mRNP complex the pre-initiation complex is formed, also called the 48S complex (Figure 3B). Hydrolysis of eIF2-GTP to eIF2-GDP occurs when the 48S complex locates the AUG start codon. Dissociation of eIF3, eIF1, eIF1A, and eIF2 occurs following eIF2-GTP hydrolysis (Figure 3C). Following dissociation of eIF3, eIF1, eIF1A, and eIF2 the 60S ribosomal subunit binds the 40S ribosomal subunit forming the 80S ribosome (Figure 3D).

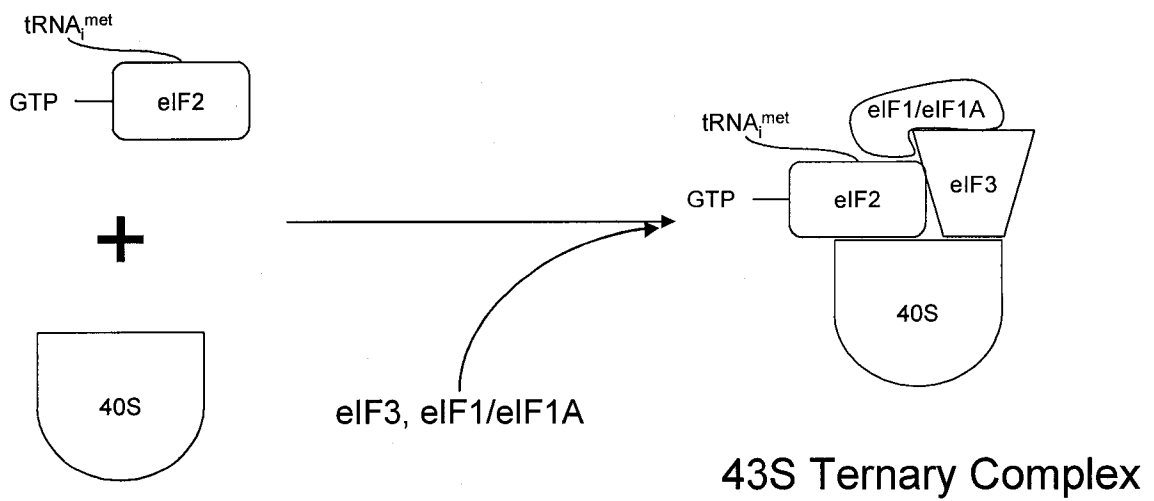


Figure 3A – Formation of the 43S ternary complex.

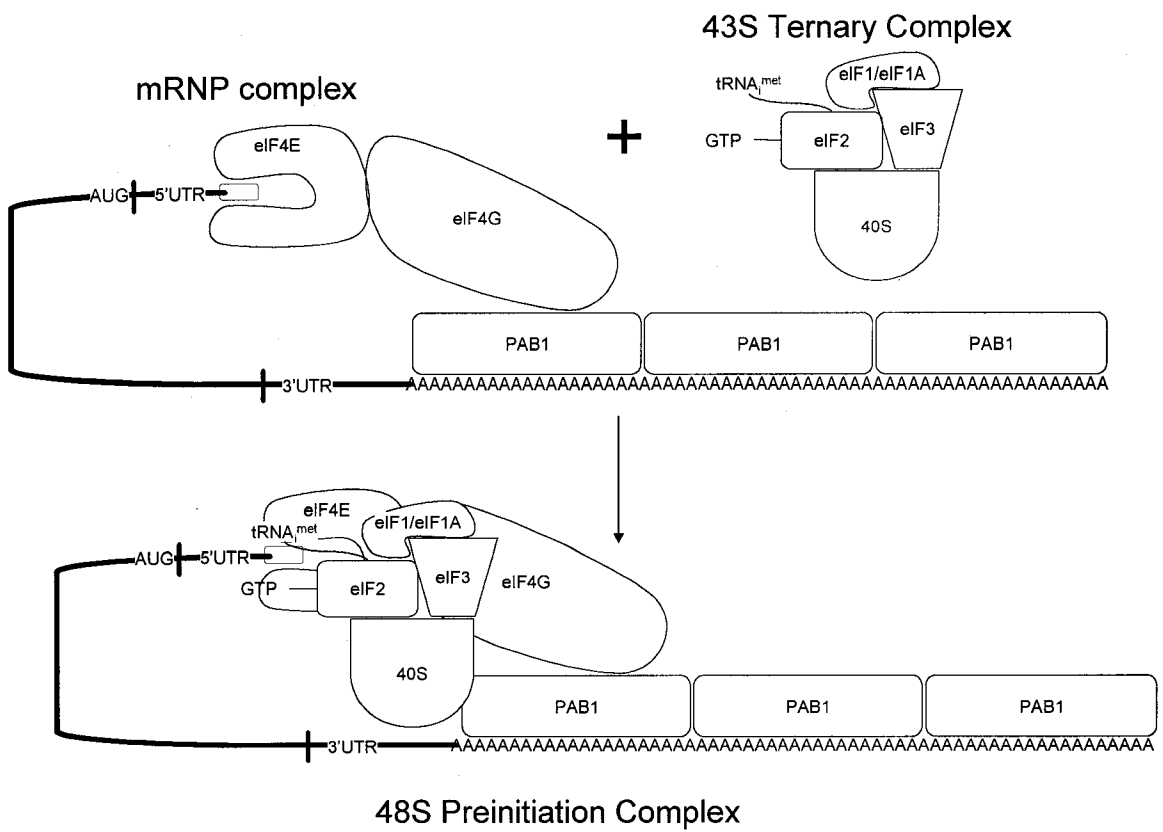


Figure 3B – Formation of the 48S preinitiation complex

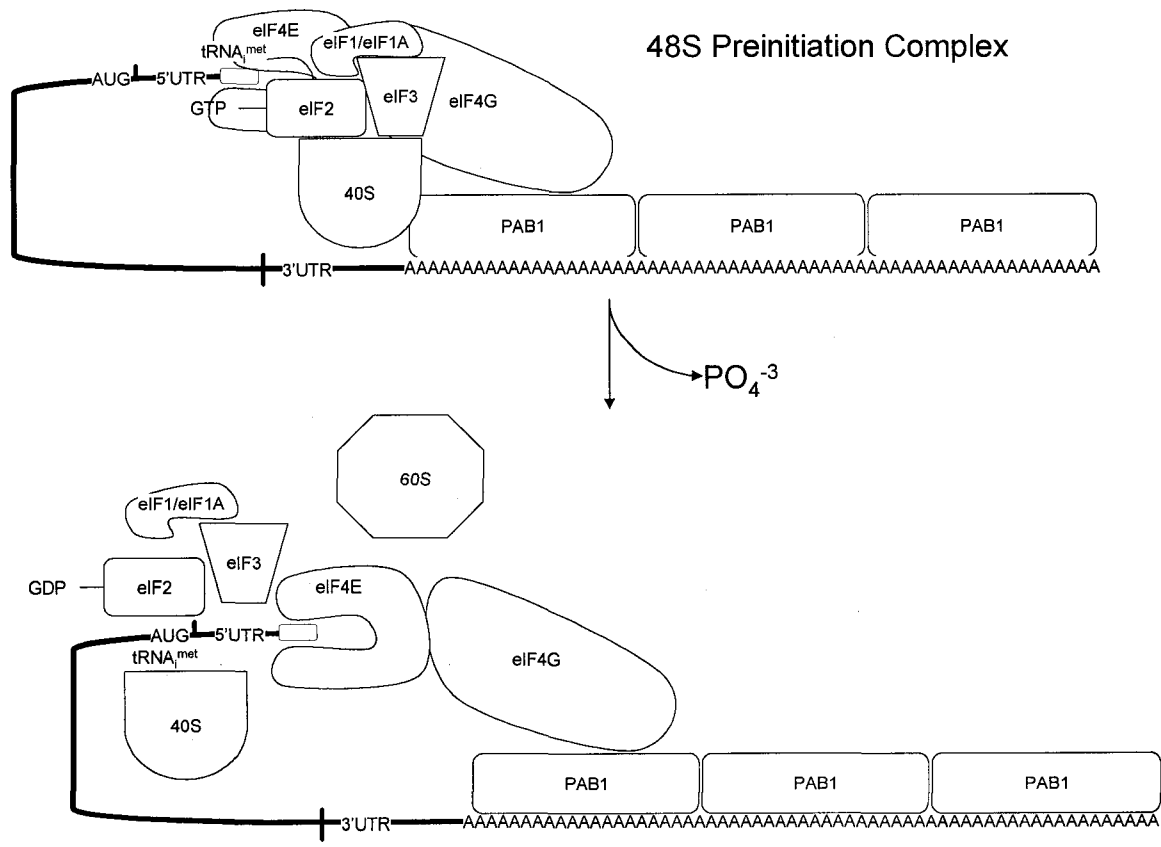


Figure 3C – Hydrolysis of eIF2-GTP to eIF2-GDP causes dissociation of eIF2, eIF1, eIF1A, and eIF3 from the ribosome.

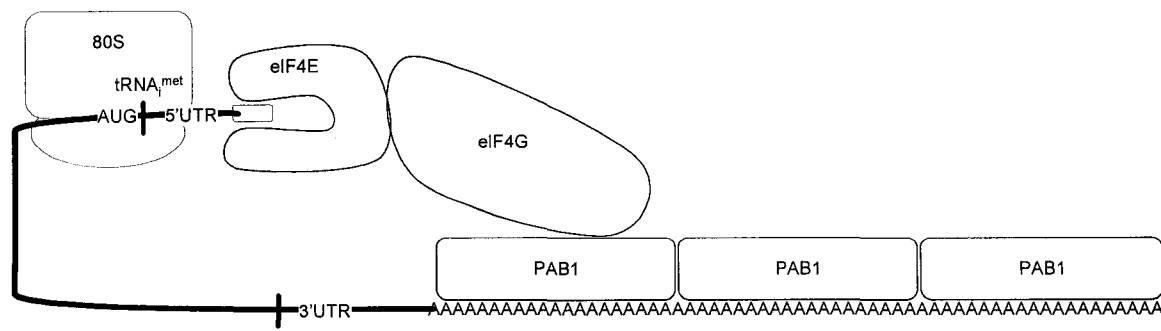


Figure 3D – Formation of the 80S ribosome.

Translational elongation of the polypeptide occurs through hydrolysis of GTP by eEF1A when an aminoacyl-tRNA of the correct anti codon is brought to the A site of the ribosome. Peptidyltransferase then catalyzes a peptide bond with the amino group of the peptide in the P site. This process continues until a stop codon is reached. A stop codon (UAA, UGA, or UAG) is recognized by eRF1 (Frolova et al., 1994), and peptidyltransferase catalyzes a bond to water instead of an aminoacyl-tRNA.

Dissociation of the complete polypeptide, 40S, and 60S ribosomal subunits from the mRNA occurs through the hydrolysis of GTP by eRF3 (Zhouravleva et al., 1995).

Deletion of the N terminal region of eRF3, which is the region responsible for contacting PAB1, blocks PAN2/3 deadenylation but also appears to affect CCR4 dependent deadenylation (Hosoda et al., 2003). Mutation of the GTP binding motif in the C-terminal region of eRF3 is required for association with eRF1 and increases mRNA degradation rates (Kobayashi et al., 2004). These last two observations link translational termination with mRNA deadenylation.

In mammals, regulatory proteins, called 4E-binding proteins (4E-BPs) bind to eIF4E. 4E-BPs blocks eIF4E contact to eIF4G which results in a block in translation. The only 4E-BP in yeast is p20 (CAF20) which has been shown to competitively bind to eIF4E and cause the similarly observed block in cap-dependent translation, as in mammalian cells (Ptushkina et al., 1998). Over-expressed CAF20 displayed synthetic lethality in backgrounds containing defect in *eif-4e*, *eif-4a*, *eif-4*, and *eif-4g* (de la Cruz et al., 1997), further indicating a CAF20 role in translation initiation. One model suggests that CAF20 inhibits translation by disrupting the mRNP complex. By over-expressing CAF20 we can determine how perturbations in the mRNP structure affect translation.

Another method to determine how the mRNP structure affects translation is by analyzing defects in components of the mRNP structure. One such mRNP component suitable for analysis is the poly(A)-binding protein, or PAB1.

PAB1

Initial identification of PAB1 in rabbit and mouse was through association with the polysome (Blobel, 1972). In subsequent studies upon isolating PABP or PAB1 (poly(A) binding protein), it was shown to bind to the 3' poly(A) tail of mRNA (Blobel, 1973). The PAB1 protein has four RNA Recognition Motifs (RRM1-4), an unstructured Proline and Methionine rich region (P), and a globular C-terminal region (C). Contact between eIF4G and PAB1 is made through RRM2, and the C terminal region makes multiple protein contacts with: PBP1, eRF3, and the deadenylase PAN2/3 ((Hosoda et al., 2003; Mangus et al., 2004; Mangus et al., 1998). A mature mRNA available for translation is bound by multiple proteins into a complex called the mRNP complex. The core components of the mRNP complex are eIF4E which contacts the 5' cap of the mRNA, PAB1 which binds the 3' poly (A) tail, and eIF4G, which contacts PAB1 and eIF4E causing the mRNA to form a loop (Wells et al., 1998; Jacobson and Peltz, 1996; von Der Haar et al., 2000).

Since PAB1 is bound to mature mRNA in the cytoplasm, it is highly suggestive that PAB1 plays a role in stability of the mRNA and/or translation of mRNA into protein. One class of mutations allowing for viability of a *pab1* deletion are mutations in mRNA degradation factors (Caponigro and Parker, 1995), suggesting a role for PAB1 in mRNA degradation. Additionally, *in vitro* evidence showed PAB1 to inhibit mRNA deadenylation (Tucker et al., 2002; Wilusz et al., 2001). However, *in vivo*, a *pab1*

deletion resulted in mRNA with long poly(A) tails, suggesting that PAB1 has multiple roles in deadenylation of mRNA (Caponigro and Parker, 1995). Since PAB1 has been shown to play a role in mRNA biogenesis (Dunn et al., 2005), proper export of mRNA from the nucleus (Brune et al., 2005), and adenylation of the 3' end of mRNA (Dheur et al., 2005), the inconsistency between the *in vivo* and *in vitro* observations could be due to epistatic effects the *pab1* deletion has on mRNA other than specific degradation of the mRNA transcript. Alternatively, it has been suggested, that in a *pab1* deletion, non-specific RNA binding proteins associated with the poly(A) tail inhibit deadenylation. The role of PAB1 in deadenylation is most likely an inhibitory one in terms of CCR4 action but it is also required for deadenylation.

Recent analysis of PAB1 has suggested a model whereby PAB1 self-association results in reduced poly(A) binding, thereby accelerating deadenylation (Yao et al., 2007). The model of PAB1 self-association to promote deadenylation is supported through three observations. First, the two PAB1 domain deletions resulting in the most severe defect in deadenylation are *PAB1- Δ RRM1* and *PAB1- Δ P* (Yao et al., 2007). Second, these PAB1 domains are also necessary for the self-association or circularization of PAB1. Third, the self-associated PAB1 binds poly(A) less efficiently compared to linear PAB1 (Yao et al., 2007). This model indicates that PAB1 has an inhibitory role in deadenylation, and when it dissociates from the poly(A), through self-association, deadenylation occurs. If PAB1 works through a general mechanism to inhibit all mRNA, then analysis of *PAB1- Δ RRM1* and *PAB1- Δ P* should show a defect in deadenylation for a PUF3 controlled mRNA such as *COX17*.

Another class of mutations allowing for the viability of a *pab1* deletion are defects in translational components, suggesting a PAB1 role in translation (Sachs and Davis, 1989). It has been shown that deletion of RRM2 and RRM4 in PAB1 cause decreased *in vitro* translation (Kessler and Sachs, 1998), furthermore substitution of PAB1 RRM2 yeast residues with human residues showed defects in *in vitro* translation (Otero et al., 1999). Tethering various PAB1 domains to a luciferase-MS2 fusion transcript has shown, *in vivo*, that specific regions of PAB1 are capable of stimulating translation (Gray et al., 2000). Clearly, further analysis of PAB1 is necessary to determine the exact role that it has on mRNA stability and translation.

One way to determine the function of PAB1 is to conduct a deletion analysis of the protein. In order to understand the effect of mRNA binding, point mutations abrogating the mRNA binding ability were created (Sachs and Deardorff, 1992). By changing the order of the domains we were also able to gain insight into any positional effects and possible linker interactions between the domains. Lastly, swaps between human PABP1 domains and yeast PAB1 domains have been constructed in order to determine which human residues have a different function in yeast. These PAB1 variants were analyzed to determine additional information about how PAB1 involvement in deadenylation and translation occurs.

CHAPTER I

MEASUREMENT OF *IN VIVO* TRANSLATION RATES IN STRAINS CONTAINING PAB1 VARIANTS, DEFECTS IN mRNA DEGRADATION COMPONENTS, AND DEFECTS IN TRANSLATION COMPONENTS

Introduction

Gene regulation at the level of translation initiation is a very complex process. By determining the exact mechanisms whereby translation occurs we hope to better understand how the process is regulated. As reviewed in Sonenberg, 2003; Sachs et al., 1997 (Sachs et al., 1997; Sonenberg and Dever, 2003), translation begins with recruitment of the eIF2-GTP- Met-tRNA_i^{Met} complex to the 40S ribosome, this is facilitated by eIF3, eIF1, and eIF1A, thus forming the 43S complex (Figure 3A). The 48S complex forms when the 43S complex binds eIF4F, mRNA, and PAB1 (Figure 3B). The 48S preinitiation complex then locates the AUG start codon which causes the hydrolysis of eIF2-GTP to eIF2-GDP (Figure 3C). Hydrolysis of eIF2-GTP to eIF2-GDP causes eIF2 to dissociate from the ribosome, followed by dissociation of the remaining eIFs (Figure 3C). After dissociation of all eIFs from the ribosome, binding of the 40S and 60S ribosomes occurs, resulting in a translationally competent 80S ribosome (Figure 3D).

It is known that the cap structure and the poly(A) structure have a synergistic relationship which allows for efficient translation (Otero et al., 1999). In order to

determine how the cap and poly(A) structure function in translation we must analyze the proteins associated with these structures. The most suitable candidates for analysis are the core components of the mRNP complex: eIF4E, eIF4G, PAB1 (Jacobson A. and Peltz S.W., 1996; von Der Haar T. et al., 2000). eIF4E and eIF4G both play a role in cap-dependent translation (Otero et al., 1999; Gross et al., 2003). However, eIF4G may present a greater challenge due to the fact that two genes encode it, making it more complicated for gene deletion analysis. As such, the temperature sensitive (ts) eIF4E protein which is encoded by the *cdc33-1* allele may be more useful in understanding cap-dependent translation. PAB1 is known to play a role in both cap-dependent and poly(A) dependent translation (Otero et al., 1999). In order to better understand cap-dependent and poly(A) dependent mechanisms we have to understand the role that PAB1 has in translation. PAB1 variants can be analyzed alone and in combination with the eIF4E defect for the purpose of gaining additional insight into the function of PAB1 in translation. By combining mutations in these two genes (eIF4E and PAB1) we provide a system in which cap-dependent and poly(A) dependent translation are both defective, thereby allowing us to better understand how these mechanisms operate to carry out the translational process.

In order to gain additional insight into the role of PAB1 on translation analysis of PAB1 defects with another translational defect other than eIF4E may be useful. A temperature sensitive (ts) allele of eIF3 (encoded by *prt1-63*) is a particularly useful strain because it represents a defect at a different site in translation than eIF4E. Additionally, this allele has been previously studied by Schwartz and Parker (Schwartz

and Parker, 1999): in the same procedure that I used to measure translation rates. The eIF3 complex is involved in the joining step between the mRNP complex, ribosome, and eIF2-GTP- Met-tRNA_i^{Met} (Jivotovskaya et al., 2006), which occurs before contact with eIF4G is made. Additional evidence suggesting that eIF3 and eIF4G have different roles in translation is supported by the observation that depleting eIF3 does not allow mRNA accumulation on ribosomes, whereas depletion of eIF4G results in mRNA bound to ribosomes but has defects in the 60S ribosomal subunit joining step (Jivotovskaya et al., 2006).

The PAB1 domains seem to have discrete functions in translation, as suggested by three observations. First, Sachs and colleagues has shown that RRM2 is involved in contacting eIF4G, (Otero et al., 1999) which is also a core component of the mRNP complex (Jacobson A. and Peltz S.W., 1996; von Der Haar T. et al., 2000; Wells et al., 1998). The fact that the PAB1 RRM2 domain contacts eIF4G and that PAB1 RRM1 is not directly involved in eIF4G contact while still having a role in translation suggests PAB1 RRM1 and PAB1 RRM2 each have different roles in translation. Second, each of the PAB1 RRMs have varying mRNA binding specificities and translational involvement (Burd et al., 1991), also indicating a modular nature to the PAB1 protein. Third, sequence similarity between domains RRM1-RRM4 within PAB1 (in yeast 25-43%) is much lower than across species (61% amongst the PAB1 RRM1 domain; 64% amongst the PAB1 RRM2 domain; 55 % for PAB1 RRM3 domain; 77% for PAB1 RRM4), suggesting discrete conserved functions among the individual domains. Since deletion of *PAB1* is lethal and the various domains seem to serve different functions from one another, analysis of the PAB1 protein containing a deletion of each domain is one way to

better understand the function of the individual PAB1 domains and hence how the PAB1 protein functions.

Another method to determine the function of PAB1 in translation would be to analyze the ability of each domain to stimulate translation, as performed by Wickens and colleagues (Gray et al., 2000). Determination of PAB1 domains capable of stimulating translational activity was done by injecting *Xenopus laevis* oocytes with a luciferase RNA reporter containing a MS2 binding site and a fusion protein containing a PAB1 variant fused to the MS2 protein (Gray et al., 2000). The MS2 protein recognizes and binds the MS2 RNA binding site in the luciferase RNA (Witherell et al., 1991), thus tethering the PAB1 variant to the luciferase RNA. This analysis determined which portions of PAB1 are capable of stimulating translation, thus implicating those portions of PAB1 that are involved in translation. If the identified portions of PAB1 are necessary for translation then deletion of those domains should show a decreased translational rate *in vivo* and *in vitro*, and PAB1 regions incapable of translation stimulation should not show any effect on the translation rate. *In vitro* analysis of PAB1 domain deletions has been conducted (Otero et al., 1999; Kessler and Sachs, 1998) and some results agree with the oocyte data, but some results do not. Another analysis of PAB1 would help clarify what exactly the role of PAB1 is with respect to translation. Using a set of PAB1 domain deletions I have measured the *in vivo* translation rates in order to gain additional insight into the role of each PAB1 domain.

Other factors may also affect translation, including the proteins involved in shortening the poly(A) tail, such as the CCR4-NOT deadenylase complex. Specifically, CAF1 may play a role in aiding translation and can be surmised through three

observations. First, lethality between a *PAB1-ΔRRM2* and *caf1Δ* has been observed (Ohn et al. 2007), indicating a genetic interaction between a core component of the mRNP complex, PAB1, and that of CAF1. Second, a screen for genes whose over expression was capable of bypassing the lethality of the *PAB1-ΔRRM2 caf1Δ* double mutant identified the following genes: *DHH1*, *STM1*, *SEC66*, *MSP1*, *OKP1*, and *POL4* (Ohn et al. 2007). Homologs of *DHH1* are involved in translational suppression (Ladomery et al., 1997; Nakamura et al., 2001; Navarro et al., 2001), and *STM1* contacts ribosomes and is involved in translation following nitrogen starvation (Van Dyke et al., 2006; Van Dyke et al., 2004). Because *DHH1* and *STM1* are capable of rescuing the *PAB1-ΔRRM2 caf1Δ* lethality and are involved in translation, this implies that a role for CAF1 in translation is possible. Third, the *cdc33-1* allele, encoding a defect in eIF4E is lethal at 37°C, can be rescued by over-expression of CAF1 (Ohn et al 2007).

In addition to CAF1, another member of the CCR4-NOT complex that may have a role in translation is CCR4. There are four observations that suggest CCR4 involvement in translation. First, a link between mRNA deadenylation and translation termination has been observed (Keeling et al., 2006). Second, the stress response slows deadenylation and suppresses translation, which suggests a link between link between deadenylation and translation (Hilgers et al., 2006). Third, deletion of the N terminal region of eRF3 blocks deadenylation and interferes with translation termination (Hosoda et al., 2003). Lastly, mutation of the GTP binding motif in the C-terminal region of eRF3 is required for association with eRF1 and increases mRNA degradation rates (Kobayashi et al., 2004). Since, the role of CAF1 seems to be more closely linked to CCR4 than with the rest of the CCR4-NOT complex, CAF1 may have multiple roles, as with CCR4,

one in deadenylation and another in translation. What is not well understood is how CAF1 operates in translation and in deadenylation.

In order to show more definitively that CAF1 and CCR4 are involved in translation and to help understand the role of each protein in translation, I have conducted *in vivo* translation assays in *caf1* Δ , *ccr4* Δ , alone and combined with *cdc33-1*, and also in a *cdc33-1* background with over-expressed CAF1 and CCR4. *In vivo* translation rates have also been measured in strains with defects in other mRNA degradatory proteins, such as DCP1, DHH1, and PAN3. In order to better understand the relationship between CAF1 and the RRM2 domain of PAB1, rates have been measured in *RRM2* mutants capable of rescuing the synthetic lethality combined with *caf1* Δ mutants. For the purpose of determining what the translational role of PAB1 is and how the interaction between PAB1 with additional factors affects translation, I measured the translation rates of the PAB1 variants combined with *prt1-63*, *cdc33-1*, *ccr4* Δ , and *caf1* Δ .

Materials and Methods

Yeast Strains and Plasmids

Yeast strains are shown in Table 1. Plasmids used are shown in Table 2. Yeast cells were inoculated into YEP medium (1% Bacto-yeast extract, 2% Bacto-peptone) containing 2% glucose, and grown to exponential phase.

³⁵S *In Vivo* Translation

In vivo translation was done in a similar fashion as previously described (Schwartz and Parker, 1999). Five mL yeast cells were grown to exponential phase in 2% glucose YEP medium, the optical density at 600 nm was measured, then cells were pelleted and resuspended in prewarmed 37°C met⁻ medium (0.67% yeast nitrogen base

without amino acids, and amino acids as required except methionine). Twenty five μCi [^{35}S] labeled methionine was then added to the resuspended cells in met⁻ medium. At 10 and 20 minute time points, cold 10% trichloroacetic acid (TCA) was added to the reaction, incubated for five minutes at 95 °C, and precipitated proteins were then collected on nitrocellulose membrane filters. Filters were then washed with 10 mL 5% TCA, and finally with 10 mL 70% ethanol. Following washing, filters were dried for 30 minutes, [^{35}S] incorporation into protein was then measured in a liquid scintillation counter. Using the Excel program, incorporated [^{35}S] per optical density was determined by dividing counts by the optical density for the strain. Rates were measured by dividing the [^{35}S] per optical density difference between time points by the difference between the time points. Standard error of the mean (SEM) was used to determine if the rates were significantly different. SEM was calculated by dividing the standard deviation by the square root of the total number of samples.

Table 1 Yeast Strains Used

Strain	Relevant Genotype	Source
AS319/pYC360	α <i>ade2 his3 leu2 trp1 ura3 pab1::HIS3</i> [PAB1-URA3]	Yao et al., 2007
AS319/pYC504	Isogenic to AS319/360 except [PAB1-TRP1]	Yao et al., 2007
AS1881/pYC504	Isogenic to AS319/360 except [PAB1-TRP1] <i>cdc33-1</i>	Ohn et al., 2007
1743-2/pYC504	75% Isogenic to AS319/360 except [PAB1-TRP1] <i>prt1-63</i>	C. Denis, UNH
1729-3/pYC504	75% Isogenic to AS319/360 except [PAB1-TRP1] <i>gst1-1</i>	C. Denis, UNH
AS319-1a-uN/pYC504	Isogenic to AS319/360 except [PAB1-TRP1] <i>ccr4::ura3::NEO</i>	Yao et al., 2007
AS319-c1-IN/pYC504	Isogenic to AS319/360 except [PAB1-TRP1] <i>caf1::ura3::LEU2</i>	Ohn et al., 2007
AS319-dhh1-uL/pYC504	Isogenic to AS319/360 except [PAB1-TRP1] <i>dhh1::ura3::LEU2</i>	Hata et al., 1998
DB267/pYC504	Isogenic to AS319/360 except [PAB1-TRP1] <i>pan3Δ</i>	Yao et al., 2007
1754-1-tN/pYC504	Isogenic to AS319/360 except [PAB1-TRP1] <i>pan2::trp1::NEO</i>	Yao et al., 2007

Strains were obtained from various sources as indicated by the reference. 1743-2 and 1729-3 were derived from crosses conducted by Clyde Denis.

Table 2 **Plasmids Used**

Plasmid	Relevant Genotype and Marker	Source
<i>pYC504</i>	<i>PAB1 [TRP1]</i>	Yao et al., 2007
<i>pYC460</i>	<i>PAB1-ΔPC [TRP1]</i>	Yao et al., 2007
<i>pYC505</i>	<i>PAB1-ΔRRM1 [TRP1]</i>	Yao et al., 2007
<i>pYC506</i>	<i>PAB1-ΔRRM2 [TRP1]</i>	Yao et al., 2007
<i>pYC507</i>	<i>PAB1-ΔRRM3 [TRP1]</i>	Yao et al., 2007
<i>pYC508</i>	<i>PAB1-ΔRRM4 [TRP1]</i>	Yao et al., 2007
<i>pYC509</i>	<i>PAB1-ΔC [TRP1]</i>	Yao et al., 2007
<i>pYC510</i>	<i>PAB1-ΔP [TRP1]</i>	Yao et al., 2007
<i>pYC513</i>	<i>PAB1-1212PC [TRP1]</i>	Yao et al., 2007
<i>pYC525</i>	<i>PAB1-Y170V [TRP1]</i>	Yao et al., 2007
<i>pYC536</i>	<i>PAB1-F83V [TRP1]</i>	Yao et al., 2007
<i>pYC537</i>	<i>PAB1-F83V Y170V [TRP1]</i>	Yao et al., 2007
<i>pYC538</i>	<i>PAB1-184 [TRP1]</i>	Yao et al., 2007
<i>pYC545</i>	<i>PAB1-134 [TRP1]</i>	Yao et al., 2007
<i>pYC551</i>	<i>PAB1-F366V [TRP1]</i>	Yao et al., 2007
<i>pYC552</i>	<i>PAB1-F263V [TRP1]</i>	Yao et al., 2007
<i>pYC562</i>	<i>PAB1-1214PC [TRP1]</i>	Yao et al., 2007
<i>pYC570</i>	<i>PAB1-hRRM3 [TRP1]</i>	Yao et al., 2007
<i>pYC571</i>	<i>PAB1-1224PC [TRP1]</i>	Yao et al., 2007
<i>pYC572</i>	<i>PAB1-1232PC [TRP1]</i>	Yao et al., 2007
<i>YE_p13</i>	<i>[LEU2]</i>	Hata et al., 1998
<i>YE_pMPT0</i>	<i>CAF1 [LEU2]</i>	Hata et al., 1998
<i>YE_pMPT2</i>	<i>CCR4 [LEU2]</i>	Hata et al., 1998
<i>pRP1007</i>	<i>GAL_p-COX17 [LEU2]</i>	Olivas and Parker, 2001

Plasmids designated *pYC* were constructed by Yueh-Chin Chiang and used for previous analysis in Yao et al., 2007. Other plasmids were obtained from the indicated source. All plasmids were transformed into the indicated strain by the LiOAc method (Ito et al., 1983).

Results

Effects of Defects in mRNA Degradative Proteins on *In Vivo* Translation

The determination of the accuracy of the *in vivo* translation assay that I performed was ascertained by comparison of the translation rate with previously determined rates, such as that determined for the *cdc33-1* allele measured by Schwartz and Parker (Schwartz and Parker, 1999). All reported translation rates represent the average of two or more samples compared to the PAB1 wild type strain and all compared strains are isogenic to the PAB1 wild type strain, except for 1729 (contains *gst1-1* allele) and 1743 (contains *pri1-63* allele) which are 75% isogenic. Defects in eIF4E are encoded by the temperature sensitive allele, *cdc33-1*, which displayed an average translation rate of 29% compared to wild type (Figure 4, Table 3). As mentioned in the introduction, eIF4E, is the mRNA cap binding protein involved in cap mediated translation (Jacobson A. and Peltz S.W., 1996; von Der Haar T. et al., 2000; Wells et al., 1998). A decreased translation rate was therefore expected in the strain carrying the *cdc33-1* allele. The slightly lower rate of translation in a *cdc33-1* strain (19%) that was determined previously (Schwartz and Parker, 1999) could be due to the differences among the strain backgrounds used. My results showed an *in vivo* translation rate of 45% for the *pri1-63* strain (Figure 4, Table 3). This decreased rate was expected, since at 37°C the *pri1-63* allele causes *in vitro* and *in vivo* defects in the translation initiation factor eIF3 (Phan et al., 2001; Jivotovskaya et al., 2006; Searfoss et al., 2001). The discrepancy between my *in vivo* translation rate (45%) and the previously reported *in vivo* translation rate (8%)

(Schwartz and Parker, 1999) could be caused by two factors. First, the wild type and *prt1-63* strains are only 75% isogenic, which could result in an error when normalizing the *prt1-63* strain to wild type. Second, the strain I used and the strain previously used by Schwartz and Parker have different backgrounds. What is important is the observation that *prt1-63* caused a decrease in translation, indicating that the assay is functioning properly to show a decreased translation rate when one is expected. The translation termination factor eRF3 is encoded by *GST1*, and the *gst1-1* allele confers temperature sensitivity (Hosoda et al., 2003). The strain, containing the *gst1-1* allele, did not show as much decrease in translation rate (62%) as did defects in the translation initiation factors (Figure 4, Table 2). Since it is known that translation termination, mRNA deadenylation, and translation initiation are related (Hosoda et al., 2003; Kobayashi et al., 2004; Schwartz and Parker, 1999), it is expected that the decreased rate in a *gst1-1* strain was observed. Similarly, with the exception of the *caf1Δ* deletion, which displayed 35% of the translation as of wild type, defects in other mRNA degradation factors all have less severe effects on the rate of translation (in the range of 45-78%, Figure 4, Table 3) compared to the rates observed for the translation initiation deficient strains (ts eIF4E: 29%, ts eIF3: 45%). Since some of these were rates comparable to *prt1-63*, it indicates that factors involved in mRNA degradation affect *in vivo* translation. These observations suggest that the assay is functioning properly and should be useful to determine if additional mutations have significant major defects in translation. CAF1 displayed the most severe defect on *in vivo* translation and was analyzed further.

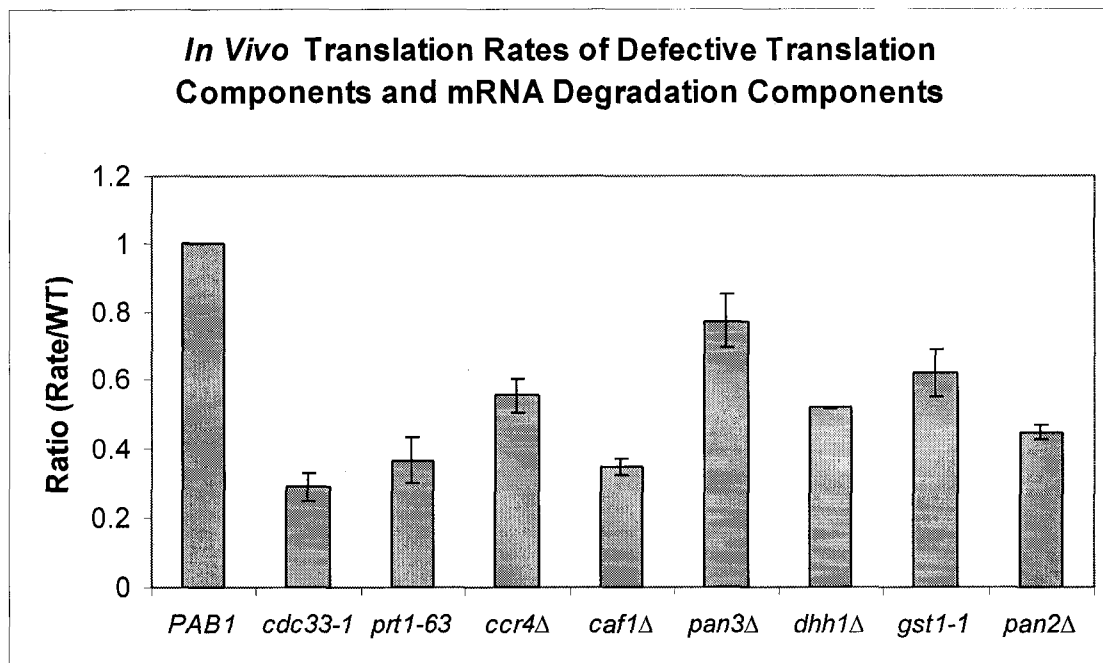


Figure 4 – *In vivo* translation rates of *cdc33-1*, *prt1-63*, *PAB1*, *dcp1Δ*, *ccr4Δ*, *pan3Δ*, *dhh1Δ*, and *gst1-1*. Values are averages of mutant relative to wild type *PAB1* and SEMs are shown as error bars. SEM is the standard error of the mean, and was calculated by dividing the standard deviation by the square root of the number of samples. All calculations were done with Microsoft Excel software.

Strain	Relevant Genotype	Mean (rate/WT)	SEM	Samples
AS319/pYC504	<i>PAB1</i>	100%		2
AS1881/pYC504	<i>cdc33-1</i>	29%	4%	12
1743/pYC504	<i>prt1-63</i>	37%	7%	2
AS319-1a/pYC504	<i>ccr4Δ</i>	55%	5%	7
AS319-c1/pYC504	<i>caf1Δ</i>	35%	2%	7
DB267/pYC504	<i>pan3Δ</i>	78%	11%	2
AS319-dhh1Δ/pYC504	<i>dhh1Δ</i>	52%	0%	2
1729-3/pYC504	<i>gst1-1</i>	62%	9%	3
1754-1-tN/pYC504	<i>pan2Δ</i>	45%	3%	2

Table 3 Absolute values and number of samples run for Figure 4.

Translation Rates in PAB1 Domain Deletions

To determine if domain deletions in PAB1 or PAB1 variants cause major defects in translation or if they have only minor effects, I measured the *in vivo* translation rate of PAB1 variants and domain deletions. If the translation rate of a PAB1 domain deletion or variant was similar to the translation rate of a defect in translation initiation factors eIF3 and eIF4E, then the PAB1 domain or variant probably had a major role in translation. However, most individual domain deletions and the PAB1 variants had only minor effects on translation (Figures 4 and 5, Tables 3 and 4). Analysis of PAB1 variants and domain deletions displayed translation rates ranging from 70% to 112% of wild type (Figure 5, Table 4). Since the translation rate of translation initiation factors, ts eIF4E and ts eIF3, was 29% and 45% of wild type, this group of PAB1 variants and domain deletions was not as deficient in translation as with a defect in eIF4E or eIF3, which suggested that deletion of individual domains of PAB1 did not have a major role in translation.

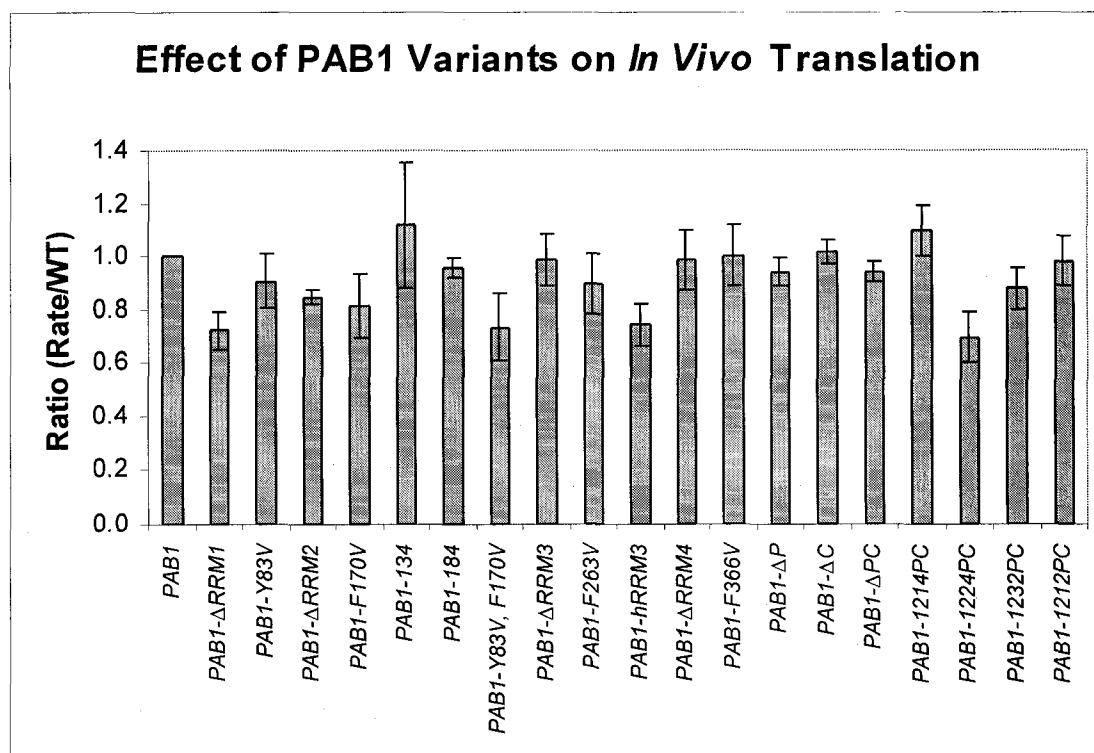


Figure 5 – Values are averages of the indicated PAB1 variant or domain deletion relative to wild type PAB1. Error bars represent SEM. SEM is the standard error of the mean, and was calculated by dividing the standard deviation by the square root of the number of samples. All calculations were done with Microsoft Excel software.

Strain	Relevant Genotype	Mean (rate/WT)	SEM	Samples
AS319/pYC504	<i>PAB1</i>	100%		4
AS319/pYC505	<i>PAB1-ΔRRM1</i>	72%	8%	4
AS319/pYC536	<i>PAB1-Y83V</i>	90%	12%	3
AS319/pYC506	<i>PAB1-ΔRRM2</i>	85%	3%	4
AS319/pYC525	<i>PAB1-F170V</i>	81%	15%	3
AS319/pYC545	<i>HPD134DKS</i>	112%	27%	4
AS319/pYC538	<i>DAL184EKM</i>	96%	5%	3
AS319/pYC537	<i>PAB1-Y83V, F170V</i>	73%	14%	4
AS319/pYC507	<i>PAB1-ΔRRM3</i>	99%	12%	3
AS319/pYC552	<i>PAB1-F263V</i>	89%	13%	4
AS319/pYC570	<i>PAB1-hRRM3</i>	74%	10%	3
AS319/pYC508	<i>PAB1-ΔRRM4</i>	99%	13%	4
AS319/pYC551	<i>PAB1-F366V</i>	100%	14%	3
AS319/pYC510	<i>PAB1-ΔP</i>	94%	6%	3
AS319/pYC509	<i>PAB1-ΔC</i>	102%	6%	3
AS319/pYC460	<i>PAB1-ΔPC</i>	94%	5%	4
AS319/pYC562	<i>PAB1-1214PC</i>	110%	11%	4
AS319/pYC571	<i>PAB1-1224PC</i>	70%	14%	3
AS319/pYC572	<i>PAB1-1232PC</i>	88%	11%	3
AS319/pYC513	<i>PAB1-1212PC</i>	98%	12%	3

Table 4 Absolute values and number of samples run for Figure 5.

Translation Rates in the PAB1 RRM1 domain

PAB1-ΔRRM1 was shown to significantly lower the *in vivo* translation rate compared to wild type (72%). When injected into oocytes, tethered PAB1 RRM1/2 domains were capable of stimulating translation more than any other domain compared to tethered full length PAB1, suggesting the PAB1 RRM1/2 domains have a greater role in translation than any other PAB1 domain (Gray et al., 2000). If PAB1 RRM1/2 domains are necessary for translation, then deletion of either PAB1 RRM1 domain or PAB1 RRM2 domain should decrease the *in vivo* translation rate, which is what I observed for the *in vivo* translation rate in the *PAB1-ΔRRM1* strain. However, *in vitro* the cap-dependent translation and poly(A)-dependent translation for *PAB1-ΔRRM1* displayed a significant increase (Otero et al., 1999; Kessler and Sachs, 1998) which is inconsistent with my results. In order to determine if the PAB1 RRM1 domain contact to poly(A) was necessary for the PAB1 RRM1 requirement for *in vivo* translation, a point mutation in PAB1 RRM1 known to make RNA contact (Y83V) was analyzed. PAB1-Y83V resulted in reduced binding to the poly(A) tail as compared to wild-type PAB1 (Yao et al., 2007; Deardorff and Sachs, 1997). PAB1-Y83V, however, did not have a significant effect on the *in vivo* translation rate (90%).

Translation Rates in PAB1 RRM2 Variants

PAB1-ΔRRM2 was shown to significantly lower the *in vivo* translation rate compared to wild type (85%). A PAB1 RRM1 domain deletion and a PAB1 RRM2 domain deletion resulted in the larger decreases for *in vivo* translation rates compared to other domain deletions (Figure 5, Table 4). This observation suggests that PAB1 RRM1 and PAB1 RRM2 have a more important role in translation than the other domains, and

should be able to stimulate translation if tethered to a luciferase reporter. When PAB1 RRM1/2 tethered to a luciferase reported, translation was stimulated more than any other tethered PAB1 domain (Gray et al., 2000). *In vitro*, PAB1- Δ RRM2 was previously shown to have a greater than four fold decrease for both cap-dependent translation and poly(A) dependent translation compared to wild type (Otero et al., 1999), which is inconsistent with my observed *in vivo* translation rate for PAB1- Δ RRM2 of 85% of wild type. Furthermore, the *in vitro* translation rate for poly(A)-dependent and cap-dependent PAB1- Δ RRM2 was previously determined to be more than two fold lower than for PAB1- Δ RRM1 (Otero et al., 1999; Kessler and Sachs, 1998), which is inconsistent with my determined *in vivo* translation rates, in which PAB1- Δ RRM2 resulted in 85% of wild type and PAB1- Δ RRM1 resulted in 72% of wild type (Figure 5, Table 4).

In order to better understand the role of PAB1-RRM2 in translation, three variants in the PAB1 RRM2 domain were analyzed. PAB1-F170V is defective in binding poly(A) (Deardorff and Sachs, 1997). PAB1-184 carrying the mutations DAL to EKM fails to bind eIF4G *in vitro* (Otero et al., 1999) although *in vivo* it is still able to make contact to eIF4G, albeit to a lesser extent (Y.C. Chiang, personal communication) and PAB1-134 has the mutations HPD to DKS. The measured *in vivo* translation rate of a point mutation in PAB1 RRM2 important for RNA binding (F170V) (Yao et al., 2007; Deardorff and Sachs, 1997) was 81%. However, the SEM of 15% indicates that additional samples are needed to determine if the decreased rate is significant. This preliminary observation suggests that the PAB1 RRM2 poly(A) contact is important for translation. *In vitro* PAB1-184 showed an increase in cap-dependent translation compared to PAB1- Δ RRM2 and was as defective in poly(A)-dependent translation as

PAB1- Δ RRM2, indicating that PAB1-184 is defective in poly(A) dependent translation and capable of stimulating cap-dependent translation (Otero et al., 1999). The *PAB1-184 in vivo* translation rate was determined to be 96%, which is in agreement with the *in vitro* translation rate for cap-dependent translation but in disagreement with the *in vitro* translation rate for poly(A)-dependent translation. The *in vitro* translation rate for PAB1-134 was similar to PAB1- Δ RRM2 for cap-dependent translation but poly(A)-dependent translation was increased more than four fold compared to PAB1- Δ RRM2 (Otero et al., 1999). The *in vivo* translation rate for *PAB1-134* was measured at 112% which is in agreement with the *in vitro* poly(A)-dependent translation rate, but disagrees with its effect on the *in vitro* cap-dependent translation rate (Otero et al., 1999).

Translation Rates in PAB1-RRM3

Deletion of the complete region of PAB1-RRM3 had no effect on *in vivo* translation (99%) compared to wild type. Tethering of PAB1 RRM3/4 to a luciferase reporter decreased translation slightly (93%) compared with tethered full length PAB1 (Gray et al., 2000). This indicates that PAB1 RRM3/4 does not have a large role in translation. If the PAB1 RRM3/4 domains do not play a role in translation, then deletion of PAB1 RRM3 or PAB1 RRM4 should show no significant change in the *in vivo* translation rate, which is what is observed for both domains (see next section for *PAB1- Δ RRM4* data). *In vitro*, the deletion increased poly(A)-dependent and cap-dependent translation (Otero et al., 1999; Kessler and Sachs, 1998). A point mutation (F263V) in the PAB1 RRM3 domain important for binding RNA (Yao et al., 2007; Deardorff and Sachs, 1997) had no significant effect on *in vivo* translation (89%), further suggesting the PAB1 RRM3 domain is unimportant for translation. However, substitution of the PAB1

RRM3 domain with the PAB1 RRM2 domain (*PAB1-1224PC*) gave 70% of the wild-type *in vivo* translation rate. This was the largest decrease for *in vivo* translation rate observed in the PAB1 domain deletions and variants (Figure 5, Table 4). In contrast, substitution of the PAB1 RRM3 domain with the PAB1 RRM1 domain (*PAB1-1214PC*) showed an *in vivo* translation rate of 110%. The PAB1-hRRM3 variant did show a decreased *in vivo* translation rate of 74%. However, as no other human PAB1 domain substitutions were available at the time, it was not possible to compare this result to other human PAB1 domain substitutions.

Translation Rates in PAB1-RRM4, PAB1-P, PAB1-C Deletions

Analysis of the translation rates for *PAB1-ΔRRM4* (99%), *PAB1-ΔP* (94%), *PAB1-ΔC* (102%), and *PAB1-ΔPC* (94%) showed no significant differences compared to the wild-type PAB1 strain (Figure 5, Table 4). Additionally, mutation of a PAB1 RRM4 residue (Y366V), important for RNA contact (Yao et al., 2007; Deardorff and Sachs, 1997) further indicates that changes in the PAB1 RRM4 domain do not affect *in vivo* translation. Previous *in vitro* translation rates indicate that deletion of the PAB1 RRM4 domain decreased cap-dependent and poly(A) dependent translation (Otero et al., 1999; Kessler and Sachs, 1998), which is inconsistent with my *in vivo* translation rate. Previous analysis of the C terminus of PAB1 was done with a truncation of both PAB1 P/C domains which showed cap-dependent translation to be higher than wild type (Otero et al., 1999) and lower than wild type for poly(A)-dependent translation (Kessler and Sachs, 1998). The *in vivo* translation rates I determined for the *PAB1-ΔP*, *PAB1-ΔC*, and *PAB1-ΔPC* strains were consistent with the *in vitro* PAB1-ΔPC cap-dependent rate but inconsistent with the poly(A)-dependent rate. *In vivo* translation rates measured with

injected oocytes showed that tethered PAB1 PC domains were less capable of stimulating translation compared to the full length PAB1, indicating the PAB1 P and PAB1 C domains have no role in translation or act to suppress translation (Gray et al., 2000). If PAB1 P and PAB1 C domains act to suppress translation then deletion of the domains should increase the rate of translation, whereas if PAB1 P and PAB1 C domains have no role in translation then deletion of either domain should not affect the *in vivo* translation rate. The latter hypothesis agrees with the *in vivo* translation rates that I observed for *PAB1-ΔP*, *PAB1-ΔC*, and *PAB1-ΔPC*.

Translation Rates in ts eIF3 with PAB1 Variants

In vivo translation rates were measured in a strain with the *prt1-63* allele and PAB1 domain deletions. The following rates of translation were observed: *PAB1-ΔRRM1* (56%), *PAB1-ΔRRM2* (44%), *PAB1-ΔRRM3* (39%), *PAB1-ΔRRM4* (44%), *PAB1-ΔP* (50%), and *PAB1-ΔC* (42%) (Figure 6, Table 5). Compared to the *prt1-63* allele alone (45%) the translation rates for the PAB1 domain deletions and almost all PAB1 variants in the presence of *prt1-63* displayed no significant difference (Figure 6, Table 5). The only significant difference was for PAB1-134 (31%) that was much lower than *prt1-63* alone (45%). More determinations would be needed to verify that observation, but it does suggest that the lesion in PAB1-134 functionally interacts with eIF3 to decrease translation.

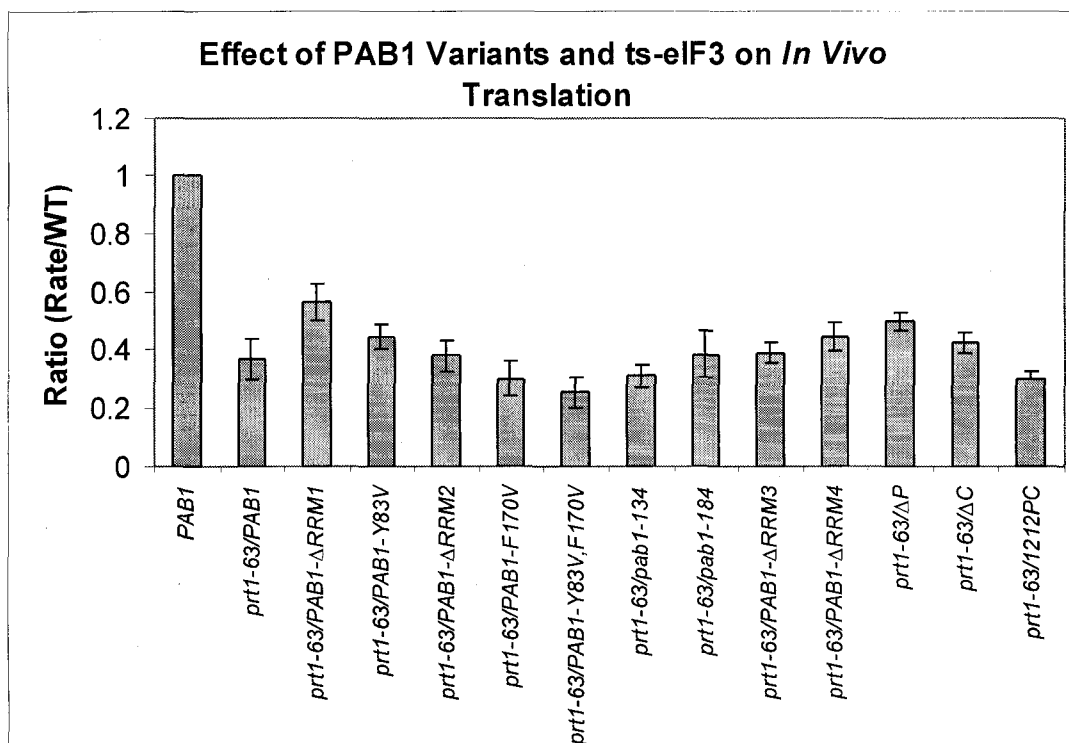


Figure 6 – Average values for *prt1-63* with *PAB1* variants and domain deletions. Values are relative to the wild-type parent strain. Error bars represent SEM. SEM is the standard error of the mean, and was calculated by dividing the standard deviation by the square root of the number of samples. All calculations were done with Microsoft Excel software.

Strain	Relevant Genotype	Mean (rate/WT)	SEM	Samples
AS319/pYC504	<i>PAB1</i>	100%		2
1743/pYC504	<i>prt1-63 PAB1</i>	37%	7%	2
1743/pYC505	<i>prt1-63 PAB1-ΔRRM1</i>	56%	6%	2
1743/pYC536	<i>prt1-63 PAB1-Y83V</i>	44%	4%	2
1743/pYC506	<i>prt1-63 PAB1-ΔRRM2</i>	38%	5%	2
1743/pYC525	<i>prt1-63 PAB1-F170V</i>	30%	6%	2
1743/pYC537	<i>prt1-63 PAB1-Y83V,F170V</i>	25%	5%	2
1743/pYC545	<i>prt1-63 HPD134DKS</i>	31%	4%	2
1743/pYC538	<i>prt1-63 DAL184EKM</i>	38%	8%	2
1743/pYC507	<i>prt1-63 PAB1-ΔRRM3</i>	39%	3%	2
1743/pYC508	<i>prt1-63 PAB1-ΔRRM4</i>	44%	5%	2
1743/pYC510	<i>prt1-63 PAB1-ΔP</i>	50%	3%	2
1743/pYC509	<i>prt1-63 PAB1-ΔC</i>	42%	4%	2
1743/pYC513	<i>prt1-63 PAB1-1212PC</i>	30%	3%	2

Table 5 Absolute values and number of samples run for Figure 6.

Translation Rates in ts eIF4E with PAB1 Variants

Translation rates measured in the *cdc33-1* background varied from day to day so in order to confirm a significant difference, the rates obtained for a sample on a specific day or set of days had to be compared against the observed *cdc33-1* rate for the same specific day or set of days as the sample being analyzed. The average *in vivo* translation rate obtained for the *cdc33-1* allele alone (29%, Figure 7, Table 6) was taken from 12 samples. Since this average is taken from a large sample size it was considered to be representative of the affect of *cdc33-1* on translation. The average *in vivo* translation rates for the remaining PAB1 variants and deletions in the *cdc33-1* background were taken from two to seven samples. If *in vivo* translation rates for a particular variant were measured outside the mean, those values were compared with rates for the wild type PAB1 in the *cdc33-1* background on the same day. *PAB1-134* (15%, Figure 7, Table 6) in a *cdc33-1* background showed an average rate significantly lower than the mean translation rate of the *cdc33-1* allele alone (29%, Figure 7, Table 6). However, as the translation rates varied from day to day, *cdc33-1 PAB1-134* rates were measured at 14% and 17% (data not shown) and on the same days the translation rates for the *cdc33-1* allele alone were measured at 12% and 14% (data not shown), indicating that the PAB1-134 protein does not affect translation significantly in a *cdc33-1* strain. *cdc33-1 PAB1-ΔRRM4* showed an *in vivo* translation rate of 15% (Figure 7, Table 6) with SEM of 4%, which is significantly lower than *cdc33-1 PAB1*. However, on the same day that *cdc33-1 PAB1-ΔRRM4* was analyzed, *cdc33-1 PAB1* showed *in vivo* translation rates of 16%, 19%, 20%, and 21%, averaged to 19% with SEM of 1% (data not shown) which indicates

that *cdc33-1 PAB1-ΔRRM4* is not significantly different from the mean. In an effort to better understand the interaction between eIF4E and the PAB1 RRM2, domain we then analyzed the *in vivo* translation rate of the PAB1-184 protein (14%, Figure 7, Table 6), which is significantly lower than the *cdc33-1* allele alone (29%, Figure 7, Table 6). In contrast, when compared against another mutation in the RRM2 domain of PAB1 (F170V, Table 2), which results in decreased mRNA contact, showed no difference in translation rate (22%, Figure 7, Table 6) compared to the *cdc33-1* allele alone (29%, Figure 7, Table 6). The remaining PAB1 variants do not show any significant difference compared to the *cdc33-1* allele (Figure 7, Table 6). These observations indicate that *PAB1-184* caused a reduced *in vivo* translation rate when combined with a defect in the cap binding protein, eIF4E. It has been shown that, *in vitro*, PAB1-184 is incapable of binding eIF4G (Otero et al., 1999) and since eIF4G is responsible for contacting additional translation initiation complexes (Neff and Sachs, 1999; Dominguez et al., 1999) it is logical that abrogating contact between both the cap (eIF4E) and poly(A) tail (PAB1-184) to eIF4G would cause a greater impairment to translation.

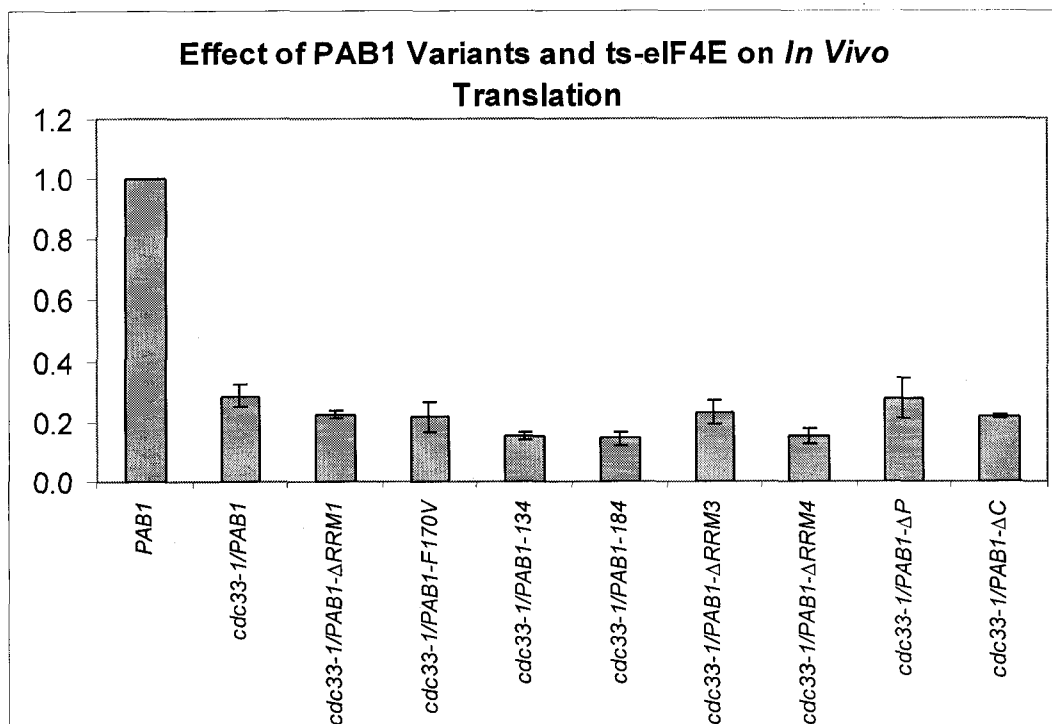


Figure 7 – Average values for *cdc33-1* with *PAB1* mutations. Values are relative to the wild-type parent strain.

Strain	Relevant Genotype	Mean (rate/WT)	SEM	Samples
AS319/pYC504	<i>PAB1</i>	100%		2
AS1881/pYC504	<i>cdc33-1 PAB1</i>	29%	4%	12
AS1881/pYC505	<i>cdc33-1 PAB1-ΔRRM1</i>	22%	2%	2
AS1881/pYC525	<i>cdc33-1 PAB1-F170V</i>	22%	5%	5
AS1881/pYC545	<i>cdc33-1 HPD134DKS</i>	15%	1%	2*
AS1881/pYC538	<i>cdc33-1 DAL184EKM</i>	14%	3%	5
AS1881/pYC507	<i>cdc33-1 PAB1-ΔRRM3</i>	23%	6%	2
AS1881/pYC508	<i>cdc33-1 PAB1-ΔRRM4</i>	15%	4%	2*
AS1881/pYC510	<i>cdc33-1 PAB1-ΔP</i>	28%	7%	2
AS1881/pYC509	<i>cdc33-1 PAB1-ΔC</i>	22%	1%	2

Table 6 – Average values for *cdc33-1* with *PAB1* mutations and domain deletions. The strains indicated by * show a significant decrease in the mean, however comparison with the *cdc33-1/PAB1* rate obtained on the same day was not significantly different.

Analysis of CCR4 and CAF1 Effects on Translation

CCR4 and CAF1 are two components of the CCR4-NOT deadenylase complex shown to play a role in deadenylation. When deleted, *caf1* and *ccr4* showed *in vivo* deadenylation defects (Tucker et al., 2001). Defects caused by a *caf1* deletion can be rescued by over-expression of CCR4. However, defects associated with a *ccr4* deletion cannot be rescued by over-expression of CAF1 (Tucker et al., 2001; Chen et al., 2002; Tucker et al., 2001). Moreover, mutation of predicted key catalytic active sites in CAF1 did not affect *in vivo* deadenylase activity (Viswanathan et al., 2004), whereas point mutations in the predicted catalytic active sites in CCR4 does cause a defect in deadenylation (Chen et al., 2002). Genetic evidence suggests that CAF1 plays a role in translation, whereas CCR4 does not (Ohn et al., 2007; Coller and Parker, 2005; Maillet and Collart, 2002). These observations suggest that CAF1 and CCR4 have different roles in deadenylation and translation.

In order to determine if in fact CCR4 and CAF1 have functions in translation we asked whether or not *caf1* Δ and *ccr4* Δ strains show a decreased translation rate. A decrease in translation rate was observed for both CCR4 and CAF1, 55% for *ccr4* Δ and 35% for *caf1* Δ . This indicates that both CCR4 and CAF1 have roles in translation, but CAF1 is more critical for translation than CCR4. Since it has been shown that a *caf1* deletion is lethal when combined with *PAB1*- Δ RRM2 and a *ccr4* deletion is viable when combined with *PAB1*- Δ RRM2, we then asked how the PAB1 RRM2 variants affect *in vivo* translation rates when combined with *caf1* Δ and *ccr4* Δ . When expressed in a *caf1* Δ strain, PAB1-184 raised the translation rate to 50%. PAB1-134, in contrast, displayed little effect when combined with *caf1* Δ and resulted in a rate of 30%. In a *ccr4* Δ

background both PAB1-184 and PAB1-134 decreased the *in vivo* translation rate to 38% and 46%, respectively (Figure 8, Table 7). These results suggest that the PAB1-184 mutations rescue the defect in translation caused by *caf1*Δ.

The synthetic lethality observed when combining a *caf1*Δ with the PAB1 domain deletion *PAB1-ΔRRM2* suggests that CAF1 has a similar translational role as eIF4E because the *cdc33-1* allele also displayed a synthetic lethality with *PAB1-ΔRRM2* (unpublished results). Because of this observation we asked how the *cdc33-1* allele affects the translation rate in a *caf1*Δ or *ccr4*Δ background. In the *cdc33-1 caf1*Δ strain we saw a much lower *in vivo* rate of translation (13%) than *cdc33-1 ccr4*Δ (24%). Compared to the *cdc33-1* allele alone (29%), *cdc33-1 caf1*Δ has a more significant effect on *in vivo* translation than *cdc33-1 ccr4*Δ, implying that CAF1 and CCR4 have different roles in translation. Previous deadenylation data regarding CAF1 and CCR4 imply the two proteins have a role in deadenylation, albeit apparently, separate roles (Ohn, et al. 2007). My data suggests that CAF1 and CCR4 do in fact have roles in translation, but those roles are separate as well as the roles in deadenylation.

Since over-expression of *CAF1* rescues the lethality of the *cdc33-1* allele (Ohn, et al. 2007), and since the *cdc33-1* allele confers a defect in the translation initiation factor eIF4E it is reasonable to imagine that *CAF1* rescues the *cdc33-1* lethality by increasing the rate in translation when over-expressed. If, in fact, CAF1 has a role in translation similar to eIF4E, then we would expect that over-expressed CAF1 would increase the rate of translation in a strain with the *cdc33-1* allele. CAF1 and CCR4 were over-expressed from a YEp13 plasmid which was introduced into a strain carrying the *cdc33-1* allele. The *in vivo* translation rate of *cdc33-1/YEp13-CAF1* was measured and normalized to the

wild type parent strain with a YEp13 plasmid, *cdc33-1/YEp13*. In order to determine if CCR4 has a different role in translation than CAF1, the *in vivo* translation rate of over-expressed CCR4 was also measured in a strain carrying the *cdc33-1* allele. The *cdc33-1* allele alone, *cdc33-1/YEp13* showed an *in vivo* translation rate of $38\% \pm 7\%$ (Figure 8, Table 7), *cdc33-1/YEp13-CCR4* ($31\% \pm 7\%$, Figure 8, Table 7) and *cdc33-1/YEp13-CAF1* ($49\% \pm 6\%$, Figure 8, Table 7) did not show a significant difference compared to the *cdc33-1* allele alone, due to overlapping SEM values. This suggests that over-expressed CAF1 does not rescue a lesion in eIF4E by increasing *in vivo* translation rates. Furthermore, this observation indicates that CCR4 does not have a significant translational effect when combined with a defect in eIF4E. Although, these results do not show that over-expressed CCR4 and CAF1 significantly increase or decrease in the rate of translation from the translation rate of the *cdc33-1* allele alone, the *in vivo* translation rates for *cdc33-1/YEp13-CCR4* and *cdc33-1/YEp13-CAF1* are significantly different from one another. This suggests that over-expressed CAF1 and over-expressed CCR4 affect the translation rate differently when combined with a defect in eIF4E, supporting the hypothesis that CAF1 and CCR4 do in fact have different roles in translation.

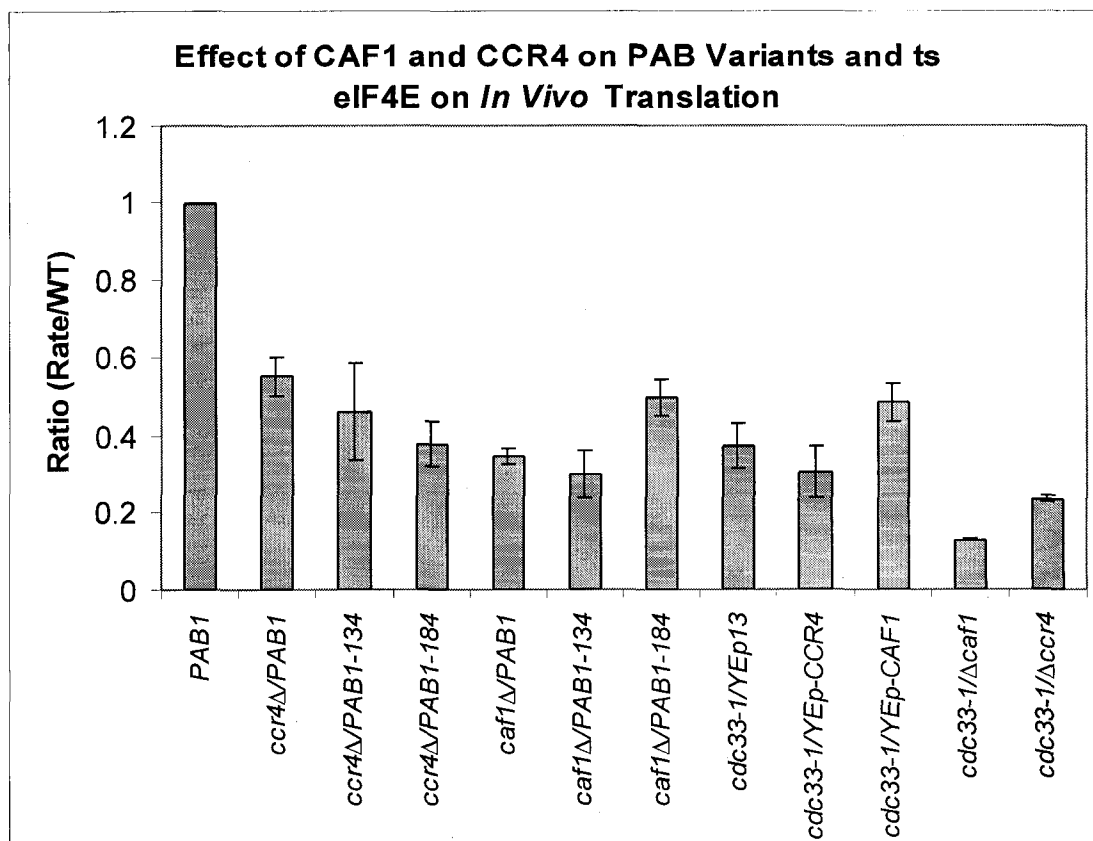


Figure 8 – Values are the average of the rate relative to the wild-type parent strain. Error bars represent SEM.

Strain	Relevant Genotype	Mean (rate/WT)	SEM	Samples
AS319/pYC504	PAB1	100%		4
AS319-1a/pYC504	ccr4Δ PAB1	55%	5%	7
AS319-1a/pYC545	ccr4Δ HPD134DKS	46%	14%	4
AS319-1a/pYC538	ccr4Δ DAL184EKM	38%	7%	3
AS1881-1a/ pYC504	cdc33-1 Δccr4	24%	4%	3
AS319-c1/pYC504	caf1Δ PAB1	35%	2%	7
AS319-c1/pYC545	caf1Δ HPD134DKS	30%	8%	3
AS319-c1/pYC538	caf1Δ DAL184EKM	50%	6%	3
AS1881-c1/pYC504	cdc33-1 Δcaf1	13%	4%	3
AS1881/YEp13	cdc33-1 YEp13	38%	7%	4
AS1881/MPT2	cdc33-1 YEp-CCR4	31%	7%	3
AS1881/MPT0	cdc33-1 YEp-CAF1	49%	6%	4

Table 7 Absolute values and number of samples run for Figure 8.

Discussion

Previous studies analyzing the role of PAB1 in translation have been limited to *in vitro* studies (Otero et al., 1999; Kessler and Sachs, 1998) and the ability of PAB1 domains to stimulate translation (Gray et al., 2000). The *in vitro* studies have been useful for determining a link between the PAB1 RRM2 domain, eIF4G, and translation but the results do not agree with the observed *in vivo* translation rates that I have observed. It is known that PAB1 is involved in various aspects of mRNA transport (Brune C, et al 2005), mRNA biogenesis (Dunn EF, et al 2005), and mRNA deadenylation (Yao, et al 2007, Caponigro G and Parker R 1995), so differences in the observed *in vivo* translation rates and *in vitro* translation rates could be due to pleiotropic effects associated with a PAB1 domain deletion or variant. Analysis of PAB1 domains able to stimulate translation in *Xenopus oocytes* is difficult to compare side by side with the analysis of translation rates in the domain deletions because different domains are examined. Specifically, the domains deleted were much smaller than the domains used for the analysis of translation stimulation. Additionally, the ability of a PAB1 domain capable of translation stimulation determines if the domain is sufficient for translation, the deletion analysis determines if the domain is necessary for translation. Despite these problems, when analyzing the PAB1 domains capable of translation stimulation, PAB1 RRM1/2, I am able to conclude that the PAB1 RRM1 and PAB1 RRM2 domains are both necessary and sufficient for translation. The domains of PAB1 unable to stimulate translation compared to full length PAB1 were PAB1 RRM3/4 and PAB1 P/C. This indicates that the only

domains sufficient for translation are PAB1 RRM1/2. Additionally, when analyzing a deletion of the domains incapable of translation stimulation, I observed that *in vivo* translation rates were unaffected, indicating that the PAB1 RRM3, PAB1 RRM4, PAB1 P, and PAB1 C domains are not necessary for translation.

One purpose of this study was to determine the role of PAB1 in translation. Based on these findings, deletions in PAB1 do not have a major role in translation. The domain deletion with the largest decrease in translation rate was *PAB1- Δ RRM1* (72%, Figure 5, Table 4). Compared against defects in known translation factors, ts eIF3 (45%, Figure 4, Table 3) and ts eIF4E (29%, Figure 4, Table 3) the PAB1 domain deletion with the largest decrease in translation rate is still much higher than defects in known translation factors. The rates for PAB1 domain deletions and variants (70-112%, Figure 5, Table 4) are more similar to translation rates observed in strains with defects in mRNA degradation, except for *caf1 Δ* (45-78%, Figure 4, Table 3).

Based on the observation that PAB1 does not have a large role in translation, combination of PAB1 domain deletions and variants with lesions in eIF3 and eIF4E should not affect *in vivo* translation rates. The only PAB1 variant that significantly lowered the *in vivo* translation rate in the ts eIF4E strain was PAB1-184 (14%, Figure 7, Table 6) compared to the ts eIF4E strain alone (29%, Figure 7, Table 6). Since PAB1-184 abrogates eIF4G contact (Otero et al., 1999) and eIF4G is responsible for contacting additional translation factors (Neff and Sachs, 1999; Dominguez et al., 1999), then combining a defect in eIF4E with PAB1-184 should decrease translation rates. In order to show more definitively that the contact between eIF4G and PAB1 is causing the decreased *in vivo* translation rate, another PAB1 variant known to decrease eIF4G

binding could be analyzed. Such a variant is PAB1-180, which carries the mutation KE to ER and diminishes *in vitro* cap-dependent, poly(A) dependent translation, and binding to eIF4G (Otero, et al., 1999). The PAB1 variant PAB1-134 decreased the *in vivo* translation rate in the ts eIF3 strain to 31% (Figure 6, Table 5), which is significantly lower than the ts eIF3 strain alone (45%, Figure 6, Table 5). How PAB1-134 interacts with the translation factor, eIF3, is unclear, but the observed results indicate that PAB1-134 acts on translation through a different mechanism than PAB1-184.

Another reason for this study was to determine if CAF1 and CCR4 have roles in translation and if those roles are similar. Since the role in deadenylation of CAF1 and CCR4 appears to be different, it would follow that the role in translation would be different as well. Based on these results it does appear that both CAF1 and CCR4 have roles in translation (Figure 8, Table 7), with the role of CAF1 being more critical than CCR4. Combining defects in eIF4E (*cdc33-1*) with a lesion in *caf1* or *ccr4* showed that a *caf1* deletion decreased translation rates (15%, Figure 8, Table 7) more than a *ccr4* deletion (24%, Figure 8, Table 7). These last observations support the hypothesis that CAF1 has a greater role in translation than CCR4, and that the two proteins act through different mechanisms. Furthermore, a PAB1-184 variant increased the *in vivo* translation rate in a *caf1* Δ strain to 50% of wild type (Figure 8, Table 7), whereas a *ccr4* Δ strain PAB1-184 lowered the rate to 38% of wild type (Figure 8, Table 7). This last observation indicates that deleting *ccr4* caused a similar decrease in translation as did a *cdc33-1* allele, since the double mutant, *cdc33-1 PAB1-184* (14%) showed a lower translation rate compared to the *cdc33-1* allele alone (29%, Figure 7, Table 6). How

CCR4 and CAF1 function in translation is unclear at this time. What is clear is that both CCR4 and CAF1 do have a role in translation and that role is different from one another.

Analysis of additional strains could provide the necessary insight to make a more conclusive summary of this work. As mentioned in the discussion PAB1-180 showed reduced *in vitro* contact between eIF4G and PAB1. If PAB1-180 shows a lowered *in vivo* translation rate, similar to PAB1-184, when combined with a *cdc33-1* allele then the hypothesis that decreasing contact between PAB1 and eIF4G is the reason for the decreased *cdc33-1 PAB1-184* translation rate would be supported. Additionally, combining PAB1-180 with PAB1-184 could be analyzed and would also support the stated hypothesis if a decreased translation rate is observed. Correspondingly, combining the PAB1-180 and PAB1-180, 184 variants with lesions in *cer4* and *caf1* would provide additional information about how CCR4 and CAF1 function in translation.

The PAB1 variants, PAB1-134 and PAB1-184, represent substitutions of yeast residues with the homologous human residues in the RRM2 domain. These variants have provided some additional insight into how PAB1 functions in translation and substitution of additional domains would provide additional information into how PAB1 functions in translation. Analysis of a human substitution of PAB1 RRM3 has been done (Figure 5, Table 4), but since no other domain substitutions were available for analysis at the time, proper comparisons were not available to determine the significance of the decreased *in vivo* translation rate for PAB1-hRRM3. If the remaining domains were to be analyzed, we could obtain more information about how the PAB1 domains are involved in translation. For example, the PAB1- Δ RRM3 domain deletion did not show a large decrease in translation, but the PAB1-hRRM3 variant did show a larger decrease in

translation. If PAB1-hRRM1 shows a large decrease in translation, then the PAB1-hRRM3 decrease is probably a general effect due to changing the yeast domain with the human domain. If PAB1-hRRM1 does not show a large decrease for the *in vivo* translation rate, then we would hypothesize that the PAB1 RRM3 domain does in fact play a role in translation, and the next step would be to make smaller substitutions in RRM3. Obviously, additional work is needed to make more definitive conclusions, and the work reported thus far has provided some observations that could be investigated further.

CHAPTER II

COX17 DEADENYLATION RATES IN PAB1 VARIANTS

Introduction

Proper and appropriate gene expression is essential for all biological organisms. Regulation of gene expression is necessary for many biological cues, such as: embryological development, metabolic changes, cell cycle stages, and viral infection. Gene expression can be controlled at many levels, ranging from transcriptional start signals, mRNA degradation, and post-translational modifications. Because the longer a mRNA transcript persists, the more protein can be translated from the transcript, and conversely, the shorter a mRNA half-life is the less protein can be produced from the corresponding mRNA transcript, one area of particular interest is gene regulation at the level of mRNA degradation.

In *Saccharomyces cerevisiae*, a mature mRNA consists of a 3' poly(A) tail, approximately, 90-110 adenines in length, a m⁷Gppp cap at the 5' ribonucleotide, and all introns spliced from the mRNA body. Degradation of the mRNA begins with deadenylation of the poly(A) tail by the PAN2/3 complex, which exonucleolytically digests approximately 20 adenines (A's). The CCR4-NOT complex then digests the poly(A) tail to a length of 8-12 adenines (Tucker et al., 2001). At this length the poly(A) binding protein (PAB1) cannot efficiently bind the poly(A) tail, thus signaling decapping (Coller et al., 1998; Sachs et al., 1987). The 5' m⁷Gppp cap structure is cleaved through

numerous proteins, such as DCP1/2, DHH1, EDC1, EDC2, LSM1-7, and PAT1 (Dunckley and Parker, 1999; Bouveret et al., 2000; Schwartz et al., 2003; Bonnerot et al., 2000). Decapping allows for degradation of the mRNA body 5' to 3' by XRN1 (Muhlrad et al., 1994). Additionally, degradation by the exosome can occur in the 3' to 5' direction (Anderson and Parker, 1998; Mitchell et al., 1997).

As mentioned in the general introduction, several observations indicate that deadenylation is the first and rate limiting step of mRNA degradation. Briefly, elements in the UTR of mRNA that promote rapid degradation also promote accelerated deadenylation rates (Decker and Parker, 1993; LaGrandeur and Parker, 1999; Caponigro and Parker, 1996), and deadenylation rates of stable transcripts are much slower than transcripts that degrade rapidly (Decker and Parker, 1993; LaGrandeur and Parker, 1999). Since deadenylation is the first and rate limiting step of mRNA degradation, understanding how deadenylation occurs is crucial to understanding how differential mRNA degradation occurs.

The PUF family of proteins, as mentioned in the general introduction, is a group of proteins responsible for translational repression and accelerating deadenylation of the target transcript. It has been shown that PUF3 accelerates deadenylation of *COX17* (Olivas and Parker, 2000). One approach to determining how deadenylation is accelerated or decelerated is to investigate the mechanism that PUF3 accelerates deadenylation.

Two models can explain how PUF3 accelerates mRNA deadenylation. The first is a simple recruitment model. It has been shown that the PUF consensus sequence in the 5' or 3' UTR is capable of shortening the half life of an mRNA (Olivas and Parker, 2000;

Graber, 2003). These flanking sequences bind proteins, such as PUF proteins, which in turn bind mRNA degradation proteins, such as the CCR4-NOT complex (Figure 2A), thus bringing the target mRNA into closer proximity to the degradation proteins. It has been shown that PUF5 is capable of contacting CAF1 (Goldstrohm et al., 2006), which contacts the major cytoplasmic deadenylase, CCR4. This observation provides support for the recruitment model. The second model involves rearrangement of the mRNP structure. The mRNP complex is composed of many proteins, but the core complex consists of a mature mRNA, the cap binding protein, eIF4E, the poly(A) binding protein, PAB1, and eIF4G, a bridging protein between eIF4E and PAB1. Through contact with eIF4E, eIF4G, or PAB1 the PUF proteins could perturb the mRNP structure, allowing mRNA degradation proteins access to the mRNA transcript (Figure 2B). Support for the mRNP disruption model is shown through PUF3 and PUF5 co-immunoprecipitation of eIF4E (Y. Chiang, personal communication). This suggests that PUF3 and PUF5 contact the mRNP complex through eIF4E. The two models are not mutually exclusive, however. It is possible PUF proteins could simultaneously recruit mRNA degradation components and destabilize the mRNP complex (Figure 2C).

The role of eIF4E in deadenylation is not completely understood, but several observations suggest it has an inhibitory role and interacts with PAB1. A *cdc33-1* allele, encoding a temperature sensitive eIF4E protein, defective in cap binding (Altmann and Trachsel, 1989), displayed an accelerated deadenylation rate for *GAL1* (T. Ohn, personal communication) and *PGK1* (Schwartz and Parker, 1999), but not *MFA2* (Schwartz and Parker, 1999), suggesting that eIF4E has an inhibitory effect on deadenylation of transcripts with an average to long half-life. Furthermore, deletion of PAB1 RRM1 and

PAB1 P domains, the two domains of PAB1 that showed the greatest defects in deadenylation and are responsible for circularization of PAB1 (Yao et al., 2007), displayed accelerated deadenylation of *GAL1* with a defect in eIF4E (T. Ohn, personal communication). Formation of the circular PAB1 promotes deadenylation, so this last observation suggests that eIF4E has a role in the circularization of PAB1. PAB1 is in close proximity to eIF4E and we have shown that PAB1 co-immunoprecipitates eIF4E (Y. Chiang, personal communication). One hypothesis is that PUF3 interacts with eIF4E to disrupt PAB1 poly(A) binding by promoting the circularization of PAB1. By understanding the role of PAB1 in deadenylation we hope to understand how the mRNP structure is involved in deadenylation.

In order to determine the role of PAB1 in the differential degradation of a mRNA transcript, deadenylation rates have been determined of an unstable mRNA and a mRNA with an average half-life. Two mRNA suitable for comparison in the PAB1 variants are, *MFA2* which displayed a half-life of 3.5 minutes (Schwartz and Parker, 1999) and is relatively fast, and *GAL1* which has a mRNA half-life of 10 minutes (Cui and Denis, 2003) and is a rate more similar to the average mRNA half-life. If one PAB1 variant had an effect on *MFA2* that did not occur in *GAL1*, then the conclusion would be that the particular PAB1 domain has a role in differential deadenylation. However, all PAB1 variants had the same effect on *GAL1* and *MFA2* (Yao et al., 2007), indicating that PAB1 does have a role in deadenylation, but that role may be a general role for transcripts with an average and a fast deadenylation rate. If, in fact, PAB1 does have a general role in mRNA deadenylation, then the deadenylation rate for the PAB1 variants should be similar for all transcripts. *COX17* encodes an mRNA with a relatively fast half-life

(Olivas and Parker, 2000), and a fast deadenylation rate (Olivas and Parker, 2000), so it would be expected that *COX17* would behave similar to *MFA2*. Since, it is known that the mRNA binding protein, PUF3, accelerates deadenylation of *COX17* mRNA (Olivas and Parker, 2000), analysis of the deadenylation rate of *COX17* in each of the PAB1 variants would provide insight into the mechanism whereby PUF3 accelerates deadenylation.

In this work I have analyzed the deadenylation rate of *COX17* in PAB1 variants containing a deletion for each of the domains, and have shown that *PAB1-ΔRRM1* blocked deadenylation of *COX17* and *PAB1-ΔP* displayed a more similar deadenylation pattern to wild-type. Previously, *PAB1-ΔRRM1* and *PAB1-ΔP* displayed a slower deadenylation rate for both *MFA2* and *GALI* (Yao, et al., 2007). The observation that deadenylation rates for *COX17* are different compared to *MFA2* and *GALI* in the PAB1 variants indicates that PAB1 has a role in deadenylation that is different for a mRNA that is dependent on PUF3 for proper deadenylation.

Materials and Methods

Strains and Plasmids Used

Strains and plasmids are listed in Tables 1 and 2.

Oligonucleotides Used

Table 8 Sequence of Probes Used for Northern Analysis

Probe Name	Sequence
COX17/dn	5' GCCATAACCCTTCATGCACTC
COX17/3' end	5' GGTTGTCGGCAGACTGTCAG
SCR1 probe250	5' ATCCCGGCCCGCCTCCATCAC

Growth Conditions and Pulse-Chase

All strains were grown in 0.67% yeast nitrogen base without amino acids, and amino acids as required (synthetic medium) containing 2% raffinose until exponential growth phase was reached. Leucine was omitted from synthetic medium (leu- medium) to ensure the presence of *pRP1007*. Cells were then resuspended in five mL of leu-medium containing 2% raffinose. One mL of 25% galactose was added to induce expression of the *GAL10* promoter. Twenty minutes after induction, one mL of 50% glucose was added, and one mL aliquots were removed and quick frozen in dry ice at the designated time points.

RNA Analysis

RNA isolation. The hot phenol method was used to isolate total RNA. 500 μ L of TES (10 mM Tris pH 7.5, 10 mM EDTA, 0.5% SDS) and 500 μ L of phenol were added to frozen cells. Following a 65°C incubation with frequent vortexing, the mixture was spun, and the aqueous layer removed. The aqueous layer was then cleared of protein and lipids with a phenol wash, followed by a chloroform wash. RNA was then precipitated with 100% ethanol and 1/10 volume 3M NaOAc pH 5.3. The pellet was washed in 70% ethanol, dried and resuspended in 50 μ L of DEPC treated water.

RnaseH digestion and analysis. 12-30 μ g of RNA was incubated with the COX17/dn probe at 75°C for ten minutes, and then one hour at 30°C. An additional sample was also hybridized to a DNA oligo-thymidine (dT), in addition to the COX17/dn probe. The dT hybridizes to the poly(A) and RNaseH digestion removes the poly(A) tail and also cleaves at the COX17/dn site. The dT sample allows for visualization of the fully deadenylated fragment following Northern analysis. One U RNaseH was added

with RNaseH buffer (40mM Tris pH 7.5, 20mM MgCl₂, 100mM NaCl, 2mM DTT, 60 µg/mL BSA) and allowed to digest at 30 °C for one hour. Two hundred and eighty µL stop buffer was added (0.04 mg/mL tRNA, 300mM NaOAc, 20mM EDTA), and the digested RNA was cleared of protein with phenol:chloroform (1:1), and chloroform. The RNA was precipitated with 100% ethanol and washed with 70% ethanol. RNA was then separated on a 6% urea polyacrylamide gel, transferred to a nitrocellulose membrane and probed using a [³²P]-labeled COX17/3' end probe. Phosphor imaging screen - K was used to detect radio-labeled *COX17* mRNA, the screens were scanned using the Bio-Rad Molecular Imager - FX, and Quantity One software was used to print and manipulate the images. Following detection of *COX17*, membranes were re-probed for *small cytoplasmic RNA 1 (SCR1)*. *SCR1* is an abundant RNA with a very long half-life and no poly(A) tail, making it a very useful loading control.

Results

COX17 Behaves as Expected in a *PAB1* Wild Type Strain and in a *PAB1* Wild Type Strain Containing a *PUF3* Deletion

Since it is known that *COX17* mRNA requires PUF3 for deadenylation of the poly(A) tail (Olivas and Parker, 2000; Jackson et al., 2004; Houshmandi and Olivas, 2005), *COX17* is a suitable mRNA for determining if PAB1 is involved in PUF3-mediated deadenylation. *COX17* expression was driven by a *GAL10* promoter allowing for synthesis of *COX17* mRNA upon addition of galactose (pulse) to exponentially growing cells. Deadenylation of the *COX17* mRNA followed after transcription of *COX17* mRNA was shut-off with glucose (chase). Deadenylation of *COX17* was analyzed in *PAB1* domain deletions with wild-type *PUF3* and a *puf3* deletion. In the wild-type *PAB1 PUF3* strain, the *COX17* poly(A) tail reached the oligo(A) form by five minutes, and by fifteen minutes there was almost no transcript remaining (Figure 9A), indicating that mRNA decapping and degradation had taken place. In contrast, in the $\Delta puf3$ background, which is known to display slowed deadenylation of the *COX17* poly(A) tail (Olivas and Parker, 2000), deadenylation was slowed and the oligo(A) form did not appear until 15 to 20 minutes (Figure 9C). Furthermore, in the *PUF3* background, at 30 minutes there is still a significant amount of *COX17* mRNA present (Figure 9C), which is indicative of slowed decapping, as previously observed (Olivas and Parker, 2000). These results reflect what has been previously shown about the effect of PUF3 on *COX17* (Olivas and Parker, 2000), and indicate that this analysis is suitable for determining any PAB1 effects on *COX17* deadenylation.

PAB1-WT

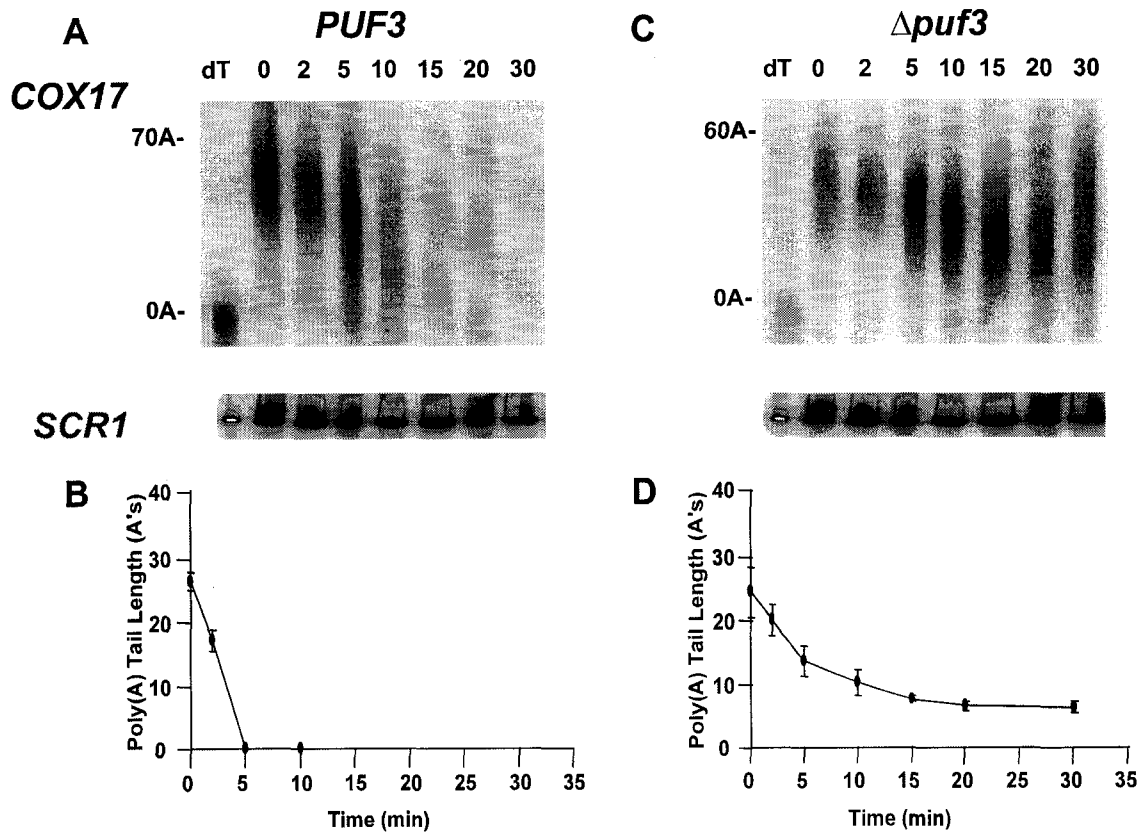


Figure 9 – Deadenylation assay of *COX17* in wild-type *PAB1* background. One sample was hybridized to an oligo-thymidine (dT) in order to identify the fully deadenylated species. *Small cytoplasmic RNA 1 (SCR1)* is used as a loading control. (A) Wild-type *PUF3*. (B and D) Average poly(A) tail length as a function of time after transcription shut off as depicted in panel A and panel C, respectively. (C) $\Delta puf3$.

The PAB1 RRM1 Domain is Necessary for Deadenylation of COX17

Previous analysis has shown that deletion of the RRM1 domain in PAB1 slows deadenylation of *GAL1* and *MFA2* mRNA but does not stop it completely (Yao et al., 2007). If PUF3 does not specifically interact with the mRNP complex or PAB1, then *COX17* should behave the same as *GAL1* and *MFA2* in respect to an RRM1 deletion. Moreover, if the deadenylation mechanism that PAB1 RRM1 works through is the same for all mRNA, then *MFA2* and *COX17* should show similar deadenylation rates relative to the wild-type PAB1. In a strain with a PAB1 RRM1 deletion, *COX17* showed extremely slow deadenylation (Figures 10A, C), in both wild type *PUF3* and the *puf3* deletion, *COX17* poly(A) tail lengths were only marginally shorter in the 30 minute time point compared with the zero minute time point. Compared to the deadenylation rate of *GAL1* and *MFA2* (Figures 11, 12 - Yao et al., 2007), the *PAB1-ΔRRM1* variant showed a block in deadenylation more severe for *COX17*. This observation indicates that the RRM1 domain of PAB1 is absolutely required for deadenylation of the *COX17* poly(A) tail.

PAB1- Δ RRM1

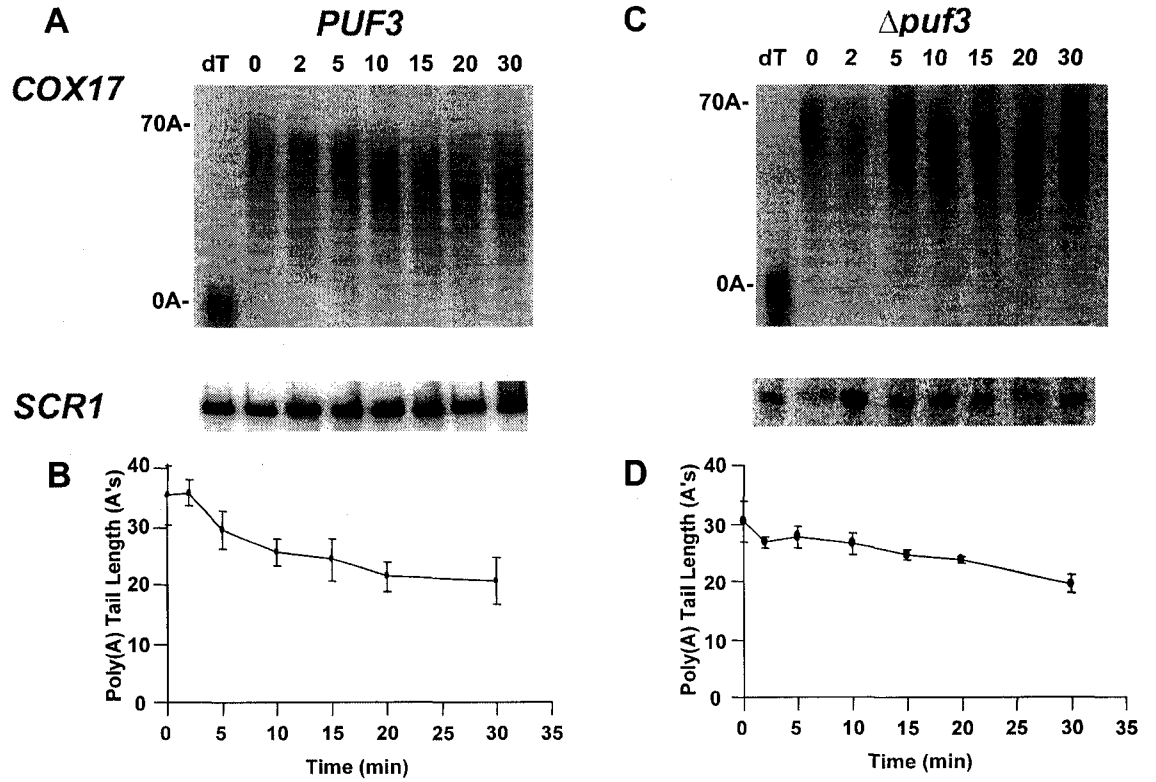


Figure 10 – Deadenylation assay of *COX17* in a *PAB1- Δ RRM1* background. One sample was hybridized to an oligo-thymidine (dT) in order to identify the fully deadenylated species. *Small cytoplasmic RNA 1 (SCR1)* is used as a loading control. (A) Wild-type *PUF3*. (B and D) Average poly(A) tail length as a function of time after transcription shut off as depicted in panel A and panel C, respectively. (C) Δ *puf3*. experiments.

GAL1-L

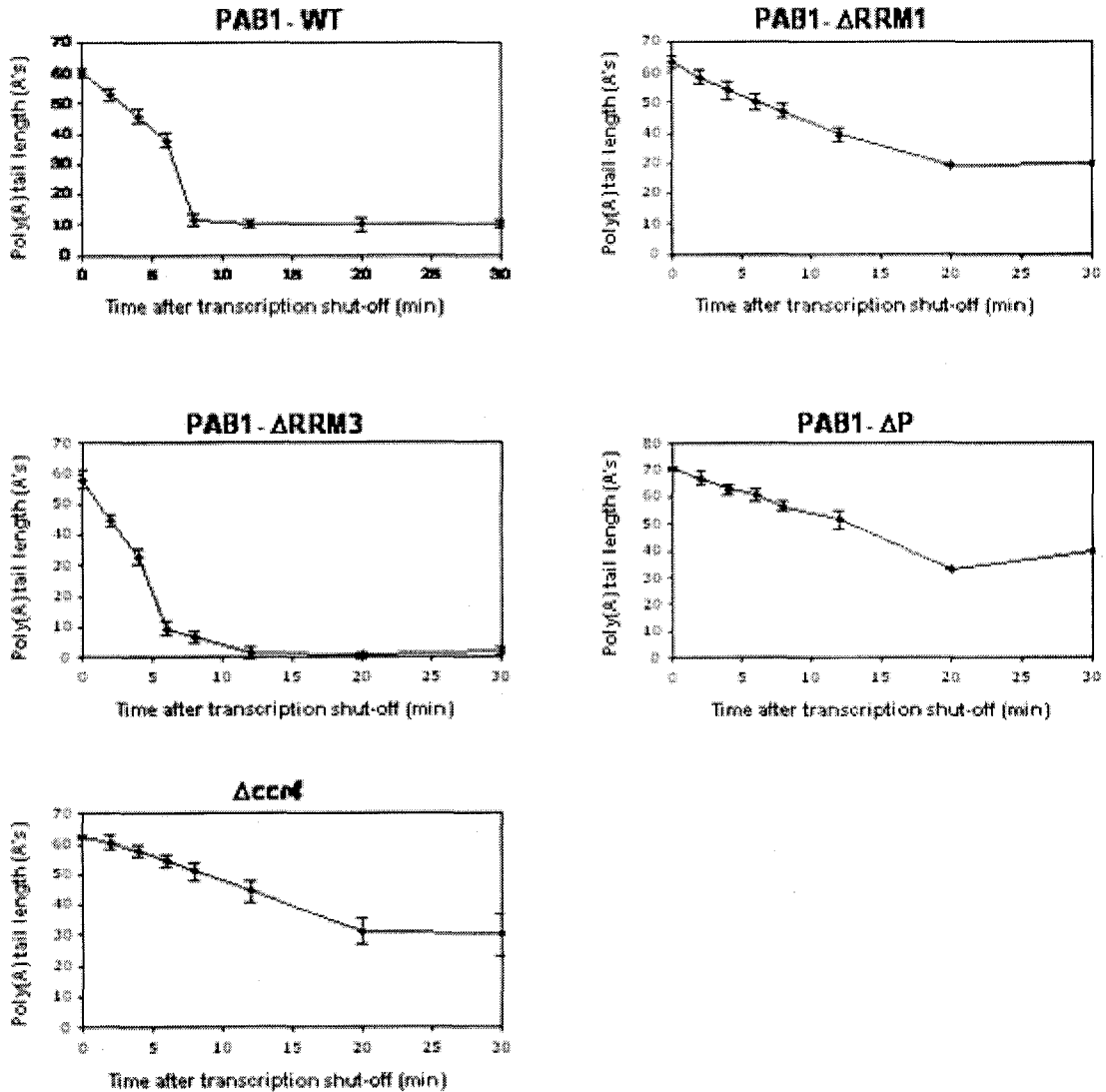


Figure 11 – Summary of *GAL1* deadenylation rates conducted by Gang Yao, and published as supplementary figures (Yao et al., 2007). *PAB1-ΔRRM1* and *PAB1-ΔP* deadenylation rates are much slower compared to wild-type and *PAB1-ΔRRM3*. Deadenylation in *PAB1-ΔRRM1* and *PAB1-ΔP* is blocked as is shown in a $\Delta ccr4$ background.

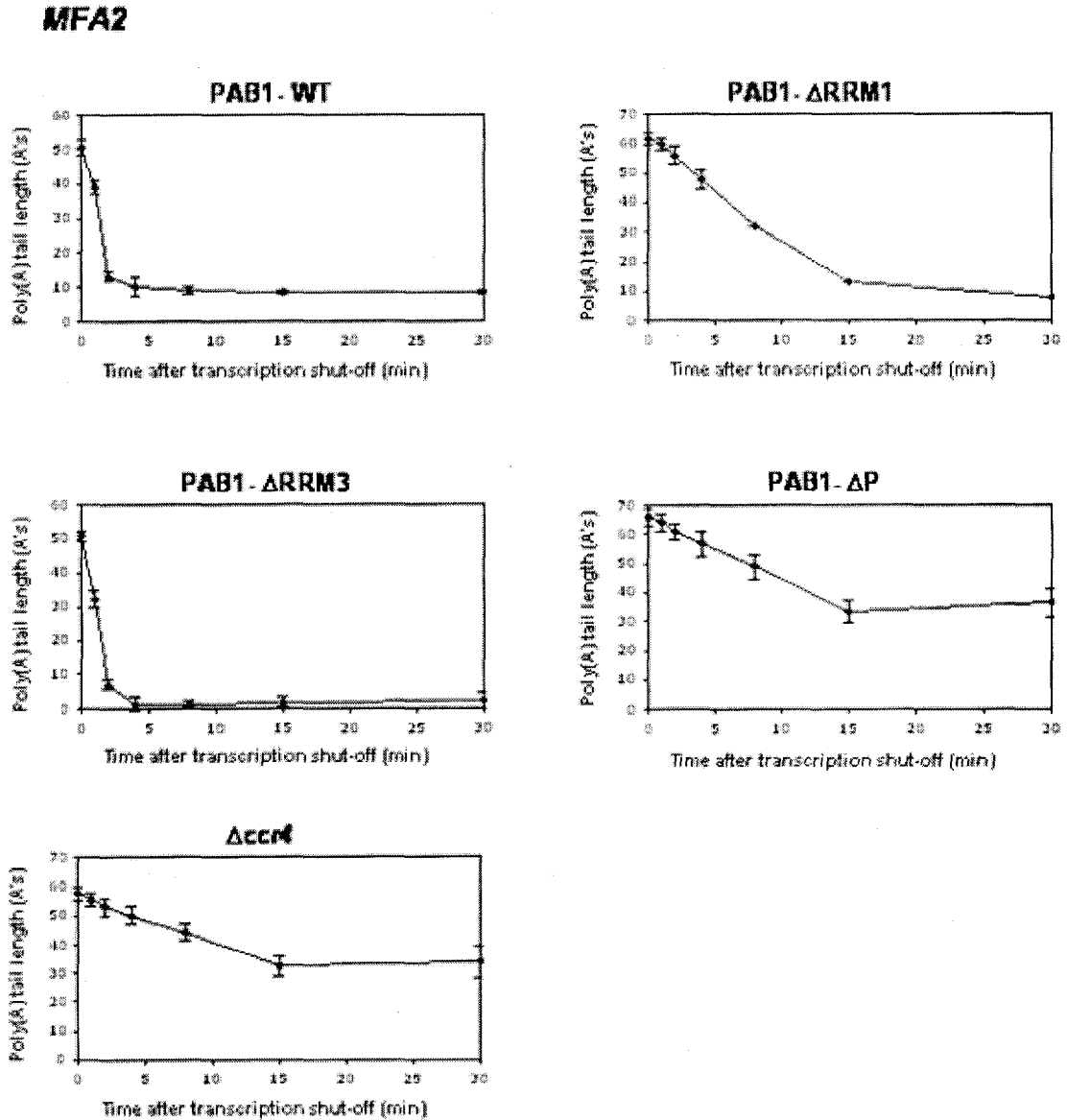


Figure 12 - Summary of *MFA2* deadenylation rates conducted by Gang Yao, and published as supplementary figures (Yao et al., 2007). *PAB1-ΔRRM1* and *PAB1-ΔP* deadenylation rates are much slower compared to wild-type and *PAB1-ΔRRM3*. Deadenylation in *PAB1-ΔRRM1* and *PAB1-ΔP* is blocked as is shown in a $\Delta ccr4$ background.

The Effect of PAB1 RRM2 on COX17

Previous analysis of *PAB1-ΔRRM2* showed a slower deadenylation rate for both *MFA2* and *GAL1* (Yao et al., 2007). The deadenylation rate of *MFA2* and *GAL1* in *PAB1-ΔRRM2* was not as slow as observed for the two domain deletions resulting in the slowest deadenylation rates, *PAB1-ΔRRM1* and *PAB1-ΔP*, but was slower compared to wild-type. My analysis of *COX17* in a *PAB1-ΔRRM2* background also showed a slower deadenylation rate with wild-type *PUF3* and a lesion in *puf3* (Figures 13A, B, C, D), but not as slow as in a *PAB1-ΔRRM1* background (Figure 10A, B). It has been shown that deletion of the RRM2 domain in PAB1 causes destabilization of the CCR4-NOT complex, resulting in about a four-fold reduction in CCR4 abundance in the cell (Yao et al., 2007). Since the CCR4-NOT complex is responsible for the majority of deadenylation of mRNA (Tucker et al., 2001), this explains the slower deadenylation rates for *GAL1*, *MFA2*, and *COX17* in a *PAB1-ΔRRM2* background compared to wild-type (Yao et al., 2007; Figures 9A, B). Additionally, this suggests that the PAB1 RRM2 domain plays the same role in deadenylation for mRNA dependent and independent on *PUF3* for accelerating deadenylation.

PAB1-ΔRRM2

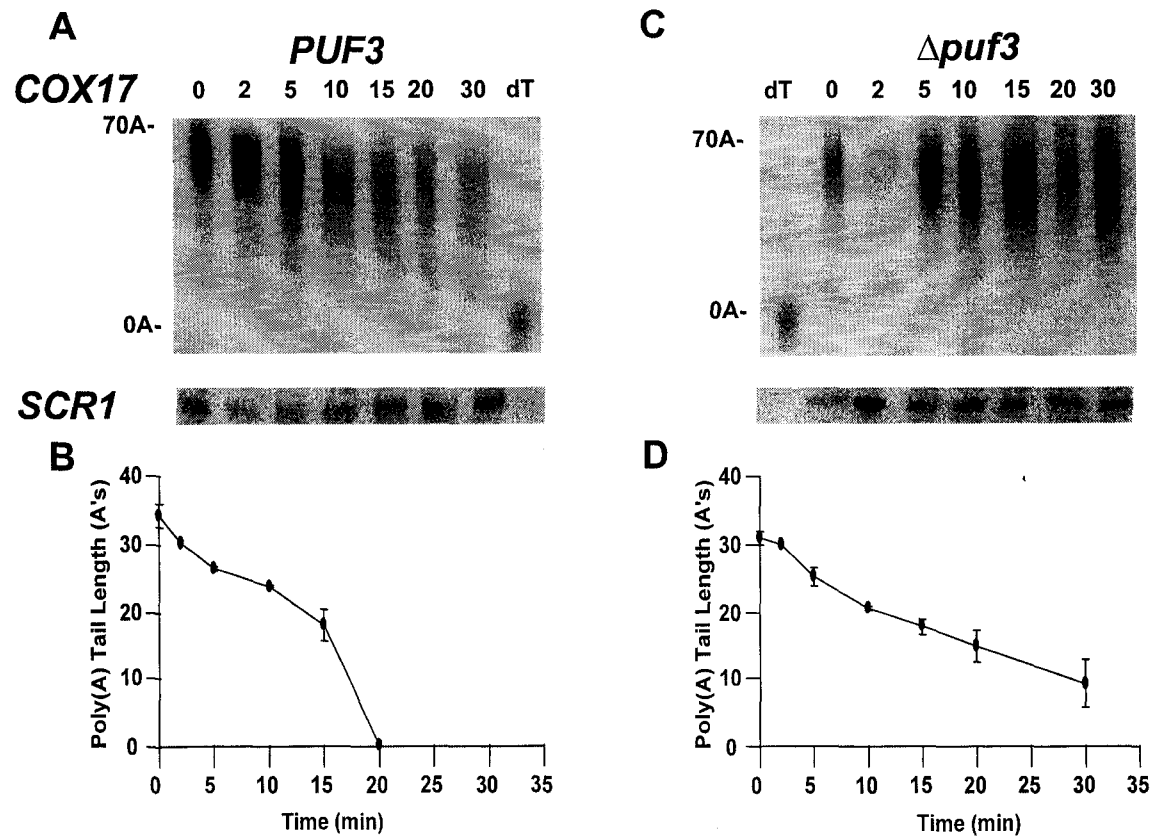


Figure 13 – Deadenylation assay of *COX17* in a *PAB1-ΔRRM2* background. One sample was hybridized to an oligo-thymidine (dT) in order to identify the fully deadenylated species. *Small cytoplasmic RNA 1 (SCR1)* is used as a loading control. (A) Wild-type *PUF3*. (B and D) Average poly(A) tail length as a function of time after transcription shut off as depicted in panel A and panel C, respectively. (C) $\Delta puf3$.

The Effect of PAB1 RRM3 on COX17

Deletion of the RRM3 domain in PAB1 was shown to accelerate the deadenylation rates of *MFA2* and *GALI* mRNA (Yao et al., 2007). The *COX17* transcript in *PAB1-ΔRRM3* with wild-type *PUF3* showed that the poly(A) tail was completely deadenylated by five minutes (Figure 14A), which in wild-type *PAB1*, *COX17* mRNA became nearly completely deadenylated by this time point. Although *COX17* poly(A) tail length at the five minute time point is the same for both wild-type *PAB1* and *PAB1-ΔRRM3*, the poly(A) tail length at the zero minute time point is much shorter in the *PAB1-ΔRRM3* background (~12 A's, Figure 14B) compared to the wild-type *PAB1* background (~28 A's, Figure 9B). In addition, *COX17* mRNA is nearly gone by ten minutes in an RRM3 deletion background in contrast to 15 minutes for wild-type. These results suggest that deleting the RRM3 domain in PAB1 accelerates deadenylation in a PUF3 background.

In the $\Delta puf3$ background, the PAB1 RRM3 deletion also accelerated *COX17* deadenylation (Figure 14C). The *COX17* mRNA does not completely deadenylate in a *puf3* deletion with wild-type *PAB1* (Figure 9C) whereas a fully deadenylated *COX17* mRNA was observed at the fifteen minute time point in a *PAB1-ΔRRM3* background, despite the absence of *puf3* (Figure 14C). This indicates that the mechanism whereby *PAB1-ΔRRM3* accelerates deadenylation is a general mechanism that works on mRNA independent and dependent on PUF3.

PAB1- Δ RRM3

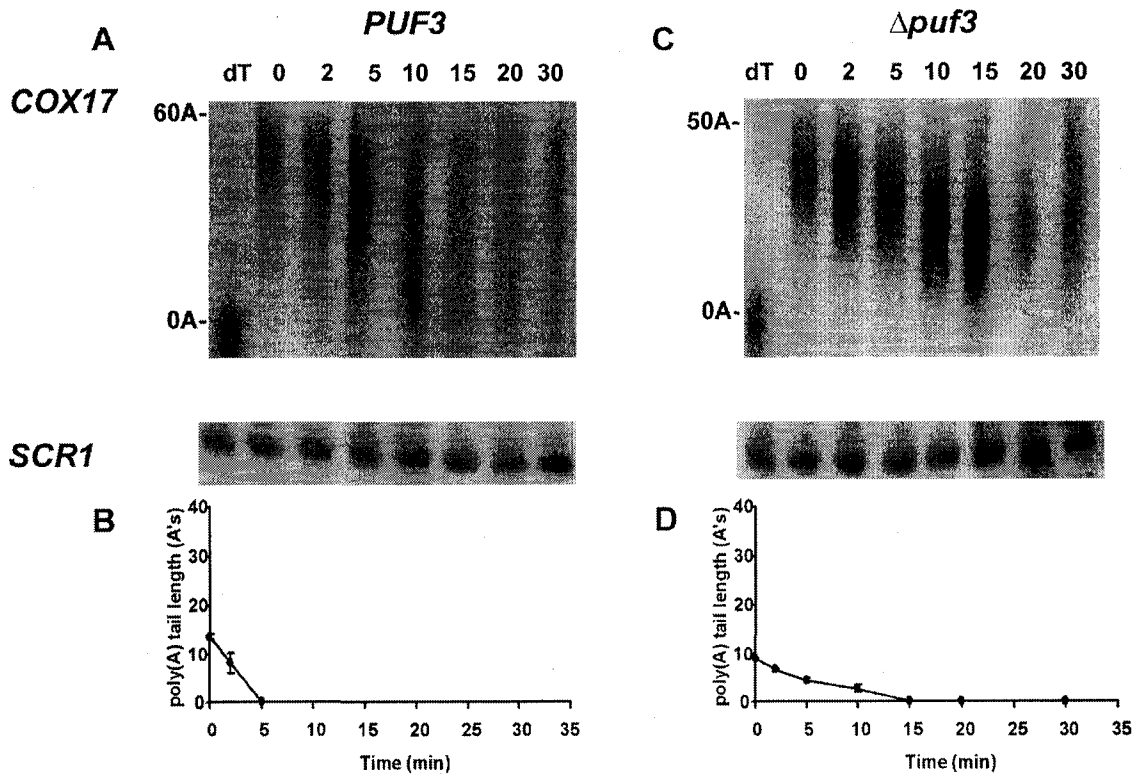


Figure 14 – Deadenylation assay of *COX17* in a *PAB1- Δ RRM3* background. One sample was hybridized to an oligo-thymidine (dT) in order to identify the fully deadenylated species. *Small cytoplasmic RNA 1 (SCR1)* is used as a loading control. (A) Wild-type *PUF3*. (B and D) Average poly(A) tail length as a function of time after transcription shut off as depicted in panel A and panel C, respectively. (C) $\Delta puf3$.

The Effect of PAB1 RRM4 on COX17

Previous analysis of *PAB1-ΔRRM4* showed no change in the deadenylation of *MFA2* and *GAL1* mRNA compared to wild-type (Yao et al., 2007). Analysis of *COX17* mRNA showed a poly(A) tail length of zero A's by five minutes in a *PAB1-ΔRRM4* background (Figure 15A), which is similar to wild-type (Figure 9A). The double mutant, *PAB1-ΔRRM4 Δpuf3*, displayed a slightly slower deadenylation rate compared to *Δpuf3* alone (Figures 15D and 9D). In the *Δpuf3* alone, the poly(A) tail length of *COX17* is deadenylated to a length of 8-10 A's by fifteen minutes and remains at that length for the remaining time points (Figure 9C). In comparison, the double mutant, *PAB1-ΔRRM4 Δpuf3*, showed the *COX17* transcript deadenylated to the same length as the *Δpuf3* alone, 8-10 A's, by twenty minutes (Figure 15C). These observations suggest that the RRM4 domain of PAB1 is at least partially responsible for proper deadenylation of PUF3 dependent transcripts when PUF3 is absent.

PAB1-ΔRRM4

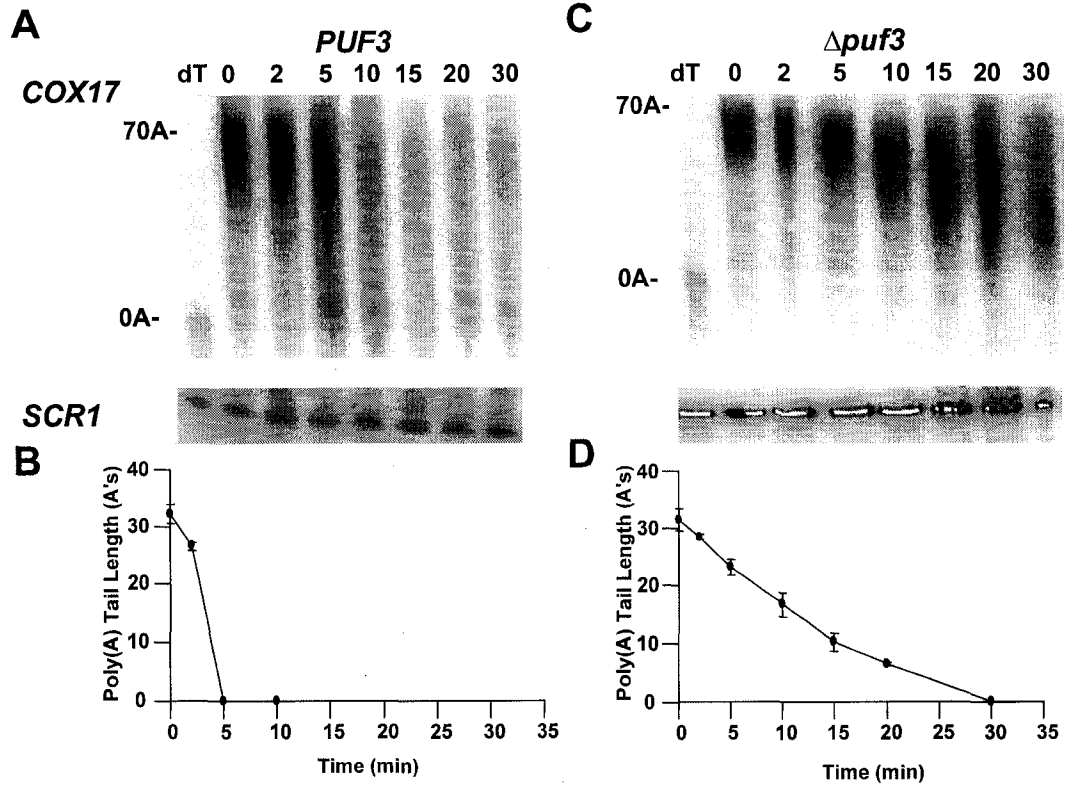


Figure 15 – Deadenylation assay of *COX17* in a *PAB1-ΔRRM4* background. One sample was hybridized to an oligo-thymidine (dT) in order to identify the fully deadenylated species. *Small cytoplasmic RNA 1 (SCR1)* is used as a loading control. (A) Wild-type *PUF3*. (B and D) Average poly(A) tail length as a function of time after transcription shut off as depicted in panel A and panel C, respectively. (C) *Δpuf3*.

PAB1 P Domain is not Necessary for Deadenylation of COX17

In a deletion of the P domain of PAB1, *GAL1* and *MFA2* showed an extreme deficiency in deadenylation (Figures 11, 12 - Yao et al., 2007). In contrast with *GAL1* and *MFA2* deadenylation data, the *PAB1-ΔP* variant showed almost complete deadenylation of *COX17* by ten minutes and no visible mRNA by fifteen minutes (Figure 16A). The *PAB1-ΔP puf3Δ* strain showed a block in deadenylation more severe than the *PAB1 puf3Δ* strain (Figure 16C), after 30 minutes deadenylation remained completely blocked in the *PAB1-ΔP puf3Δ* strain. This observation indicates that the block in deadenylation observed by a PAB1 P deletion is bypassed by PUF3, and that deletion of both *PUF3* and the *PAB1 P* domain severely impairs deadenylation.

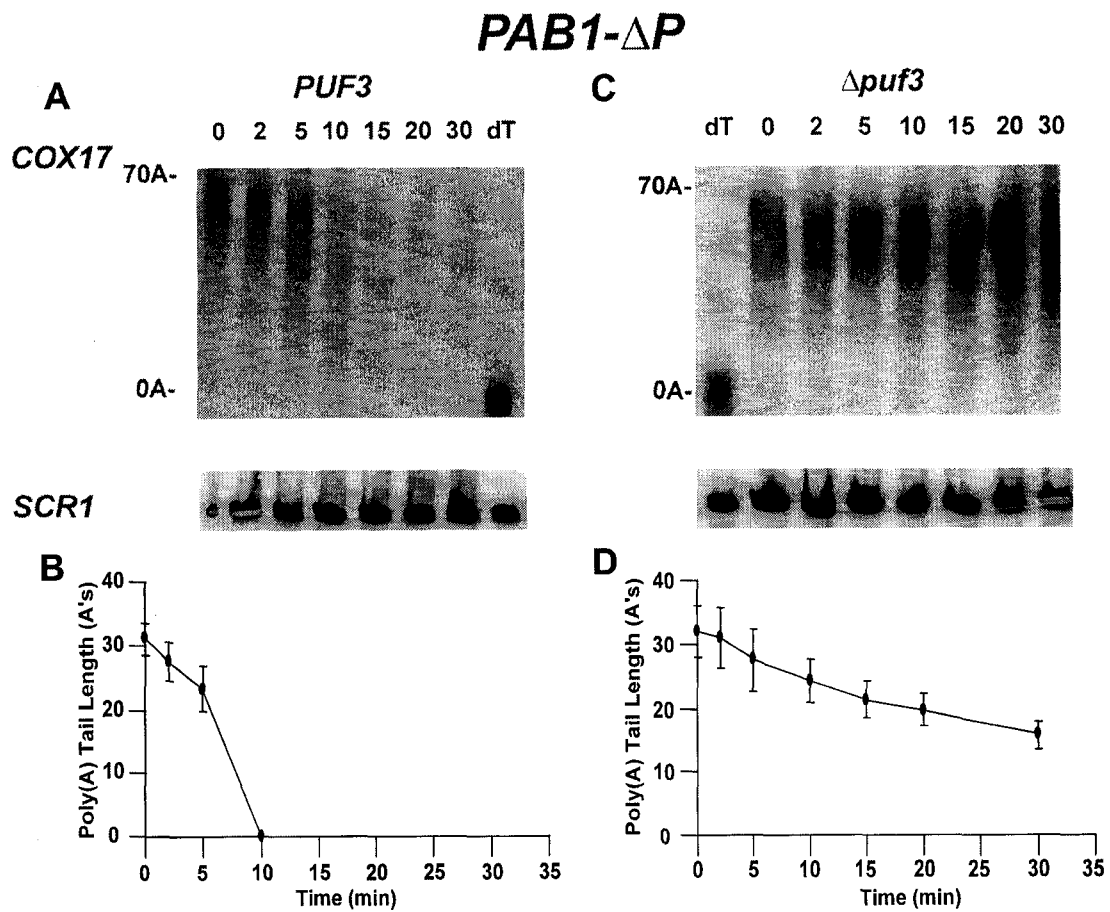


Figure 16 – Deadenylation assay of *COX17* in a *PAB1- Δ P* background. One sample was hybridized to an oligo-thymidine (dT) in order to identify the fully deadenylated species. *Small cytoplasmic RNA 1 (SCR1)* is used as a loading control. (A) Wild-type *PUF3*. (B and D) Average poly(A) tail length as a function of time after transcription shut off as depicted in panel A and panel C, respectively. (C) Δ *puf3*.

The Effect of PAB1 C on COX17

Analysis of *MFA2* and *GAL1* in *PAB1-ΔC* showed a slight decrease in the rate of deadenylation (Yao et al., 2007). The translation termination factor eRF3 and PAN2/3 complex are two proteins that contact the C terminal domain of PAB1 (Mangus et al., 1998; Hosoda et al., 2003; Kobayashi et al., 2004). Since eRF3 and deadenylation has been shown to be linked (Hosoda et al., 2003; Kobayashi et al., 2004) and PAN2 is a known deadenylase, it is likely that the role of the C terminal domain in PAB1 is a general one that affects all or most mRNA. In the *PAB1-ΔC* background the deadenylation rate of *COX17* is slightly slower (Figure 17A) compared to wild-type *PAB1* (Figure 9A) and when *puf3* is deleted in the *PAB1-ΔC* variant (Figure 17C) the deadenylation rate is much slower compared to the wild-type *PAB1 Δpuf3* strain (Figure 9C). By ten minutes the *COX17* poly(A) tail length has reached zero A's in wild-type *PAB1* (Figure 9A) and in the *PAB1-ΔC* variant at ten minutes there is still *COX17* mRNA present (Figure 17A), indicating that deletion of the C domain in PAB1 slows deadenylation slightly. The *COX17* poly(A) tail length in the double mutant, *PAB1-ΔC Δpuf3*, was deadenylated to a length of approximately 20 A's by the fifteen minute time point (Figures 17C, D) and remained at a length of approximately 20A's until the last time point was taken at thirty minutes, which is twice as long as the terminal poly(A) tail length of *COX17* in the wild-type *PAB1 Δpuf3* strain (Figure 9C, D). This indicates that *COX17* mRNA shows a similar decrease in the deadenylation rate as *MFA2* and *GAL1*, which suggests that the C terminal domain of PAB1 plays a PUF3-independent role in deadenylation.

PAB1-ΔC

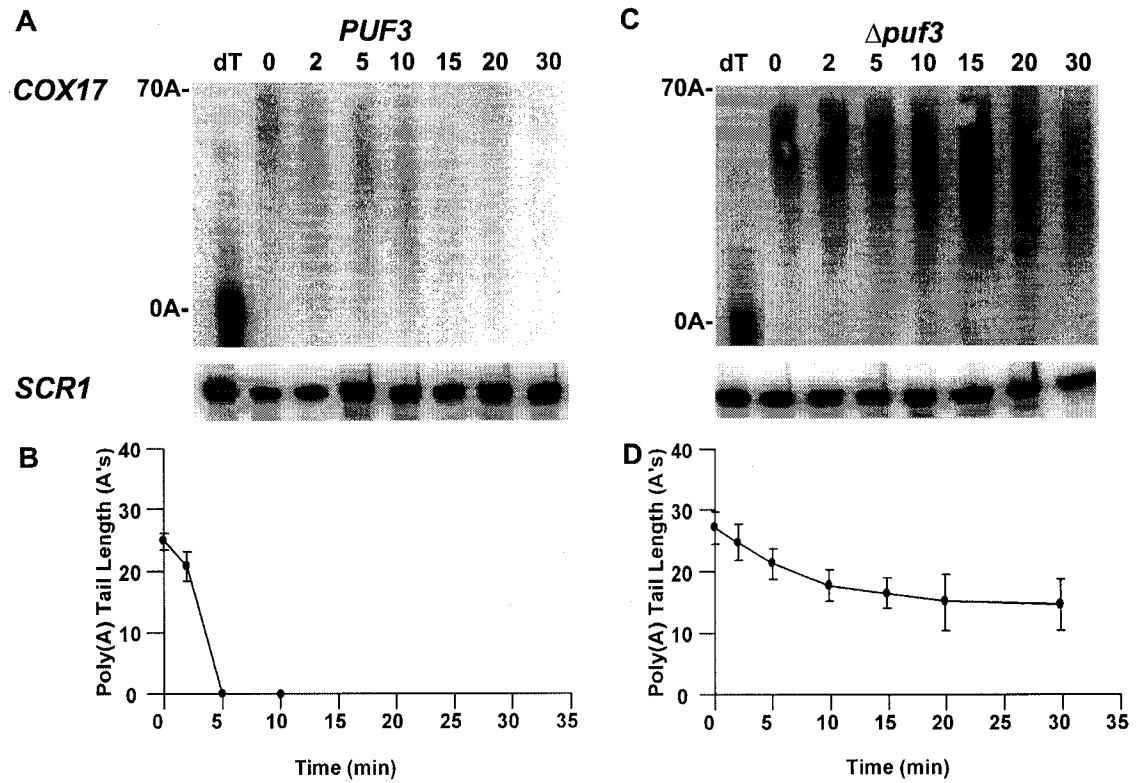


Figure 17 – Deadenylation assay of *COX17* in *PAB1-ΔC* background. One sample was hybridized to an oligo-thymidine (dT) in order to identify the fully deadenylated species. *Small cytoplasmic RNA 1 (SCR1)* is used as a loading control. (A) Wild-type *PUF3*. (B and D) Average poly(A) tail length as a function of time after transcription shut off as depicted in panel A and panel C, respectively. (C) $\Delta puf3$.

Decapping of *COX17* is not Affected by PAB1 Deletions

In the major mRNA degradation pathway, deadenylation is the initial step, followed by decapping and degradation of the mRNA body. Since decapping can be inhibited by PAB1 (Morrissey et al., 1999; Caponigro and Parker, 1995), it is possible that mutations in PAB1 have an effect on decapping and eventual degradation of the mRNA. If a decapping defect is present then two events could occur. First, mRNA degradation could be blocked despite deadenylation of the poly(A) tail. Second, mRNA degradation could occur independently of deadenylation. The first event would be observed if *COX17* mRNA does not degrade following deadenylation. A wild type PUF3 background accelerates *COX17* deadenylation in the following PAB1 domain deletions: *PAB1-ΔRRM2*, *PAB1-ΔRRM3*, *PAB1-ΔRRM4*, *PAB1-ΔP*, and *PAB1-ΔC* (Figures 13A, 14A, 15A, 16A, 17A), and since *COX17* mRNA has degraded following deadenylation, none of these PAB1 domain deletions caused a defect in decapping. Furthermore, when *COX17* deadenylation is blocked as observed in *PAB1-ΔRRM1* we do not see any *COX17* mRNA being degraded (Figure 10A). This last observation does not support the second event, that mRNA degradation could occur independently of deadenylation.

A *puf3* deletion is known to block *COX17* mRNA decapping. This can be observed in Figure 9C in which *COX17* mRNA does not become degraded after the oligo (A) form of *COX17* is reached at about 15 minutes. None of the PAB1 variants that I analyzed bypassed this affect of *puf3Δ* on the decapping of *COX17*. These observations indicate that the analyzed PAB1 domain deletions do have a role in deadenylation but not in decapping.

The decapping step has been shown to occur in discrete locations in the cytoplasm, labeled P bodies (Sheth and Parker, 2003). PAB1 and the deadenylases have been shown to localize to the cytoplasm (Sheth and Parker, 2003), suggesting that deadenylation occurs in the cytoplasm and the deadenylated mRNA then moves to P bodies where decapping and further degradation occurs. Since deadenylation occurs in a *puf3* deletion but degradation does not occur (Figures 9C, 10C, 13-17C) it is likely that PUF3 has a role in the degradation and possibly decapping of *COX17* mRNA. Because PUF3 is localized in the cytoplasm (Sheth and Parker, 2003), while PUF3 itself does not localize to P bodies (Sheth and Parker, 2003) it is possible that PUF3 is involved in the transport of *COX17* mRNA from the cytoplasm to P bodies. This could be easily observed by examining the localization of *COX17* mRNA. A GFP-MS2 fusion has been shown allow visualization of an mRNA containing a MS2 binding site within the transcript (Sheth and Parker, 2003). Determination of the localization of *COX17* in *PUF3* and Δ *puf3* strains could be accomplished by examining if PUF3 has a role in the transportation of *COX17-MS2* from the cytoplasm to P bodies.

The Effect of eIF4E on *COX17*

A defect in the cap binding protein, eIF4E, was shown to accelerate the deadenylation rate of the stable mRNA, *PGK1*, which has an average half-life of 17 minutes in a wild-type background (Schwartz and Parker, 1999). The unstable mRNA, *MFA2*, which has a half-life of 3.5 minutes, did not show an accelerated deadenylation rate when a defect in eIF4E was present (Schwartz and Parker, 1999). The first observation indicates that wild-type eIF4E slows deadenylation by some unknown mechanism. The second observation could be explained by three possibilities. First, the

deadenylation mechanism that eIF4E acts on does not affect *MFA2*. The second possibility is that the deadenylation rate of *MFA2* is so fast that the deadenylation mechanism that eIF4E acts on does not affect *MFA2* or any mRNA with a fast deadenylation rate. Third, the defect in eIF4E does accelerate the deadenylation rate, but the change is undetectable by this assay.

Our previous results showed that PUF3 is capable of co-immunoprecipitation of eIF4E (Y.C. Chiang, personal communication). If PUF3 makes contact with eIF4E or another translation initiation factor to mediate deadenylation then a defect in eIF4E should show a change in the deadenylation rate of *COX17*. Since it has been shown that a defect in eIF4E is capable of accelerating the rate of deadenylation for *GAL1* and *PGK1* (T. Ohn, personal communication; Schwartz and Parker, 1999), we would expect that deadenylation of *COX17* would be accelerated as well. In order to test if the eIF4E defect affects deadenylation of *COX17*, I analyzed the *COX17* deadenylation rate in a strain carrying the *cdc33-1* allele, which encodes a temperature sensitive eIF4E protein. The *cdc33-1* allele, at 37°C, did not cause a significant increase in the deadenylation rate compared to the wild-type strain at 37°C (Figure 18A, 19A), and both wild-type and *cdc33-1* strains showed oligo-adenylated poly(A) tail length by two minutes and the *COX17* mRNA was gone by five minutes (Figure 18A, 19A). This observation is similar to what was observed for *MFA2* (Schwartz and Parker, 1999) and the same three possibilities apply. It is possible that the mechanism whereby eIF4E accelerates deadenylation does not work on *COX17*. However, since both *COX17* and *MFA2* are both very unstable mRNA, it is likely that the mechanism that eIF4E works through is

just not apparent for these two mRNA, as the deadenylation rate is already so fast that a defect in eIF4E would not accelerate it even further.

If the mechanism whereby eIF4E works to accelerate deadenylation is a general mechanism affecting all mRNA, then deadenylation of *COX17* in a *puf3* deletion background should be accelerated as well. Analysis of the double mutant, *cdc33-1 Δpuf3*, showed deadenylation of *COX17* stop at ten minutes (Figures 19B, D), which is the same time point when deadenylation of *COX17* stopped in the strain carrying the lesion in *puf3* alone at 37°C (Figure 18B, D). The difference in poly(A) tail length may be due to decapping occurring much faster due to the defect in eIF4E, which has been observed previously to inhibit the decapping reaction (Schwartz and Parker, 1999; Schwartz et al., 2003). A defect in eIF4E does appear to affect the final length of the *COX17* poly(A) tail if *puf3* is absent (Figures 18B, 18D, 19B, 19D), so it is likely that eIF4E does affect *COX17* mRNA. However, these observations indicate that PUF3 is required to accelerate deadenylation of *COX17*, despite the defect in eIF4E (Figures 19A, B, C, D). This indicates that the mechanism that PUF3 acts through, is downstream of the mechanism that eIF4E works through.

PAB1-WT 37°C

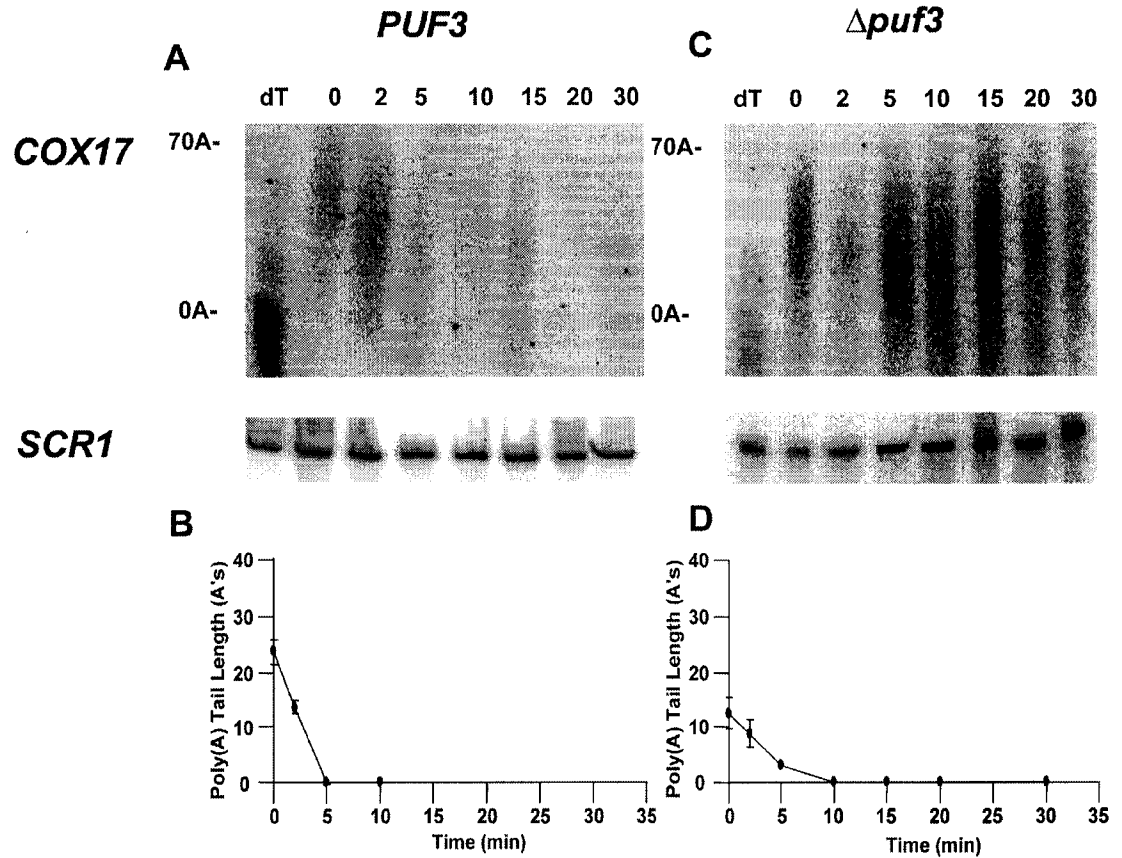


Figure 18 – Deadenylation of *COX17* at 37°C. Assay was conducted at 37°C, as a control for the *cdc33-1* allele. One sample was hybridized to an oligo-thymidine (dT) in order to identify the fully deadenylated species. *Small cytoplasmic RNA 1 (SCR1)* is used as a loading control. (A) Wild-type *PUF3*. (B and D) Average poly(A) tail length as a function of time after transcription shut off as depicted in panel A and panel C, respectively. (C) $\Delta puf3$.

PAB1-WT 37°C

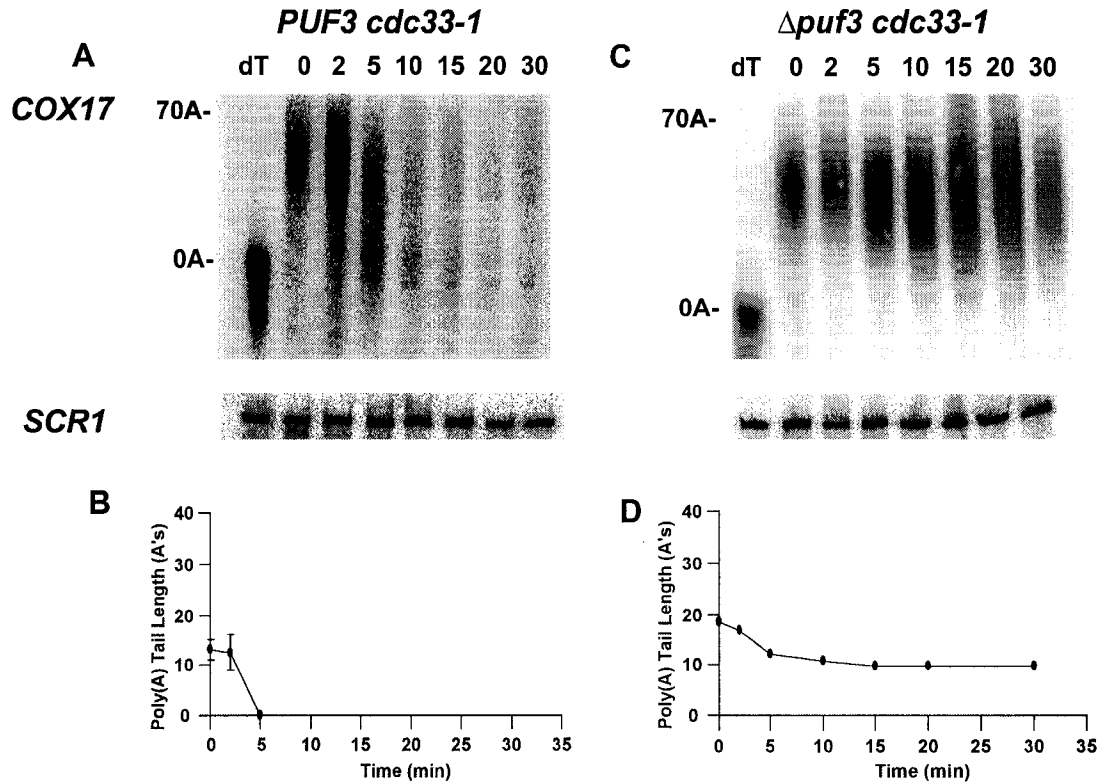


Figure 19 – Deadenylation assay of *COX17* in *PAB1-WT cdc33-1* background. Assay was conducted at 37°C, the non-permissive temperature for the *cdc33-1* allele. One sample was hybridized to an oligo-thymidine (dT) in order to identify the fully deadenylated species. *Small cytoplasmic RNA 1 (SCR1)* is used as a loading control. (A) Wild-type *PUF3*. (B and D) Average poly(A) tail length as a function of time after transcription shut off as depicted in panel A and panel C, respectively. (C) $\Delta puf3$.

Strain	Relevant Genotype	Rate (A's/min)	t for 0 A's	SEM	Total Samples
319/504	<i>PAB1-WT</i>	5.4	5	0.3	4
319/505	<i>PAB1-ΔRRM1</i>	0.5	>30	0	4
319/506	<i>PAB1-ΔRRM2</i>	1.4	20-30	0.3	2
319/507	<i>PAB1-ΔRRM3</i>	3.5	5	0.6	2
319/508	<i>PAB1-ΔRRM4</i>	5.3	10	1	3
319/510	<i>PAB1-ΔC</i>	3.1	10	0.2	5
319/509	<i>PAB1-ΔP</i>	5.7	5	0.3	3
319/504 37°C	<i>PAB1-WT 37°C</i>	4.7	5	0.4	3
1881/504	<i>cdc33-1</i>	1.7	5	0.4	2
1738/504	<i>PAB1-WT Δpuf3</i>	0.9	30	0.2	4
1738/505	<i>PAB1-ΔRRM1 Δpuf3</i>	0.4	>30	0	3
1738/506	<i>PAB1-ΔRRM2 Δpuf3</i>	0.6	>30	0.1	2
1738/507	<i>PAB1-ΔRRM3 Δpuf3</i>	0.5	15	0.1	2
1738/508	<i>PAB1-ΔRRM4 Δpuf3</i>	1	20	0	3
1738/510	<i>PAB1-ΔC Δpuf3</i>	0.6	>30	0	4
17.8/509	<i>PAB1-ΔP Δpuf3</i>	0.7	>30	0	3
1738/504 37°C	<i>PAB1-WT Δpuf3 37°C</i>	1.3	10	0.1	2
1777/504	<i>cdc33-1 Δpuf3</i>	0.4	>30	0.1	2

Table 9 – Summary table of *COX17* deadenylation rates. SEM is the standard error of the mean of the rate, calculated by dividing the standard deviation by the square root of the total number of samples. For strains with a very fast deadenylation rate it was difficult to obtain a precise measure of the true deadenylation rate. In order to analyze the deadenylation rate, a qualitative approach was used. The qualitative approach that I used was to describe at what time point the *COX17* poly(A) tail was fully deadenylated as indicated by “t for 0 A’s”.

Discussion

This data provides three observations supporting the model that PUF3 interacts with components of the mRNP complex for proper deadenylation. First, the RRM1 domain of PAB1 is required for deadenylation of *COX17* (Yao et al., 2007). Second, PUF3 is able to bypass the requirement of the P domain in PAB1, which was shown to be required for deadenylation of *MFA2* and *GAL1* (Yao et al., 2007). Third, we have also shown that PUF3 co-immunoprecipitates multiple components of the CCR4-NOT complex (Y. Chiang, personal communication), and *COX17* requires CAF1, CCR4, and PAN2 for deadenylation (Tucker et al., 2002). These observations suggest that PUF3 works to simultaneously perturb the mRNP complex and recruit deadenylases.

Although this data shows an interaction between PAB1 and PUF3, the exact mechanism whereby PUF3 communicates with PAB1 is still a mystery. Since PUF3 did not co-immunoprecipitate PAB1 (data not shown), nor did a poly(A) sepharose pull-down identify PUF3 (G. Quigley, personal communication), it is likely that PUF3 makes unknown proteins contacts which are responsible for PAB1 contacts. In *Drosophila*, *Pumilio* contacts *Nanos* and *Brat* to repress *hunchback* mRNA (Edwards et al., 2001; Wang et al., 2002; Wang et al., 2001; Edwards et al., 2003). This observation, supports the model that PUF3 in *Saccharomyces* could function in a similar manner by contacting additional proteins that are responsible for contacting PAB1.

Since eIF4E did co-immunoprecipitate with PUF3, it is possible that some sort of communication between PUF3 and PAB1 is mediated through the cap-binding protein.

In order to show that PUF3 does in fact contact eIF4E, the pull down would need to be conducted in the presence of RNaseA to rule out RNA contacts. If the eIF4E and PUF3 contact is RNA independent then a yeast two-hybrid experiment, or an *in vitro* binding experiment could be used to show that the two proteins do in fact interact. Additionally, to show that eIF4E does act on PUF3 controlled mRNA, a mRNA with a slower deadenylation rate would need to be used to show that a defect in eIF4E does in fact accelerate deadenylation of a PUF3 controlled mRNA. *CYT2* or *TUF1* have recently been shown to be controlled by PUF3 (Olivas W, poster #380, RNA meeting 2007). These mRNA would be suitable candidates to show that a defect in eIF4E does accelerate deadenylation of PUF3 controlled mRNA. Alternatively, a chimeric *GALI-COX17* 3' UTR could be used for further analysis. This could be used to show that the PUF3 consensus sequence is sufficient to accelerate deadenylation of a non-PUF3 controlled mRNA. If the chimeric reporter has a slower deadenylation rate then we could analyze the mRNA in a *cdc33-1* background, which could show more definitively that a defect in eIF4E does or does not require PUF3 to accelerate deadenylation.

This work and previous work has shown that the various PAB1 domains each play different roles in deadenylation. It is likely that since the RRM2, RRM3, and C domains in PAB1 act on general mechanisms that they affect all mRNA, or at the very least that they affect both PUF3-dependent and PUF3-independent transcripts. The P domain in PAB1 is necessary for proper deadenylation of transcripts that do not require PUF3. The requirement for this domain in *COX17* deadenylation was bypassed by the PUF3 protein to allow rapid deadenylation and degradation of the *COX17* mRNA. PUF3 and the P domain function may act through a similar mechanism in the deadenylation of

transcripts. This work has shown that the PAB1 RRM1 domain is absolutely necessary for deadenylation of *COX17*, indicating that PUF3 somehow works through PAB1 RRM1 in some way to accelerate deadenylation. Although further work is necessary to definitively prove that eIF4E requires PUF3 for proper deadenylation, these results suggest that PUF3 works downstream of eIF4E in the deadenylation process.

This work provides support for the model that PUF3 does in fact work through the mRNP complex to accomplish deadenylation of its target transcripts. Our studies and those of others have also provided evidence that PUF proteins recruit deadenylases to the target transcript, suggesting that PUF proteins both recruit and perturb the mRNP complex to accomplish deadenylation and eventual degradation of the transcript.

GENERAL DISCUSSION

In this work I have investigated the biological roles of the mRNP complex, focusing specifically on the poly(A) binding protein (PAB1) and its role in mRNA deadenylation. The two processes that I have examined are: the relationship of to mRNA deadenylation and the role of PAB1 in controlling regulated mRNA deadenylation.

The *in vivo* translation analysis of PAB1 was conducted for two reasons. First, *in vivo* translation rates were measured in each of the PAB1 variants to determine which, if any of the domains have a role in translation *in vivo*. The second reason for this analysis is to determine if any of the PAB1 domains have pleiotropic effects, specifically on translation and deadenylation. It has been shown that translation and mRNA degradation are linked (Schwartz and Parker, 1999), and it is also known that PAB1 plays a role in both translation (Sachs and Deardorff, 1992; Otero et al., 1999; Gray et al., 2000) and mRNA deadenylation (Tucker et al., 2002; Wilusz et al., 2001; Caponigro and Parker, 1995). Hence, a defect that a PAB1 variant may have on mRNA deadenylation could also cause a defect in translation or a defect in translation could cause a defect in deadenylation.

The first conclusion of the *in vivo* translation analysis is that individual domains of PAB1 do not have a major role in translation. Because none of the PAB1 variants displayed a decrease in translation rate as severe as defects in known translation initiation components, such as eIF3 or eIF4E (Figure 4, Table 3), this analysis suggests that PAB1 has a minor role in translation. However, the minor role that PAB1 does play in translation appears to be mediated by the RRM1 and RRM2 domains. Of the PAB1

domain deletions, only deleting RRM1 and RRM2 showed a significant decrease in the rate of *in vivo* translation (Figure 5, Table 4), suggesting that only two of the six domains are involved in translation. Previous experiments support this observation. Analysis of PAB1 domains capable of translational stimulation showed that only domains RRM1 and RRM2 were sufficient to stimulate translation (Gray et al., 2000). Since PAB1 lethality can be rescued by RRM1/2 but not other domains, these observations suggest that the PAB1 translational role of RRM1/2 is important. But since individual deletions of PAB1 RRM1 and RRM2 had a minor effect on translation, it is likely that the PAB1 RRM1 and RRM2 domains do not have direct roles in translation, and appear to support the function of additional factors.

Analysis of the PAB1 variants in combination with other factors indicates that PAB1 RRM2 has roles in translation related to eIF4E and eIF3. eIF4E and eIF3 are involved in different aspects of translation initiation, suggesting that PAB1 is involved in different processes of translation. These observations do not indicate a definitive mechanism for PAB1 involvement in translation but does indicate that PAB1 does in fact play multiple roles. Additionally, this data confirms previous *in vitro* experiments which indicate PAB1 RRM2 plays a key role in translation (Kessler and Sachs, 1998; Otero et al., 1999).

My work provides additional evidence linking translation and deadenylation. CAF1 involvement in deadenylation is known (Tucker et al., 2002), albeit, in a partially separate role from the major deadenylase, CCR4 (Ohn et al., 2007). My analysis has confirmed that CAF1 has a role in translation and that role is more important and different from CCR4 (Figure 8, Table 7). The evidence suggesting that CAF1 and CCR4

have separate roles in translation is supported by the observation that a lesion in *caf1* caused a greater defect in translation combined with the *PAB1-184* variant, whereas a lesion in *ccr4* caused a greater defect in translation when combined with the *PAB1-134* variant (Figure 8). Because a deletion of *caf1* has a greater defect on translation with *PAB1-184*, whereas a deletion of *ccr4* did not, it is likely that CAF1 is involved in the mechanism that *PAB1-184* participates in, whereas CCR4 is involved in a similar mechanism as *PAB1-134*. *PAB1-184* functions to aid eIF4G contact to PAB1 and *in vitro* it blocks translation. *PAB1-134* in contrast, destabilizes the mechanism of translation initiation that eIF3 is involved in. This indicates that PAB1 is involved in translation and that involvement requires many additional factors. Furthermore, because defects in PAB1 alone do not cause major defects in translation, and a large decrease in translation is observed when PAB1 variants are combined with other defects, this suggest that PAB1 may not have a direct role in translation. Instead, PAB1 may merely serve as a scaffold for the translation initiation factors to assemble on. More specifically, the PAB1 RRM1 and RRM2 domains are the location for the translation initiation factors to bind.

In the second part of my thesis, I analyzed the effect of PAB1 defects on on the PUF3 regulated deadenylation of *COX17*. Transcriptional pulse-chase analysis of PAB1 domain deletions, displayed a decreased deadenylation rate for *GAL1*, *MFA2*, and *COX17* in *PAB1-ΔRRM2* and *PAB1-ΔC* backgrounds (Yao et al., 2007; Figures 13A, B, 17A, B; Table 9), whereas, a *PAB1-ΔRRM3* background displayed an accelerated deadenylation rate (Yao et al., 2007; Figure 14A, B; Table 9). These observations suggest that PAB1 acts positively and negatively on deadenylation, multiple PAB1 domains are involved in deadenylation, and these domains act through mechanisms that are similar for PUF3

dependent and independent mRNA. The most severe deadenylation defect of *GALI* and *MFA2* was in *PAB1-ΔRRM1* and *PAB1-ΔP* backgrounds (Figure 11, 12 - Yao et al., 2007). These domains have also been shown to be important for PAB1 self association (circular form) and formation of PAB1 multimers. More importantly, these self-associated PAB1 species bind poly(A) less efficiently than single, linear PAB1 (Yao et al., 2007). This suggests that formation of circular PAB1 causes dissociation of PAB1 from the poly(A) tail, which stimulates deadenylation (Figure 20A). Because deletion of the RRM1 domain caused the most severe deadenylation defect of *GALI*, *MFA2*, and *COX17* (Figures 11, 12 - Yao et al., 2007; Figure 10A, B; Table 9) it is likely that this domain is the most crucial for the PAB1 role in deadenylation. A likely model is that PAB1 RRM1 is responsible for PAB1 dissociation and binding of poly(A). Since roughly equivalent amounts of circular and linear PAB1 have been observed (Yao et al., 2007), it is likely that the disassociated PAB1 (circular) and bound PAB1 (linear) are in equilibrium. In order for rapid deadenylation to occur, some sort of stabilizing mechanism is necessary to maintain the PAB1 circular form.

In addition to the PAB1 RRM1 domain, the P domain in PAB1 is also necessary for formation of the PAB1 circular species. This suggests that the PAB1 P domain is necessary for formation of circular PAB1, and therefore would be necessary for deadenylation. It has been demonstrated that the PAB1 P domain is necessary for deadenylation of *GALI* and *MFA2* but not *COX17* (Figures 11, 12 - Yao et al., 2007; Figure 16A, B; Table 9). Since PUF3 is involved in deadenylation of *COX17*, it is likely that PUF3 bypasses the requirement of the PAB1 P domain. If the PAB1 P domain is acting to promote formation of circular PAB1 then PUF3 must also promote formation of

circular PAB1. Since it has not been shown that PUF3 contacts PAB1 (G. Quigley, personal communication; results not shown), it is likely that PUF3 contacts another protein which is responsible for contacting and stabilizing the circular form of PAB1.

Several observations suggest that eIF4E and other TIFs are involved in preventing formation of circular PAB1. The first observation is that eIF4E co-immunoprecipitates with PUF3. Second, PAB1- Δ RRM2 can still interact with TIFs (Y. Chiang, personal communication). Since PAB1 RRM2 is known to contact eIF4G, which in turn binds eIF4E, these results imply that the TIFs are making contact to other domains of PAB1, such as to RRM1. Third, deleting the RRM1 domain reduces by two to three fold the amount of eIF4G associated with PAB1, which suggests that eIF4E contacts PAB1 through contacts in addition to the RRM2 domain of PAB1. This suggests that PUF3 could contact PAB1 through eIF4E. Fourth, a defect in eIF4E caused accelerated deadenylation of *GAL1* and *PGK1* (Schwartz and Parker, 1999; T. Ohn, personal communication). This suggests that the TIF complex blocks or slows deadenylation, perhaps by inhibiting the formation of the circular PAB1. One model that incorporates these observations is that eIF4G contacts PAB1 through the RRM1 domain to prevent PAB1 from circularizing and dissociating from poly(A). In this model, dissociation of PAB1 from poly(A) occurs through circularization of PAB1, which happens freely and is the favored structure, but, eIF4G, or any TIFs, contact to PAB1 prevents circularization. Through binding of the PAB1 P domain or PUF3 to eIF4E/eIF4G/eIF4A the connection between PAB1 and the TIFs is broken or diminished, thus allowing for more circular PAB1 and making the poly(A) to be more accessible to CCR4 (Figure 20B).

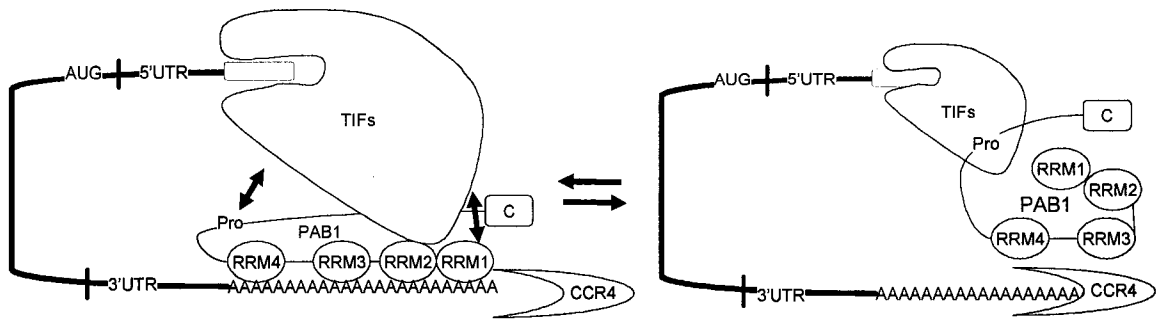


Figure 20A – Model for PAB1 self-association and disassociation from poly(A). TIFs contact PAB1 RRM1, which prevents PAB1 circularization. The PAB1 P domain contacts the TIFs to prevent the contact to RRM1, thus allowing for circularization of PAB1 through RRM1.

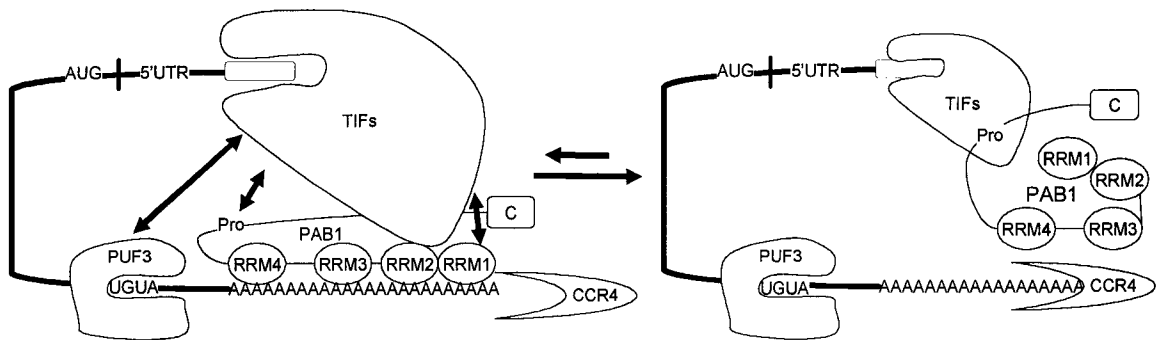


Figure 20B – Competition model for PUF3 mediated mRNP disruption. PUF3 plays a similar role as the PAB1 P domain. PUF3 contacts the TIFs to reduce TIF contact with the PAB1 RRM1 domain, thus favoring circularization of PAB1 and dissociation of PAB1 from poly(A).

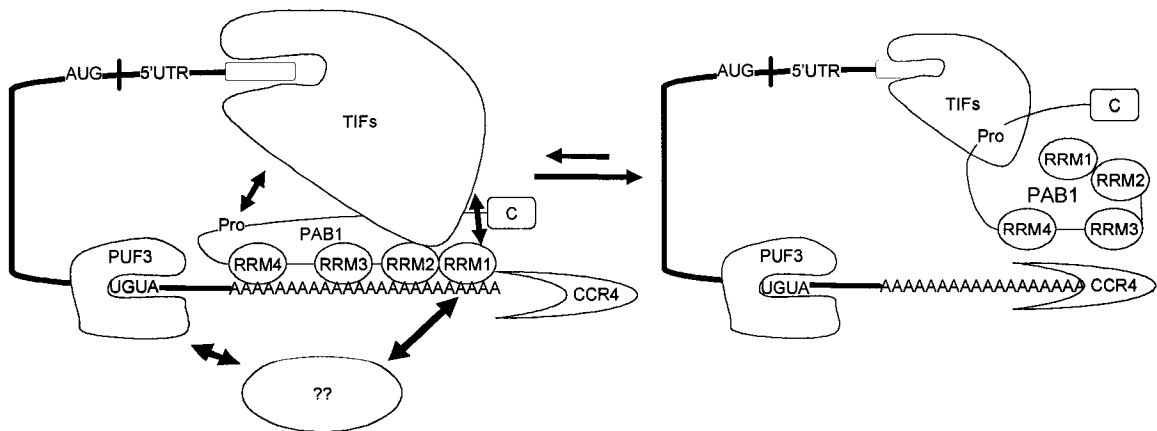


Figure 20C – Alternative competition model for PUF3 mediated mRNP disruption. Instead of PUF3 contacting the TIFs which contact PAB1, PUF3 contacts an unknown protein which contacts PAB1.

Obviously, more work needs to be done to support this model. If TIFs are responsible for inhibiting formation of circular PAB1, the amount of circular PAB1 should be higher in a *cdc33-1* background compared to wild-type. This could be shown by comparing the relative abundance of the circular form in a *cdc33-1* background versus a wild-type.

In order to prove the competition model, whereby the P domain of PAB1 or PUF3 competes with PAB1 RRM1 for TIF binding, three observations would need to be confirmed. First, it must be shown that PUF3 contacts TIFs directly, which could be done through *in vitro* binding assays. Second, it must be shown that addition of PUF3 disrupts or diminishes the TIF PAB1 contact. If the first observation is correct, then this could be done by adding PUF3 to purified eIF4G and purified PAB1, and determining if less eIF4G binds to PAB1. Third, contact between eIF4G and PAB1 must be completely disrupted and shown that this contact has a role in formation of circular PAB1 and accelerates deadenylation. Since PAB1-180 and PAB1-184 have been shown to contact eIF4G (Otero et al., 1999), a combination of these two mutations must be analyzed. Alternatively, the CAF20 protein is known to compete with eIF4G for eIF4E (Altmann et al., 1997; Ptushkina et al., 1998). Over-expression of CAF20 could therefore be used to disrupt the eIF4E contact to PAB1.

If it cannot be demonstrated that PUF3 communicates with PAB1 through the TIFs, it is possible that another protein mediates the connection (Figure 20C). It is possible that an unknown protein (not a TIF) contacts PUF3 which in turn contacts PAB1 to promote circularization and dissociation from the poly(A) tail. This hypothetical protein could be identified through PUF3 purification and mass spec analysis of

associated proteins. An alternative method would be through a two-hybrid screen for proteins that interact with PUF3. Since this protein would need to contact PAB1, likely candidates would be proteins that contact PUF3 who are known to also contact PAB1.

This work shows that PAB1 has multiple roles, both in deadenylation and translation. Two observations suggest it is likely that PAB1 has a larger role on deadenylation than translation. First, every PAB1 domain, except RRM4, had an effect on deadenylation, whereas, only RRM1 and RRM2 had an effect on translation. Second, PAB1 variants did not show a decrease in translation rate comparable to the decrease in translation associated with a defect in eIF4E or eIF3. These observations support the hypothesis that PAB1 has a larger role in deadenylation than in translation. In addition this work shows that the role of PAB1 in deadenylation is both general and specific for PUF3 controlled mRNA.

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