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ANALYSIS OF THE ROLE OF POLY (A) BINDING PROTEIN (PAB1) IN

THE mRNA DEGRADATION PROCESS IN YEAST

BY

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DISSERTATION

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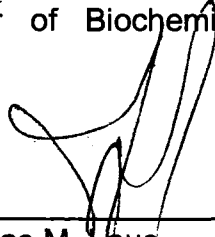Copyright 2007 by ProQuest Information and Learning Company.

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DEDICATION

MY WIFE YUTING LIU (刘禹婷), WHO IS THE MOST PRECIOUS PERSON IN MY LIFE. I GREATLY APPRECIATE YOUR LOVE, UNDERSTAND, SUPPORT, AND EVERYTHING THAT YOU SACRIFICED FOR ME.

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ABSTRACT

ANALYSIS OF THE ROLE OF POLY (A) BINDING PROTEIN (PAB1) IN THE mRNA DEGRADATION PROCESS IN YEAST

by

GANG YAO

University of New Hampshire, December, 2006

The mRNA deadenylation process influences multiple aspects of protein synthesis and is known to be the major factor controlling mRNA decay rates. My data demonstrates that yeast PAB1 plays both positive and negative roles in controlling deadenylation, and I have identified particular regions of PAB1 involved in controlling different aspects of the mRNA degradative process. I have found that yeast PAB1 does not play a simple, obstructionist role in regulating CCR4 deadenylation. Instead, PAB1-PAB1 protein interactions, as mediated by the PAB1 proline-rich region (P domain) and the RRM1 domain, are required for the CCR4 deadenylase activity. The P and RRM1 domains were shown to mediate PAB1-PAB1 binding, suggesting that enhancing CCR4 function entails the rearrangement of the PAB1-mRNP structure. I have also established that PAB1 contacts to the poly (A) tail made by the RRM2 domain are critical to stabilizing the CCR4-NOT complex and promoting deadenylation. The C-terminal globular domain of PAB1 through its contacts to eRF3 is also required for CCR4 deadenylation. In contrast, the RRM3 domain of PAB1 inhibits deadenylation and decapping. mRNP structures involving the terminal

PAB1 bound to poly (A) are also affected by RRM3 and control the end to deadenylation and apparently the commencement of decapping. These results indicate that PAB1 integrates and controls the transition from deadenylation to decapping and from a translationally competent state to an mRNA degradative state.

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CHAPTER 1

INTRODUCTION

mRNA degradation is a process involving the interaction and exchange of multiple multi-subunit complexes and RNA binding proteins (Coller and Parker 2004). Central to mRNA degradation is the removal of the poly (A) tail (deadenylation) that is controlled by a number of proteins associating with the mRNA in a structure termed the mRNP. Principal among these factors present in the mRNP, in the cytoplasm, are the poly (A) binding protein (PAB1), translation initiation and termination factors, the cytoplasmic deadenylases, and the factors that bind to the mRNA and elicit alterations in the mRNA degradative rate. The processes of mRNA degradation and deadenylation and the protein complexes that are involved are highly evolutionarily conserved from yeast to humans.

The principal pathway for mRNA degradation in yeast proceeds through several steps (see Figure 1). First, there is an initial trimming of about 15-20 nt of the poly (A) tail to a length of about 60-80 nt that is specific for each mRNA and that is carried out by PAN2/PAN3, presumably a cytoplasmic process (Brown and Sachs 1998; Tucker et al 2001; Dheur et al 2005). This trimming requires PAB1 and the translation termination factors eRF1 and eRF3 (Hosoda et al 2003; Caponigro and Parker 1995), and all these factors appear to be in a complex (Hosoda et al 2003; Hoshino et al 1999). Second, the major part of

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deadenylation utilizes the CCR4-NOT deadenylase complex (Tucker et al 2001). CCR4 is the catalytic component of this complex (Tucker et al 2002; Chen et al 2002) and shortens the poly (A) tail of mRNA to an end-point size of about 8-12 nt (Decker and Parker 1993). For more rapidly deadenylated mRNA, CCR4 deadenylation initially proceeds distributively, and at about 35-50 A's CCR4 switches to a processive mode of action (Decker and Parker 1993; Viswanathan et al 2004). Poly (A) tail shortening down to an oligo (A) form (8-12 A's) apparently leads, in turn, to the reduced ability of poly (A) binding protein (PAB1) to bind the poly (A) tail that presumably alters the translation initiation complex association with the mRNA cap structure (Mangus et al 2003; Tucker and Parker 2000). Changes in the mRNP structure around the cap lead to decapping by DCP1/DCP2 (Tharun and Parker 2001; Caponigro and Parker 1995; Tucker and Parker 2000) and 5'-3' degradation of the RNA by the exonuclease XRN1 (Muhlrad et al 1994). The transition from deadenylation to decapping occurs with apparent sequestering of the oligoadenylated mRNA in P-bodies where decapping takes place (Sheth and Parker 2003). The yeast exosome can also degrade the deadenylated mRNA in a 3'-5' direction, a process that may take place in nonsense mediated decay, for instance (van Hoof and Parker 1999; Mitchell and Tollervey 2003; Cao and Parker 2003). In higher eukaryotes both these pathways exist, as does a poly (A)-independent decapping pathway that leads to 5' to 3' decay (Tucker and Parker 2000; Parker and Song 2004). Poly (A)-independent decapping has also recently been demonstrated in yeast for at least one mRNA (Muhlrad and Parker 2005).

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DEADENYLATION-DEPENDENT DECAY

m^7G | AUG   UAA | AAAAA LONG

POLY(A) TAIL SHORTENING

PAN2/3
CCR4/NOT

m^7G | AUG   UAA | AAAAA OLIGO

DECAPPING / DCP1/2

EXOSOME *3' TO 5' DECAY*

AUG   UAA | AAAAA OLIGO

m^7G | AUG   UAA |

XRN1 *5' TO 3' DECAY*

{ AUG   UAA | AAAAA OLIGO
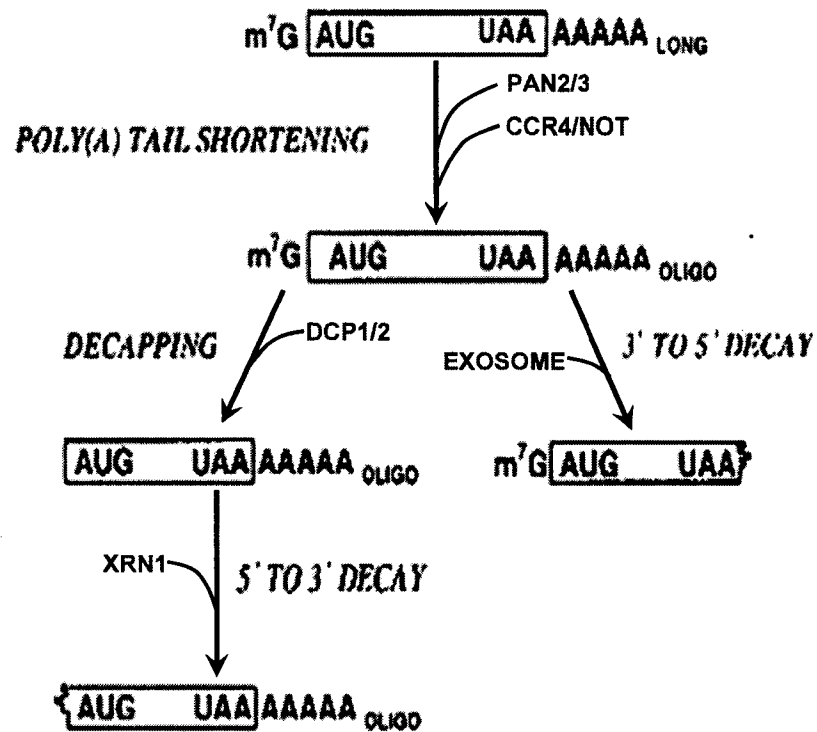
FIGURE 1: mRNA Deadenylation dependent decay pathway in eukaryotes. The mRNA degradation starts with the poly (A) tail shortening (deadenylation), followed by decapping and 5'-3' decay, or 3'-5' decay by exonucleases and/ or endonucleases. Adapted from Beelman & Parker (1995).

The rate of deadenylation of the mRNA is to the stability of most mRNA. Analyses of the relative contributions to total mRNA degradation of deadenylation, decapping, and 5'-3' degradation, clearly indicates that changes in rates of deadenylation lead to the largest changes in mRNA degradation (Cao and Parker 2001). Specific and global analysis of mRNA deadenylation rates (Decker and Parker 1993; Wang et al 2002) indicated that they vary over a 20-fold range. CCR4, the catalytic component of the CCR4-NOT deadenylase (Chen et al 2002; Tucker et al 2002), has been shown to be necessary for both rapid and slow deadenylation for a number of mRNA (Daugeron et al 2001; Tucker et al 2001, 2002; Chen et al 2002; Viswanathan et al 2004), suggesting that it affects deadenylation of most, if not all, mRNA in yeast.

Several factors have previously been shown to control the deadenylation process. Of the two deadenylases in yeast, defects in PAN2/3 alone do not affect the deadenylation rate whereas defects in the CCR4-NOT complex, specifically CCR4 and CAF1, slow the deadenylation process (Tucker et al 2001; Daugeron et al 2001). The substrate recognition features of CCR4 indicate that its in vivo activity may be directly controlled by its accessibility to a certain length of RNA and that mRNP structures involving the poly (A) tail and its adjacent 3' UTR sequences may limit these interactions and thereby significantly affect CCR4 activity (Viswanathan et al 2003, 2004).

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In addition to the deadenylases, a number of components of the poly (A) mRNP structure have been implicated in controlling deadenylation. Notable among these proteins is PAB1. PAB1 consists of four RNA binding motifs (RRM domains) and a C-terminal region comprising the penultimate proline-rich (P) domain and a terminal structured region (C) (see Figure 2). The C region binds PAN3, which is required for PAN2 activity, eRF3, and other proteins (Mangus et al 2004; Hosoda et al 2003; Kuhn and Wahle 2004). The unstructured Proline rich domain of human and xenopus PAB1 is responsible for PAB1-PAB1 cooperative interactions (Kuhn and Pieler 1996; Melo et al 2003). While RRM1 and RRM2 of PAB1 appear to bind most strongly to poly (A), RRM3 and RRM4 can also make critical contacts and may bind U-rich regions located adjacent to the poly (A) tail (Mullin et al 2004; Sladic et al 2004). RRM2 contacts eIF4G, which is believed to be important in forming the closed loop structure between the mRNA cap, eIF4E, eIF4G, PAB1 and the poly (A) tail (Tarun et al 1997; Sachs and Variani 2000). Some of the key residues for this interaction are 184-187 of RRM2, whose alterations in vitro block PAB1-eIF4G contacts and also translation (Otero et al 1999). The functions of the other RRM domains remain less clear, although deletion of RRM4 has been shown recently to reduce by 30% mRNA transport to the cytoplasm (Brune et al 2005). RRM1, 2, 3, and the P and C-terminal regions play no apparent role in mRNA transport (Brune et al 2005; K. Weis, pers. comm.).

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Previous studies concerning the role of PAB1 in deadenylation indicated that PAB1 is inhibitory to CCR4 in vitro (Tucker et al 2002). In contrast, a *pab1* deletion strain has been shown to severely block CCR4 deadenylation in vivo (Capronigro and Parker 1995), supporting a positive role for PAB1 in this process. More recent studies indicate that part of the *pab1* deletion defect on deadenylation may result from a delay in mRNA transport into the cytoplasm (Dunn et al 2005). A couple of other less comprehensive PAB1 defects have also been shown to block deadenylation (Morrissey et al 1999), supporting a positive role for PAB1 in promoting deadenylation.

In addition to PAB1, the translation initiation status of the mRNA, and, hence, the mRNP structure involving these proteins can also inhibit deadenylation (Schwartz and Parker 1999; Lagrandeur and Parker 1999). In addition, a number of specific RNA binding proteins are known or presumed to make contact to 3' UTR sequences of certain mRNA and thereby accelerate the mRNA degradative process (Tran et al 2004; Lykke-Andersen and Wagner 2005; Smeotok et al 2005). Several of these proteins appear to enhance the rate of deadenylation (Olivas and Parker 2000; Smeotok et al 2005).

In this study we addressed the role of PAB1 in the deadenylation process. Using a set of precise domain deletions and point mutations in PAB1, we find that particular regions of PAB1 play either negative or positive roles in the deadenylation process. Different aspects of the deadenylation process are

affected by the interaction of PAB1 with itself, with translation termination and initiation factors, with the CCR4-NOT deadenylase, and with decapping factors. The multiple roles of PAB1 implicate it in the integration of the deadenylation to decapping process and the conversion of a translationally competent mRNA to one that is bound for degradation.

PAB1

PAB1 consists of an N-terminal region comprising four RNA recognition motifs (RRM domains) and a C-terminal region comprising the penultimate P domain and a terminal globular C region (see Figure 2). Different regions of PAB1 play different roles in the deadenylation process. The C domain binds PAN2/3 and is required for PAN2/3 deadenylation activity. eRF3, also contacts the C region, regulating CCR4 deadenylation (Prelim. Res.). The P domain, which has a critical role in regulating deadenylation, is responsible for PAB1-PAB1 cooperative interactions, and also binds poly (A) binding protein binding protein 1, PBP1, which negatively regulates PAN2/3 activity. While RRM1 and RRM2 of PAB1 appear to bind most strongly to poly (A), RRM3 and RRM4 can also make contacts to Poly (A) and bind U-rich regions located adjacent to the poly (A) tail (Mullin et al 2004; Sladic et al 2004). Multiple PAB1 regions appear to be involved in contacting the translation initiation complex. RRM2 contacts eIF4G, which is believed to be important in forming the closed loop structure between the mRNA cap, eIF4E, eIF4G, PAB1 and the poly (A) tail. Our data, however, indicates the eIF4E can also contact PAB1 and RRM2 is not required

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for this interaction (Y. Chiang, pers. comm.) Deleting RRM4 has been shown to reduce mRNA transport to the cytoplasm, although RRM1, 2, 3, and the C-terminal region play no apparent role in mRNA transport (Brune et al 2005; K. Weis, pers. comm.). Other functions for the RRM domains remain unknown.
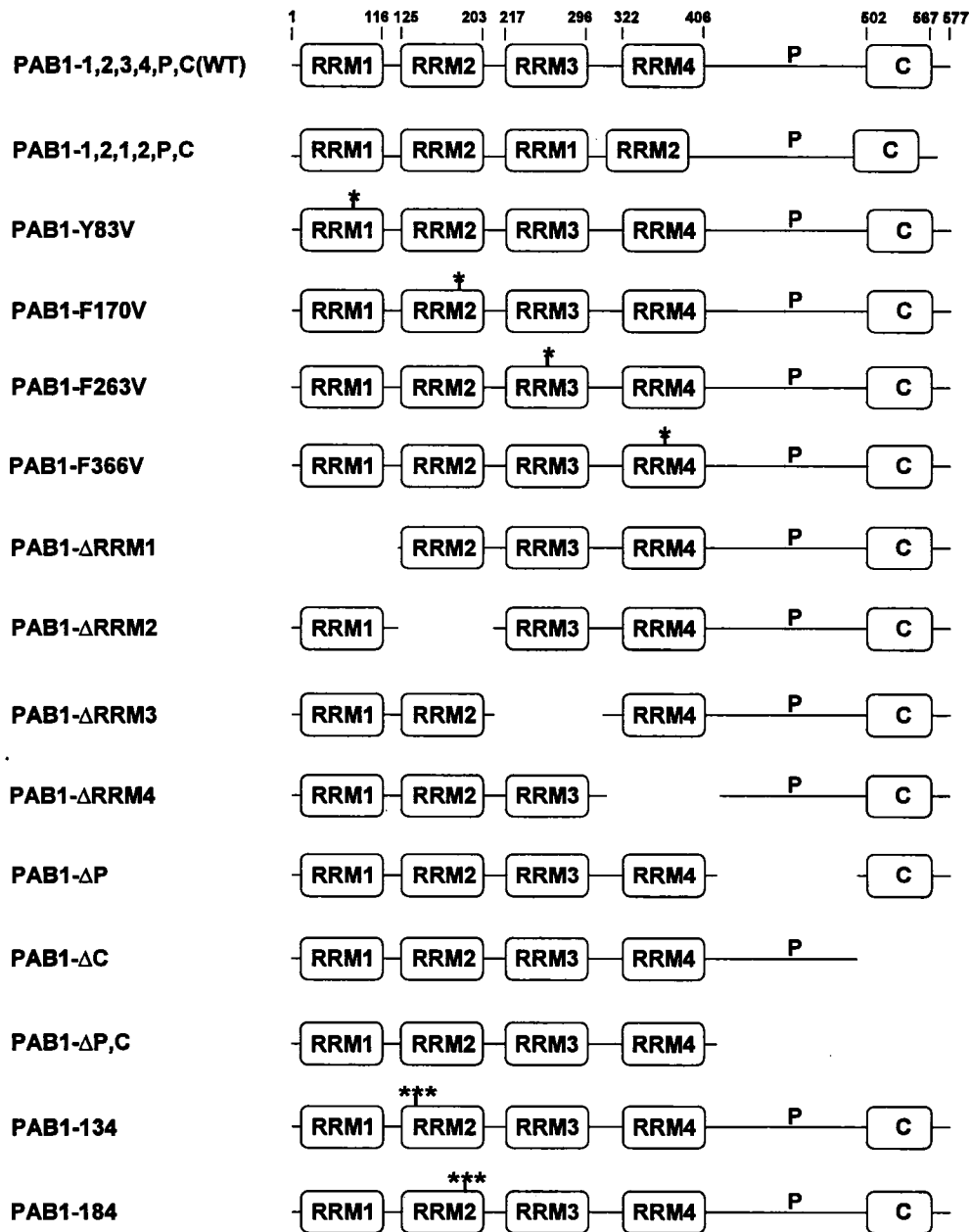
FIGURE 2: PAB1 variants that are discussed in this study. Residues for each domain are indicated at the top. Progenitors to PAB1-184, that has residues 184DAL187 replaced with EKM, and PAB-134, which has 134HPD137 replaced with DKS (Otero et al 1999), were provided by D. Mangus.

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The CCR4-NOT deadenylase complex

The CCR4-NOT proteins are evolutionarily conserved across eukaryotes (Denis and Chen 2003). Two forms of the complex exist in yeast: 1 .0 and 1 .9 MDa (Liu et al 1998). The smaller core complex contains the following proteins: CCR4, CAF1, five NOT proteins (NOT1 –NOT5), CAF4O, CAF130, and BTT1. (Chen et al 2001; Cui et al 2006) Several other proteins, which associate with the CCR4-NOT complex, may be present in the 1 .9 MDa complex, but they are not integral components of the 1 .0 MDa complex (Hata et al 1998; Maillet and Collart 2002; Liu et al 1997, 2001). Of these latter proteins, DHH1 is related to CCR4-NOT functions in mRNA degradation in that it also associates with factors involved in mRNA decapping and can enhance decapping in vitro (Cotter et al 2001; Fischer and Weis 2002).

mRNA deadenylation rates can vary over a 20-fold range between different mRNAs in yeast cells. CCR4, the catalytic component of the CCR4-NOT deadenylase, has been shown to be required for both rapid and slow deadenylation processes for most, if not all, mRNA in yeast (Daugeron et al 2001; Tucker et al 2001, 2002; Chen et al 2002; Viswanathan et al 2004). While yeast CAF1 protein, expressed from *E.coli* cell, displays deadenylase activity in vitro (Daugeron et al 2001; Thore et al 2003), we have established that CAF1 deadenylase function is not required in vivo for its role in mRNA deadenylation (Viswanathan et al 2004). CAF1 probably acts in other ways to aid the

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degradation process. Defects in other NOT proteins also affect mRNA deadenylation, albeit to a much lesser extent (Tucker et al 2002).

In mammalian cells, the deadenylation is still the key first step that triggers decay of both wild-type stable and nonsense cordon-containing unstable mRNAs (Amashita and Shyu 2005). Following the initial trimming by PAN2, CCR4 replaces the PAN2 and continuously shortens the poly (A) tail to reach the oligo form. DCP2-mediated decapping takes place after deadenylation and may serve as a backup mechanism for triggering mRNA decay when initial deadenylation by PAN2 is compromised (Amashita and Shyu 2005).

The mRNA Decapping Process

In eukaryotes, the second step of mRNA degradation process following deadenylation is the decapping process. This process is carried out by the Dcp1p and Dcp2p decapping holoenzyme. Dcp2p has been shown to be the catalytic subunit (Dunckley and Parker 1999; Lykke-Andersen 2002; She et al 2004). Dcp1p primarily functions to enhance Dcp2p activity by an unclear mechanism (She et al 2004). Dcp1p is also conserved in eukaryotes, and two human homologs, hDcp1a and hDcp1b, have been identified (Lykke-Andersen 2002). The human homologs appear functional because hDcp1a copurifies with decapping activity (Lykke-Andersen 2002). Mapping the sequence conservation on the molecular surface of Dcp1p has revealed two prominent sites, one of which is required for function of the Dcp1p/Dcp2p complex, and a second,

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corresponding to the the proline-rich sequence (PRS) binding site, that is likely to be a binding site for decapping regulatory proteins (She et al 2004). Moreover, a conserved hydrophobic patch has been revealed to be critical for decapping.

The decapping process is also regulated by some other proteins. The poly(A)-binding protein (Pab1p) inhibits the decapping process and is required to couple decapping to prior deadenylation (Caponigro and Parker 1995. Morrissey and Sachs 1999.). In addition, components of the translation initiation complex also impede decapping (Schwartz and Parker 2003. Schwartz and Parker 1999.). Most notably, the cap-binding protein, eIF-4E, inhibits decapping both in vitro and in vivo. Several proteins are required for the efficient decapping of most normal mRNAs in vivo, but they are not absolutely required for decapping per se. These proteins include the LSM1–7 complex, which is a presumed RNA-binding complex, Pat1p, which is of unknown biochemical properties, and Dhh1p, which is a member of the DEAD box ATPase family (Boeck and Sachs 1998, Bouveret and Se´raphin 2000. Fischer and Weis 2002. Tijsterman and Plasterk 2002.).

In eukaryotes, the proteins involved in mRNA decapping and 5' to 3' exonucleolytic decay are found in specific cytoplasmic foci, referred to as P bodies. In yeast, GFP-tagged Dcp1p, Dcp2p, Lsm1p, Pat1p, Dhh1p, and Xrn1p have been localized to P bodies (Sheth and Parker 2003) In contrast, Ccr4p, Ski7p, Puf3p, and translation factors are not concentrated or show only small concentrations in P bodies (Sheth and Parker 2003). In mammalian cells, the

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Lsm1–7 complex, Xrn1p, Dcp1p, and Dcp2p have been localized to analogous structures (Bashkirov and Heyer 1997, Ingelfinger and Achsel 2002, Lykke-Andersen 2002, Van and Seraphin 2002).

Two experimental observations in yeast cells indicate that P bodies are specific sites wherein mRNAs can be decapped and degraded 5' to 3' (Sheth and Parker 2003). First, the size and number of P bodies varies in a manner correlating with the flux of mRNA molecules through the decapping step. Inhibiting mRNA decay at the deadenylation step in a ccr4Δ strain leads to a reduction in P-body size and number. Similarly, inhibiting decapping by deleting the PAT1 gene or by adding cyclohexamide leads to a reduction or loss of P bodies. In contrast, inhibiting the enzymatic steps of decapping or 5' to 3' exonuclease digestion leads to an increase in the size and number of P bodies (Sheth and Parker 2003). The second key observation is that mRNA decay intermediates, trapped either by the insertion of strong secondary structures or by deletion of the gene for the 5' to 3' exonuclease Xrn1p, can be specifically localized to P bodies. The simplest interpretation of these observations is that P bodies are sites of mRNA decapping and 5' to 3' exonucleolytic decay. However, because the mRNA decapping factors are also found distributed throughout the cytoplasm, decapping and degradation may also occur outside of P bodies.

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Translation Initiation in Eukaryotes

The synthesis of a protein is one of the most central and basic events in the life of every cell. It contains three main steps: translation initiation, elongation and termination. Among these steps, translation initiation has been shown to be the overall rate-limiting step (Legocki and Marcus 1970; Wolin and Walter 1988) and also the most important target of regulation.

In eukaryotes, cap-dependent initiation is the major translation initiation pathway. Most mRNAs in yeast are capped at the 5' end and polyadenylated at the 3' end. The initiation factor eIFG binds eIF4E and associates with the cap structure and poly(A)-binding protein (PABP) which is itself bound to the poly (A) tail leading to circularization of the mRNA. Recruitment of eIF4A and eIF4B prepares the 5' end of the mRNA for ribosome binding through ATP-dependent local mRNA unwinding. 80S ribosomes dissociate into 40S and 60S subunits, 40S subunits bind a protein complex containing eIF1, eIF3, eIF5 and the ternary complex eIF2-GTP-Met-tRNAi. The resulting 40S preinitiation complex then associates with the mRNA through interaction of eIF3 with eIF4G at the cap structure. From there the initiation complex moves in the 5' to 3' direction (scanning) and recognizes the AUG initiator codon through AUG-Met-tRNAi base-pairing. The factors eIF5 and eIF5B then trigger GTP hydrolysis, eIF2-GDP release, ejection of bound factors and joining of the 60S subunit to the 40S initiation complex to form an 80S ribosome competent for polypeptide elongation.

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The cap structure plays an important role in initiation of translation on most mRNAs. It serves as an assembly site for a translation initiation factor complex. eIF4E complexed to eIF4G binds directly to the cap structure. This interaction requires functional eIF4A (Svitkin and Sonenberg 2001) and is blocked by cap analogues such as m7GDP. Bound eIF4E-eIF4G-eIF4A complex binds eIF4B and PAB1 and melts mRNA secondary structure close to the cap to generate a ribosome binding site. This requires ATP-binding to eIF4A and ATP hydrolysis by eIF4A.

The poly(A) tail also plays a role in translation initiation. It acts as an enhancer of translation (Gallie and Tanguay, 1994). PABP interacts with the amino terminal domain of the initiation factor eIF4G. This interaction is required for efficient translation of mRNA (Tarun and Sachs.,1997), and it stimulates 40S ribosome binding to mRNA (Tarun and Sachs, 1995).

Since in the circularized mRNP structure in the cytoplasm, the 5' cap translation initiation complex contacts the poly (A) tail through PAB1, it is believed that the translation initiation machinery may play a role in regulating the deadenylation process. First, severe defects in translation initiation factors, such as eIF4E and eIF4G, accelerate deadenylation for both CCR4 and PAN2 (Schwartz and Parker 1999, unpublished data), Second, the deadenylase domain of CCR4 interacts with eIF4G in two-hybrid analysis (Y.-C.Chiang, pers. comm.). Third, the CCR4-NOT complex makes contacts to DHH1 and DCP1.

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These proteins help displace the translation initiation complex and finally removing the 5' cap structure from the mRNA. It would therefore be expected that the mRNP structure involving translation initiation factors, decapping factors and PAB1 all influent either positively or negatively the CCR4/PAN2 deadenylation process.

eRF3

Translation termination in eukaryotes is mediated by two factors, eRF1 and eRF3. eRF1 recognizes each of the three stop codons (UAA, UAG, and UGA) and facilitates release of the nascent polypeptide chain. eRF3 is a GTPase that stimulates the translation process by a poorly characterized mechanism. Blocking the contact between eRF1 and eRF3 or the contact between eRF3 and PAB1 results in increased mRNA stability and decreased deadenylation rates (Hoshino, et al 1999). The eRF3 protein contains three distinct regions. The N and M regions, which occupy amino acid residues 1 to 253, and which contacts the C-terminus of PAB1, are dispensable for both translation termination and cell viability. In contrast, the C region, amino acid residues 254 to 685, contains the GTPase fold, and associates with eRF1 in a GTP-dependant manner. (Tetsuo, et al 2004). Defects in the GTPase activity of eRF3 not only compromises cell viability and affects the proper translation termination, but increases the stability of *PGK1* mRNA and significantly slows the deadenylation rate of *GAL1-S* mRNA (Joe et al 2004; Tetsuo, et al 2004 and Prelim. Res).

In this dissertation research, I studied PAB1 functions in the mRNA degradation process, especially in deadenylation and decapping processes, by using a series precisely mutated PAB1 variants. We observed the following results:

1. Proper poly (A) contact by PAB1 is required for CCR4-NOT deadenylation in vivo.

2. PAB1-PAB1 interactions, through the RRM1 and P domains, are required for the deadenylation.

3. The CCR4-NOT complex is destabilized in the cell by loss of RNA contact by RRM2.

4. RRM3 of PAB1 inhibits deadenylation and decapping and affects the mRNP structure of the terminal PAB1 bound to poly (A).

5. eRF3 contacts to the C-terminus of PAB1 and the proper translation termination are required for deadenylation.

CHAPTER 2

MATERIALS AND METHODS

Yeast strains and growth conditions

Saccharomyces cerevisiae strain AS319 (MAT . ade2 ura3 leu2 trp1 his3 pab1::HIS3 pAS77 [PAB1-CEN-URA3]) (Kessler et al 1998) was used for transforming PAB1 variants expressed under their own promoter on plasmid YC504 (pRS314: PAB1-CEN-TRP1) as indicated in Figure 2. Other strains isogenic to AS319 were DB267tL/pAS77 (contains pan3::trp1::LEU2), AS319-1a-uN/pAS77 (contains ccr4::ura3::Neo), and AS319-d1-uL/pAS377 (contains dhh1::ura3::LEU2). Strain AS319 with the RP485 plasmid was used for quantitating rates of deadenylation for MFA2pG (Tucker et al 2001). Strain 1716-1 (MATa ura3 leu2 trp1 his3 pab1::HIS3 dcp1::Neo pAS77) was used for analyzing the effect of PAB1 derivatives on suppression of dcp1 and is 75% identical to AS319. Strain 1729-3-wt (MAT . gst1-1 his3 trp1 ura3 pab1::HIS3 YCplac22-Flag-SUP35/WT YC504) and 1729-3-N106I (isogenic to 1729-3-wt except YCplac22-Flag-SUP35/N406I) (Kobayashi et al 2004) and strains YDB498/pDB663 (MATa ura3-1 leu2-3,112 his3-11,15 trp1-1 ade1-14 sup35::HIS3 [psi-] pDB663 [wt SUP35]) (Salas-Marco and Bedwell 2004), YDB498/pDB783 (isogenic to YDB484 except pDB783 [sup35-R419G]), and YDB498/pD670 (isogenic to YDB484 except pDB670 [sup35-H348L]) were used for analyzing eRF3 effects on deadenylation.

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Plasmids

PAB1 variants were constructed by PCR techniques and were expressed in yeast on a pRS314 vector (YC504) as Flag-tagged versions under control of the *PAB1* promoter, except for PAB1-ΔP,C which was expressed from a pRS424 plasmid under the control of a G3PDH promoter in order to obtain PAB1 levels comparable to the other PAB1 variants. The following amino acid residues were removed with each PAB1 variant: PAB1-ΔRRM1 (removed 1-124), PAB1-ΔRRM2 (removed 124-211), PAB1- RRM3 (removed 211-309), PAB1-ΔRRM4 (removed 309- 416), PAB1-ΔP (removed 416-492), PAB1-ΔC (removed 492-577), PAB1-ΔP,C (removed 416-577), and PAB1,2,1,2,P,C (removed 211-416 and replaced it with 1-211).

RNA analysis

Transcriptional pulse-chase and steady-state analyses for *GAL1* and *MFA2pG* mRNA were conducted as previously described (Viswanathan et al 2004; Tucker et al 2001). Briefly, for the transcriptional pulse-chase analysis, cells were grown to middle-log phase in medium containing 2% raffinose, and then induced the transcript by addition of galactose to a final concentration of 2% galactose for 10 min. The transcription was shut off with addition of glucose to a final concentration of 4% at time 0 min, and then harvested cells at each indicated time point. For the steady-state analysis, cells were grown to middle-log phase in medium containing 2% raffinose, and then induced the transcript by addition of galactose to a final concentration of 2% galactose for 3 hours.

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Harvested cells and frozen in -80°C. Total RNA was isolated by hot acid phenol method. Analysis of poly (A) tail lengths was performed by running 10ug of total RNA on a 6% polyacrylamide/ 7 M urea gel that was electroblotted and probed by standard techniques. The dT sample is RNA sample subjected to an oligo dT/RNase H cleavage to indicate the fully deadenylated species, and it marks the 0 A's position. Rates of deadenylation were determined on only those poly (A) tails that were being distributively deadenylated, that is, which were being shortened as a synchronous pool.

In vitro gel shift assays

In vitro gel shift assays were conducted as described (Deardorff et al 1997) using PAB1 variants expressed in *E. coli* from a pET28 vector. Recombinant PAB1 variants were purified to greater than 90% purity using Ni-agarose chromatography (Kessler et al 1998).

Non-denaturing gel electrophoresis was conducted on purified PAB1 proteins to identify the presence of non-specific RNAs that might interfere with the RNA binding measurements. No such interactions were detected. BSA was used as a standard to determine PAB1 protein abundance, and the K_D values were determined as described (Deardorff et al 1997).

For the in vitro gel shift assay, a fix amount of 5' labeled 7N+23A RNA substrate was incubated with purified PAB1 protein at indicated PAB1

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concentration at room temperature for 10 min, and then fractionated on a 4% non-denaturing polyacrylamide/ 0.5 X TBE gel at 4%. The RNA sequence is 5'-UCU AAA U + 23A-3'.

Native Gel Electrophoresis and Western Blot Analysis

Yeast PAB1 variants were purified by Flag immunoprecipitation as previously described (Chen et al 2002) and analyzed by nondenaturing PAGE as described (Denis and Young 1983) except that the bisacrylamide/acrylamide ratio was 1:60. Prior to non-denaturing PAGE, the PAB1 samples were incubated with either 0.1 mg/ml RNase A or 1U/μl RNase I at room temperature for 45 min to remove endogenous mRNA attached to the PAB1. PAB1 species were identified by western blotting with anti-Flag antibody.

CHAPTER 3

RESULTS

Increased PAB1 binding to poly (A) does not inhibit CCR4 deadenylation in vivo

Previous analysis of the role of PAB1 in CCR4 deadenylation showed that PAB1 was inhibitory to CCR4 activity in vitro, presumably by restricting CCR4 access to the poly (A) tail (Tucker et al 2002). We tested whether PAB1 binding to the poly (A) tail plays an inhibitory role for CCR4-NOT deadenylation in vivo by constructing a PAB1 protein that would be predicted to bind much better to poly (A) than the wild-type protein (PAB1-1,2,3,4,P,C: contains 4 RRM domains and the C-terminal P and C regions (Figure 2). Since RRM domains 1 and 2 bind better to poly (A) than RRM3,4 (Kessler and Sachs 1998), we replaced the RRM3,4 cassette with that of RRM1,2, resulting in the construction of PAB1-1,2,1,2,P,C (Figure 2). This PAB1 version and other PAB1 plasmid variants constructed below were subsequently swapped into a strain deleted for the *PAB1* gene (Kessler and Sachs 1998). The expression of each PAB1 version in yeast was monitored by Western analysis, and all versions were found to be comparably expressed (Yueh-Chin Chiang, pers. comm).

The ability of each of these PAB1 variants to bind poly (A) in vitro was monitored using a gel shift assay (Kessler and Sachs 1998; Deardorff and Sachs 1997). Wild-type PAB1 was found to bind poly (A) with a K_D of 11 nM (Figure 3;

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Table 1) that is within the range previously observed for yeast PAB1 binding to poly (A) (7-30 nM) (Kessler and Sachs 1998; Deardorff and Sachs 1997; Sachs et al 1987). As predicted, PAB1-1,2,1,2,P,C bound to poly (A) six-fold better than PAB1, with a K_D of 1.9 nM (Figure 3; Table 1). We subsequently analyzed in vivo the effect of the increased binding ability of PAB1-1,2,1,2,P,C on CCR4-NOT deadenylation.

We examined directly the effect of PAB1-1,2,1,2,P,C on *MFA2pG* and *GAL1* mRNA deadenylation rates by using pulse-chase experiments (Tucker et al 2001; Viswanathan et al 2004). *MFA2pG* mRNA was chosen for analysis because of its extensive characterization, and it is rapidly turned over (Decker and Parker 1993). Two *GAL1* mRNA species are produced in vivo that result from differential poly (A) site usage and differ by 110 nt in their 3' UTR (Miyajima et al 1984; Cui and Denis 2003). *GAL1-L* (long) deadenylates more rapidly than *GAL1-S* (short) (Viswanathan et al 2004). Following a brief induction of *MFA2pG* and *GAL1* mRNA synthesis with the addition of galactose to the medium, mRNA synthesis was shut off with glucose, and the length of *MFA2pG* and *GAL1* mRNA poly (A) tails was followed as a function of time by Northern analysis. As shown in Figure 4 and Table 1, we found that PAB1-1,2,1,2,P,C did not reduce the rate of deadenylation for *MFA2pG* or *GAL1*. The results of these experiments argue against a simple obstructionist, inhibitory model for PAB1 control of CCR4-NOT deadenylation in vivo.
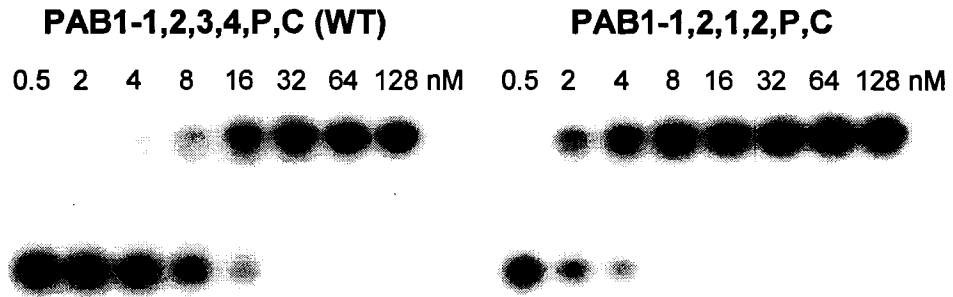
23

FIGURE 3: In vitro PAB1-poly (A) binding analyses. In vitro gel shift assays were conducted between recombinant PAB1 and the 7N+23A substrate. The concentration of each PAB1 variant was determined by Coomassie staining following SDS-PAGE using BSA as a standard. The lower band represents the RNA substrate and the upper band represents the RNA-PAB1 product.

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A. *GAL1-S*

PAB1-WT

dT 0 2 4 6 8 12 20 30 dT

80A-

0A-

PAB1-1,2,1,2,P,C

dT 0 2 4 6 8 12 20 30 dT

80A-

0A-

B. *GAL1-L*

PAB1-WT

dT 0 2 4 6 8 12 20 30 dT

80A-

0A-

PAB1-1,2,1,2,P,C

dT 0 2 4 6 8 12 20 30 dT

80A-

0A-

C. *MFA2pG*

PAB1-WT

dT 0 1 2 4 8 15 30 dT

80A-

0A-

PAB1-1,2,1,2,P,C

dT 0 1 2 4 8 15 30 dT

80A-

0A-
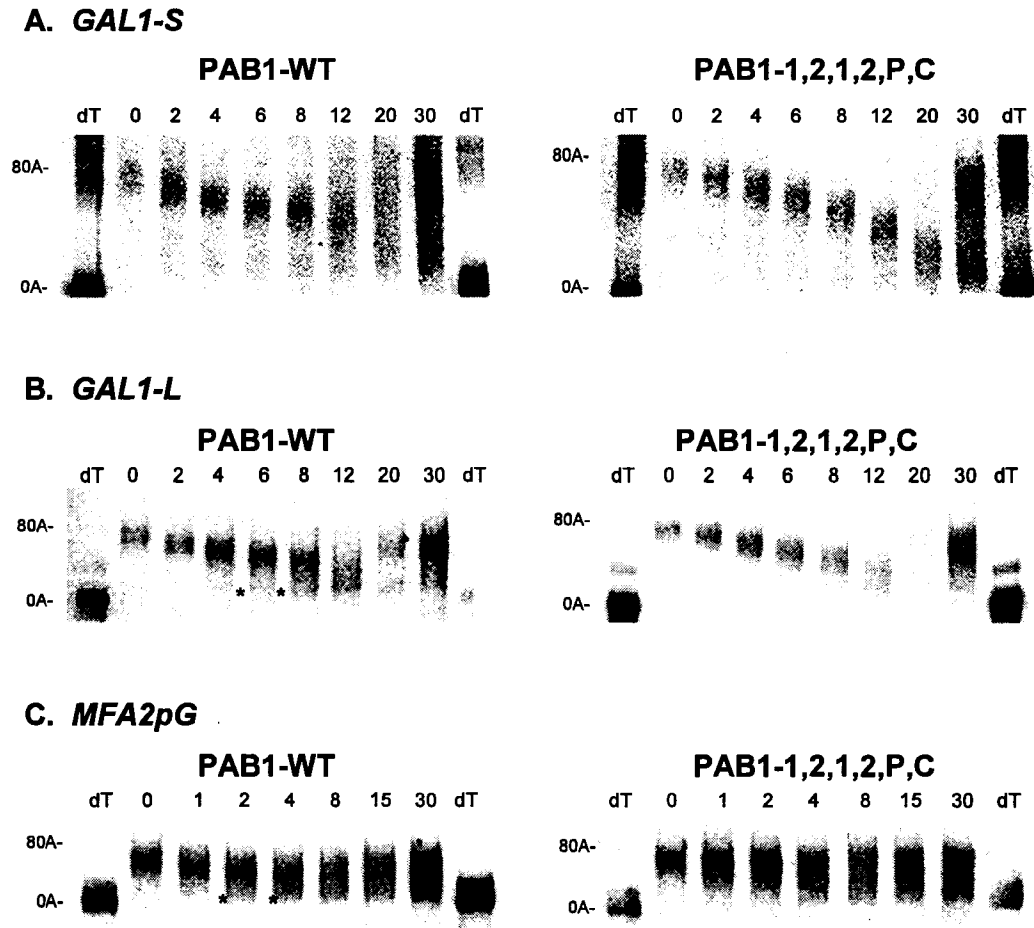
FIGURE 4. Increased PAB1 binding to poly (A) does not inhibit CCR4 deadenylation in vivo. Pulse-chase analysis was used for determination of rates of mRNA deadenylation. Northern analysis of *GAL1* and *MFA2pG* mRNA was conducted following induction of synthesis with galactose for 8 min and shutting off of synthesis at time zero with glucose. Times are in min. The poly (A) tail lengths are indicated on the left. dT- RNA sample, which had been pretreated with oligo d(T) and RNase to remove the poly (A) tail prior to Northern analysis. A- *GAL1-S*; B- *GAL-L*; and C- *MFA2pG*.

Decreased PAB1 binding to poly (A) inhibits CCR4 deadenylation in vivo

To further test the PAB1 inhibitory model, we analyzed the effect of several PAB1 variants that contained mutations in their RRM domains that have been previously found to reduce PAB1 association with poly (A) (Deardorff and Sachs 1997). If PAB1 were simply inhibiting CCR4-NOT function, we would predict that decreased PAB1 binding to the poly (A) tail would be expected to alow increased rates of deadenylation. Using PAB1-Y83V, containing an RNA binding mutation in RRM1, and PAB1-F170V, a mutation in RNA binding for RRM2, we found that in vitro binding to poly (A) was reduced (Table 1) as previously shown (Deardorff and Sachs 1997). The effect of PAB1-Y83V and PAB1-F170V on in vivo deadenylation was initially visualized by analyzing their effects on the steady state levels of GAL1-L mRNA (Figure 5A). Mutations in RRM1 or RRM2 that reduced RNA binding in vitro caused the shortest poly (A) tails to be longer than that observed for wild-type PAB1 (Figure 5A, lanes 2 and 3 as compared to lane 1), suggesting that the rate of deadenylation was being slowed. Similar results were obtained for GAL1-S (data not shown).

Although the vast majority of the deadenylation observed in Figure 5A is due to CCR4-NOT, the PAB1 mutants could also be affecting PAN2/3. We subsequently tested whether these effects on deadenylation were solely mediated by CCR4. In a strain carrying a pan3 deletion in which PAN2 is apparently not functional (Mangus et al 2004) and CCR4 is the only deadenylase (Tucker et al 2001), mutating the RNA binding residue in RRM1 (PAB1-Y83V) or

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in RRM2 (PAB1-F170V) still caused the shortest lengths of the poly (A) tails to be longer than observed for wild-type PAB1 (Figure 5A, lanes 5 and 6 as compared to lane 4). In contrast, in a Δccr4 strain where PAN2/3 is the only functional deadenylase, PAB1-Y83V (lane 8) and PAB1-F170V (lane 9) had no effect on the tail length (compared to lane 7, PAB1, Δccr4), implying that they were not affecting PAN2/3. These results support a role for PAB1 binding to poly (A) in CCR4 deadenylation.

Pulse-chase analysis of *GAL1* and *MFA2pG* RNA deadenylation was used to determine if these PAB1 binding mutants did affect the deadenylation rate. As shown in Figure 5B, 5C, and Table 1, PAB1-Y83V and PAB1-F170V significantly slowed the rate of *MFA2pG* and *GAL1* mRNA deadenylation. Single point mutations in the RNA contacts in RRM3 (F263V) or RRM4 (F366V) had lesser effects on the deadenylation rate (Table 1) or steady state mRNA levels (data not shown). The RRM1 and RRM2 effects appeared specific to PAB1 defective in contacting the poly (A) tail: PAB1-184 and PAB1-134 (each contains 3 amino acid alterations in RRM2 that affect its ability to associate with eIF4G in vitro and/or to affect translation initiation in vitro, Otero et al 1999) were unaffected in the *MFA2pG* mRNA deadenylation rate (Table 1) or in the poly (A) length for *MFA2pG* or *GAL1* steady state mRNA levels (data not shown). The above results and those described below with other RNA binding mutants indicate that PAB1 does not play a primarily inhibitory role for CCR4-NOT deadenylation but actually must make proper contacts to the poly (A) tail to aid CCR4-NOT action.

This effect also appears specific to the CCR4-NOT deadenylase, suggesting that there may exist some type of communication between CCR4-NOT and PAB1 (see below).
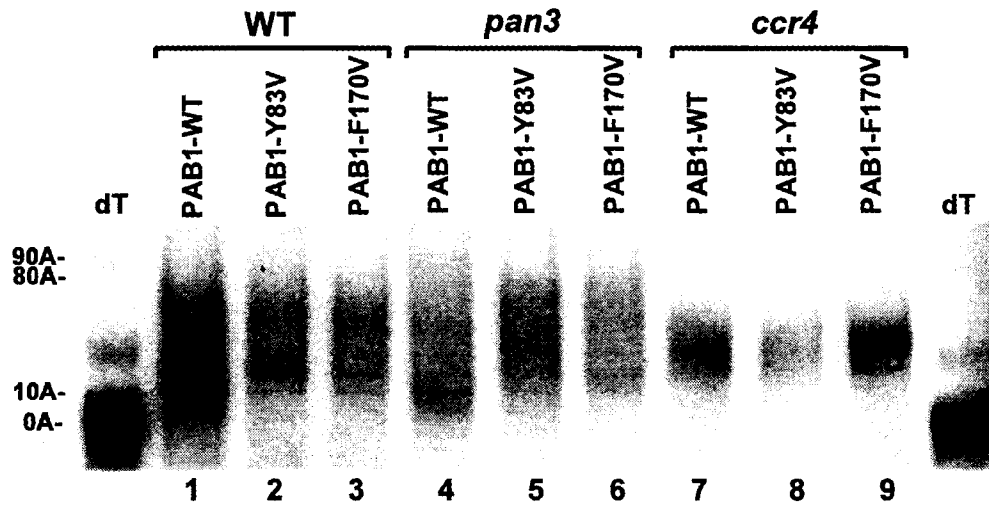
FIGURE 5A. Steady state analysis of GAL1-L mRNA poly (A) lengths as a function of PAB1 defects. Steady state levels of the mRNA were obtained following a 3 hr growth under galactose growth conditions. WT- AS319; pan3-DB267-tL; and ccr4- AS319-1a-uN. Poly (A) lengths are given on the sides.

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B. *GAL1-L*

PAB1-WT

PAB1-Y83V

PAB1-F170V

FIGURE 5B. Decreased PAB1 binding to poly (A) inhibits CCR4 deadenylation in vivo. Pulse-chase analysis was used for determination of rates of mRNA deadenylation. Northern analysis of *GAL1* mRNA was conducted following induction of synthesis with galactose for 8 min and shutting off of synthesis at time zero with glucose. Times are in min. The poly (A) tail lengths are indicated on the left. dT- RNA sample, which had been pretreated with oligo d(T) and RNase to remove the poly (A) tail prior to Northern analysis. The asterisks in PAB1-WT indicate the presence of the oligo poly (A) species present at the same time as the longer poly (A) species indicative of processive deadenylation.

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C. *MFA2pG*

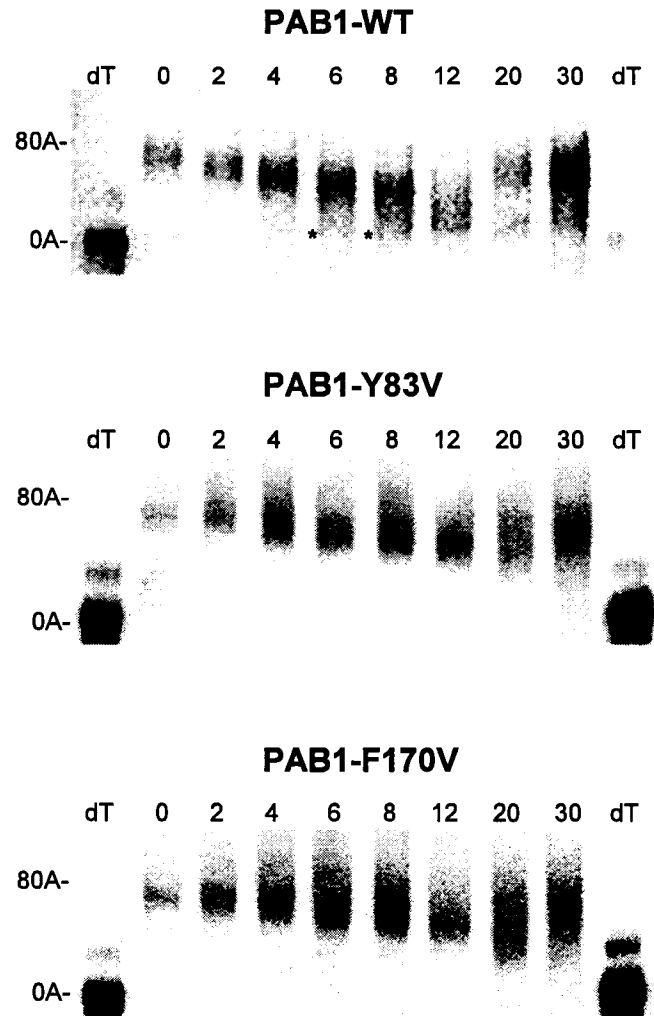### PAB1-WT

PAB1-Y83V

PAB1-F170V

FIGURE 5C. Decreased PAB1 binding to poly (A) inhibits CCR4 deadenylation in vivo. Pulse-chase analysis was used for determination of rates of mRNA deadenylation. Northern analysis of *MFA2pG* mRNA was conducted following induction of synthesis with galactose for 8 min and shutting off of synthesis at time zero with glucose. Times are in min. The asterisks in PAB1-WT indicate the presence of the oligo poly (A) species present at the same time as the longer poly (A) species indicative of processive deadenylation.
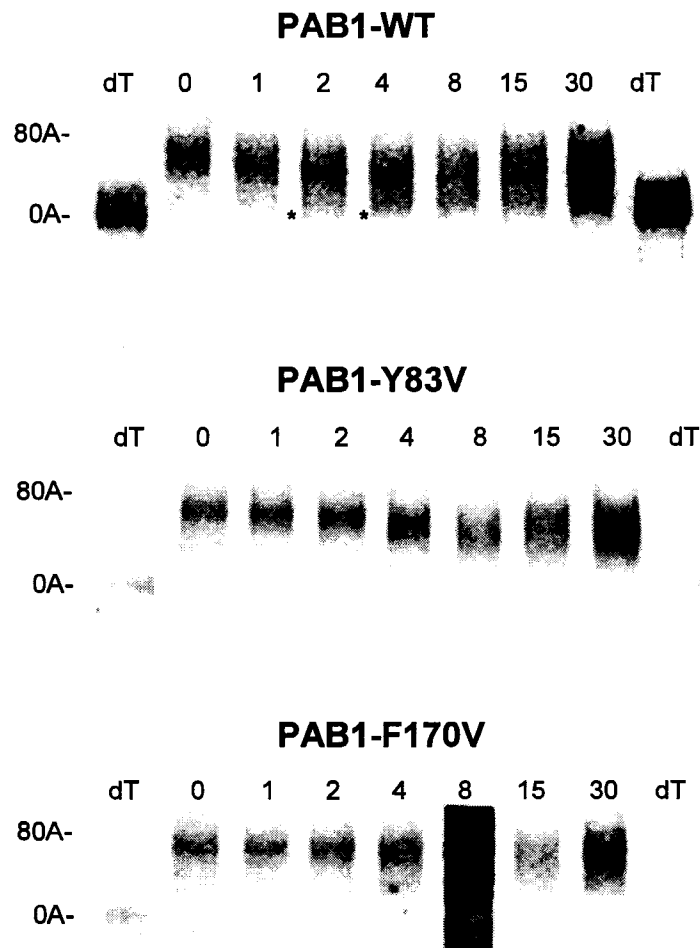
31

The proline-rich C-terminal region of PAB1 is required for CCR4 deadenylation and its transition from a distributive to a processive state

To further address the roles of PAB1 in mRNA deadenylation, we chose to delete each of the six known domains in the protein. This systematic approach would allow us to determine if individual regions of PAB1 displayed particular functions. RRM domains 1 through 4 were individually deleted (Kessler and Sachs 1998), and we additionally constructed PAB1 lacking the complete C-terminus (PAB1-ΔP,C), lacking the proline-rich penultimate region (PAB1-ΔP), and lacking the globular, terminal C-domain (PAB1-ΔC) (Figure 2). Each of these seven PAB1 alleles was analyzed for in vitro poly (A) binding (Table 1), effect in vivo on steady state *GAL1* and *MFA2pG* mRNA levels (Figures 6A and 6B; data not shown), and effect in vivo on the rate of deadenylation (Figure 7A and 7B; Table 1; data not shown).

Deleting RRM1 or RRM2 was found to slow the rate of deadenylation (Figure 8A and 8B, Table 1). The effects of ΔRRM1 and ΔRRM2 on deadenylation were attributable to CCR4-NOT and not PAN2/3 as ΔRRM1 and ΔRRM2 did not affect the steady state distribution of poly (A) tails of *GAL1* mRNA in a Δccr4 background but did affect the distribution in a Δpan3 strain (data not shown). As we noted above, PAB1-184 (defective in binding eIF4G) did not affect CCR4-NOT deadenylation (Table 1), and thus the effect of ΔRRM2 on CCR4-NOT deadenylation is most likely attributed to its decreased binding to the poly (A) tail. Interestingly, we observed that both the RRM1 deletion and the

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Y83V alteration in RRM1 displayed slower rates of deadenylation than the corresponding defects in RRM2. One implication of this is that RRM1 has a different function in deadenylation than does RRM2 (see below).

Deleting the complete C-terminus of PAB1 very significantly slowed the rate of deadenylation (data not shown), indicating that contacts that this region makes play a required role for stimulating CCR4-NOT activity. The majority of this effect was localized to the P region, as PAB1-ΔP caused a most significant block in both the distributive and processive deadenylation for both *GAL1* and for *MFA2pG* (Figure 7A and 7B; Table 1). Significantly, deleting the P region did not reduce poly (A) binding, and, in fact, the P region appeared inhibitory for PAB1 contacts to the poly (A) tail (Table 1). As the P domain is known to be required for cooperative interactions among higher eukaryotic PAB1 molecules (Melo et al 2003; Kuhn and Pieler 1996), its effects on deadenylation might be occurring through a separate mechanism than that observed for the RNA binding mutations. PAB1-ΔC also reduced the rate of deadenylation, suggesting that the C-terminal globular domain of PAB1 plays a role in this process (see below). Since it has been previously shown that deleting RRM1, RRM2, or the P and C-terminus of PAB1 does not affect the transport of mRNA into the cytoplasm (Brune et al 2005, K. Weis, pers. comm.), we interpret these results to indicate that multiple regions and functions of PAB1 are required for CCR4 deadenylation.

The results shown in Figure 7A and 7B indicate that CCR4 transitions from a distributive to a processive deadenylase when the poly (A) tail length reaches about 35-40 nt for *MFA2pG* (at about 1-2 min, wild-type, Figure 7B) and *GAL1-L* (at about 6-8 min for wild-type, Figure 7A). This result is in agreement with previous observations for rapidly deadenylated species (Decker and Parker 1993; Viswanathan et al 2004). It is therefore noteworthy that PAB1-ΔP failed to transition well from distributive to processive deadenylation for either mRNA (Figure 7A and 7B), even though the poly (A) tail lengths reached 30-36 A's (20 min for *GAL1-L* and 8-15 min for *MFA2pG*). For the RNA binding defects PAB1-Y83V and PAB1-F170V, even when the length of *GAL1-L* poly (A) reached about 30-35 A's, 12-20 min for PAB1-Y83V, and 12 min for PAB1-F170V (Figure 5A) there was no clear processive reaction occurring. Similarly, when the poly (A) tail length of *MFA2pG* was at about 30 nt for PAB1-Y83V (8 min) and for PAB1-F170V (15 min) there is little processive deadenylation (Figure 6B). Similar observations were obtained for PAB1-ΔRRM1 and PAB1-ΔRRM2 (Figure 7A and 7B). These results in general suggest that these regions of PAB1 are necessary for the formation of an mRNP structure that aids CCR4 activity in its transition from distributivity to processivity, as well as for its distributive activity.

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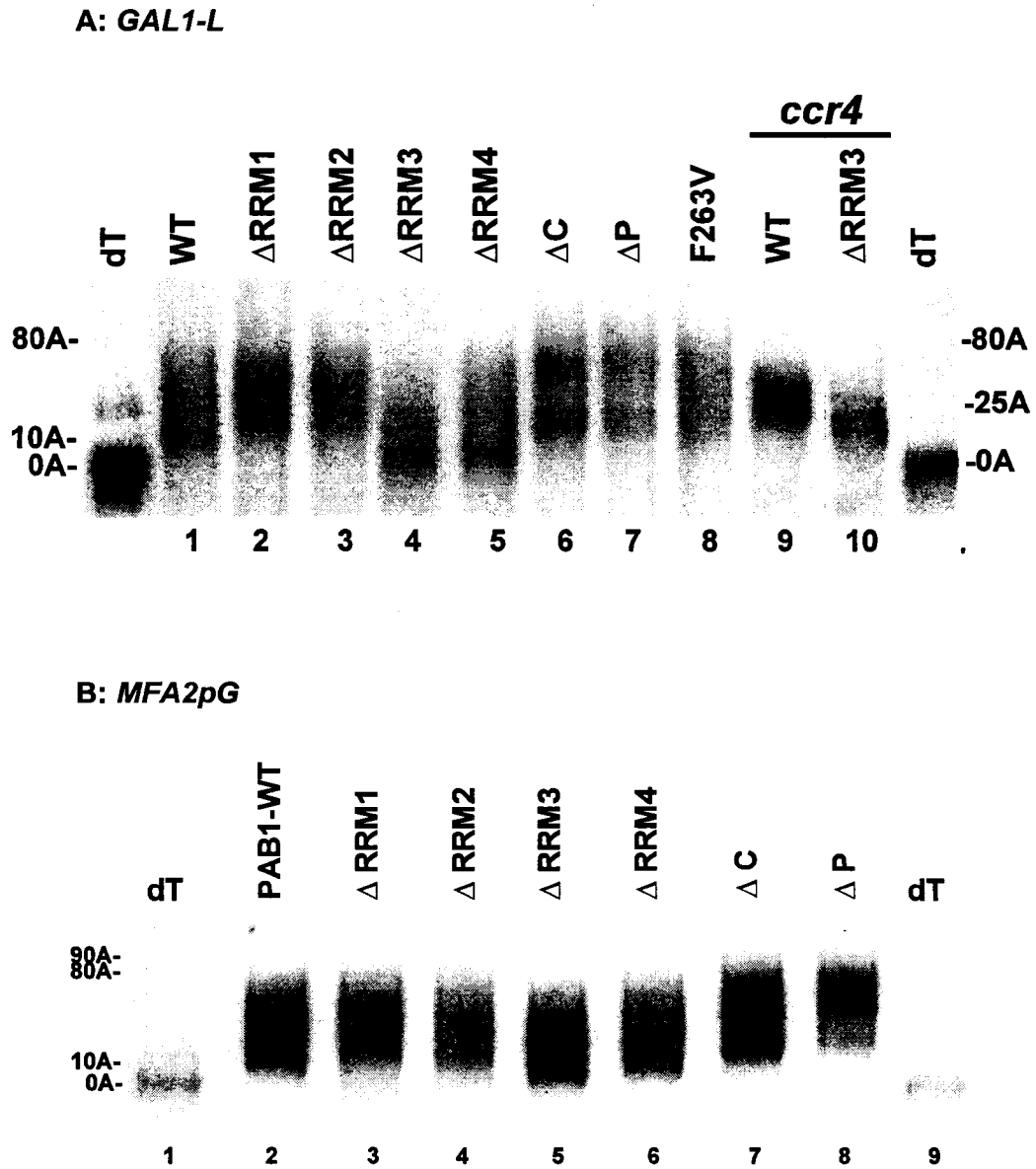
A: *GAL1-L*

B: *MFA2pG*

FIGURE 6. Steady state analysis of *GAL1-L* and *MFA2pG* mRNA poly (A) lengths as a function of PAB1 defects. Steady state levels of the mRNA were obtained following a 3 hr growth under galactose growth conditions. Poly (A) lengths are given on the sides. A- *GAL1-L*; B- *MFA2pG*

A: *GAL1-L*
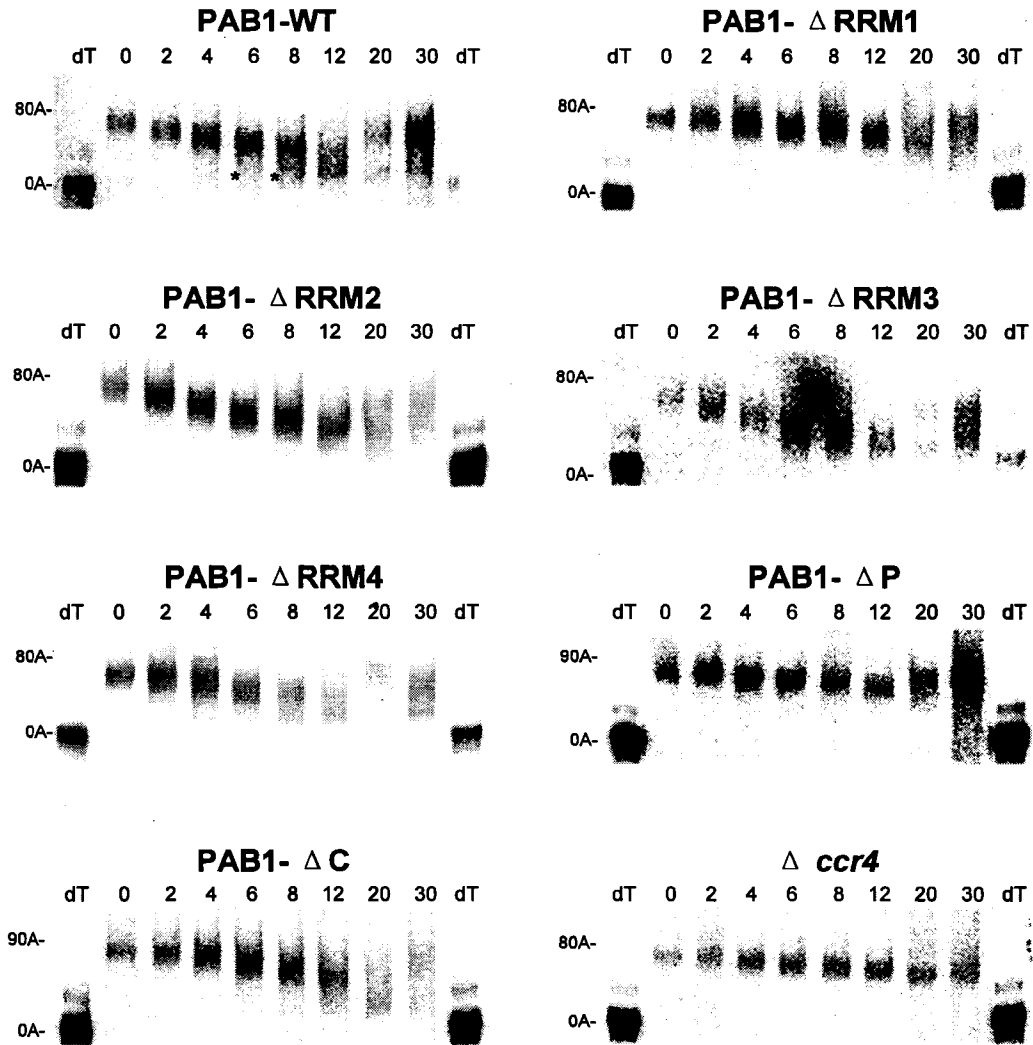
FIGURE 7A. Multiple PAB1 defects affect CCR4 deadenylation. Pulse-chase analysis was used for determination of rates of mRNA deadenylation. A- *GAL-L*. The asterisks in PAB1-WT indicate the presence of the oligo poly (A) species present at the same time as the longer poly (A) species indicative of processive deadenylation.

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B: *MFA2pG*

PAB1-WT

| dT | 0 | 1 | 2 | 4 | 8 | 15 | 30 | dT |

80A-

0A-

PAB1- Δ RRM1

| dT | 0 | 1 | 2 | 4 | 8 | 15 | 30 | dT |

80A-

0A-

PAB1- Δ RRM2

| dT | 0 | 1 | 2 | 4 | 8 | 15 | 30 | dT |

80A-

0A-

PAB1- Δ RRM3

| dT | 0 | 1 | 2 | 4 | 8 | 15 | 30 | dT |

80A-

0A-

PAB1- Δ RRM4

| dT | 0 | 1 | 2 | 4 | 8 | 15 | 30 | dT |

80A-

0A-

PAB1- Δ P

| dT | 0 | 1 | 2 | 4 | 8 | 15 | 30 | dT |

90A-

0A-

PAB1- Δ C

| dT | 0 | 1 | 2 | 4 | 8 | 15 | 30 | dT |

90A-

0A-

Δ *ccr4*

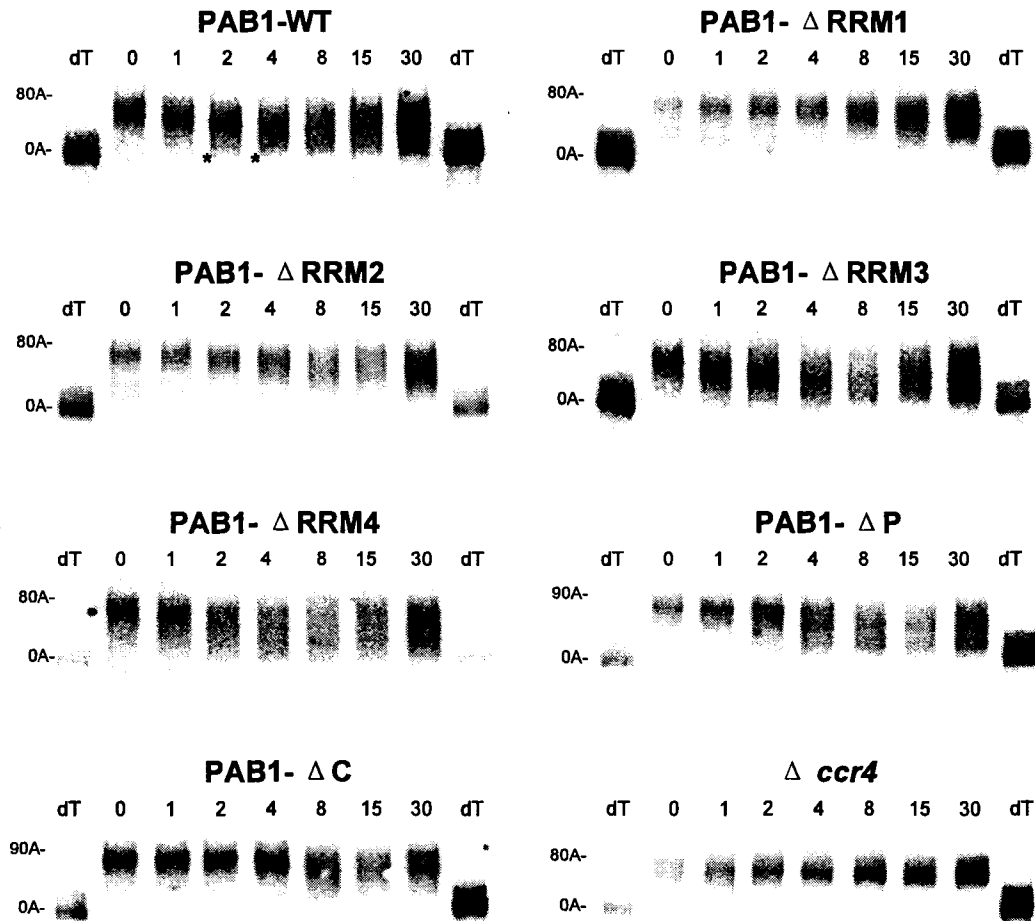| dT | 0 | 1 | 2 | 4 | 8 | 15 | 30 | dT |

80A-

0A-

FIGURE 7B. Multiple PAB1 defects affect CCR4 deadenylation. Pulse-chase analysis was used for determination of rates of mRNA deadenylation. B-*MFA2pG*. The asterisks in PAB1-WT indicate the presence of the oligo poly (A) species present at the same time as the longer poly (A) species indicative of processive deadenylation.

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PAB1-PAB1 protein interaction is mediated by the P and RRM1 domains, implicating it in the control of CCR4 deadenylation

Because deleting the P domain reduced the rate of deadenylation, we subsequently tested the model that the reduction in the rates of deadenylation caused by deletion of certain PAB1 domains was the result of PAB1 unable to interact with itself. We examined yeast PAB1 self-association following non-denaturing gel electrophoresis. Purified PAB1-wt was found as analyzed by SDS-PAGE to migrate as a single band of the expected molecular weight (Figure 8A), but under non-denaturing gel conditions, PAB1-wt was found to form a series slower migrating species (Figure 8B). Western analysis with antibody specific to PAB1 identified eight distinct species of PAB1 (Figure 8B). These slower migrating species are indicative of the formation of multimeric PAB1 species as previously observed with higher eukaryotic PAB1 (Kuhn and Pieler 1996; Melo et al 2003). Four additional observations support the identification of these species as multimers of PAB1: 1) the eight identified species of PAB1 migrate relatively linearly in relation to the expected logarithmic value of their molecular weights (data not shown), 2) the original PAB1 sample was 90% pure, 3) PAB1 migrates as a single, cohesive species following SDS-PAGE that is inconsistent with multiple phosphorylation of PAB1 (Vallari et al 1992), and 4) other experiments indicate that post-translational modifications such as acetylation do not result in the multiple species displayed by PAB1 that are observed following non-denaturing gel electrophoresis (Denis and Young 1983; Denis 1982).

We subsequently analyzed whether PAB1-PAB1 association was important for deadenylation by determining whether deletions in PAB1 that affected deadenylation were defective in PAB1-PAB1 interactions. As shown in Figure 8A and 8B, PAB1-ΔP failed to form the multimeric species that PAB1-wt did, forming only the dimeric form, albeit in much reduced amounts. This result is in agreement with previous observations demonstrating that the P domain of higher eukaryotic PAB1 is required for PAB1 multimerization (Kuhn and Pieler 1996; Melo et al 2003). Surprisingly, deleting the RRM1 domain also blocked the formation of the multimeric species (Figure 8B). As PAB1-ΔP and PAB1-ΔRRM1 were specifically defective in both the distributive and processive deadenylation, we interpret these results to indicate that PAB1-PAB1 self-association is one factor required for deadenylation by CCR4 to occur. Other PAB1 deletions that affected the rate of deadenylation to a lesser extent, such as ΔRRM2 and ΔC, did not affect the ability of PAB1 to form multimers (Figure 8B). These other domains are probably acting by other means to affect deadenylation, as observed below.

A. SDS-PAGE

B. WESTERN BLOT NATIVE GEL
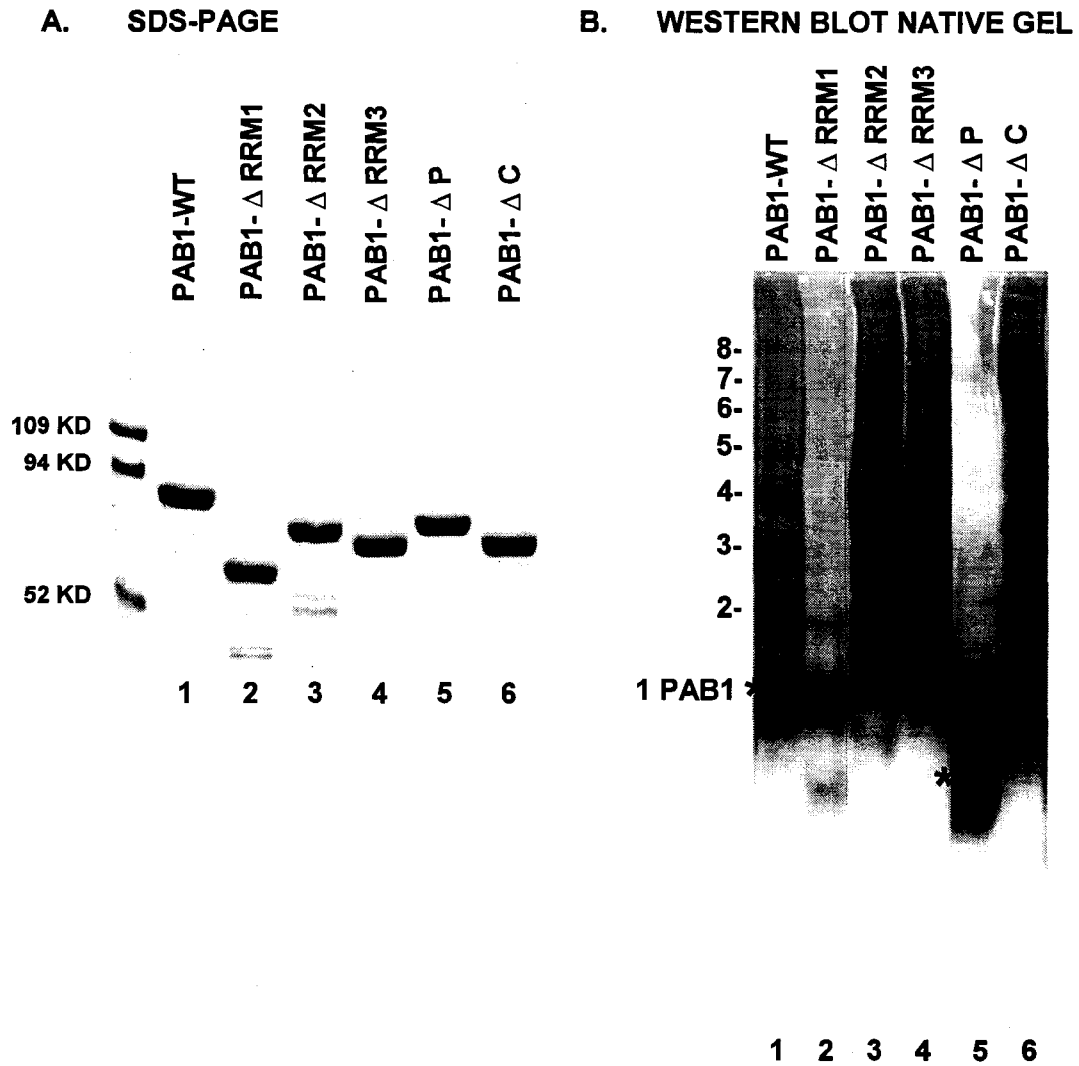
Figure 8. Yeast PAB1 can form multimers. A. Coomassie stained PAB1 proteins following SDS-PAGE. The PAB1 proteins (7 μg each) following purification by Flag immunoprecipitation were subjected to SDS-PAGE. B. Western analysis of the PAB1 purified proteins (6 μg each) using Flag antibody following non-denaturing PAGE. Samples were treated with RNase prior to separation by PAGE. Asterisks mark the multimeric species identified for wild-type PAB1. Similar profiles as that of PAB1-wt were observed for PAB1-ΔRRM3 and PAB1-ΔRRM4 (data not shown).

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The CCR4-NOT complex is destabilized in the cell by loss of RNA contact by RRM2.

Because a defect in RRM2 contact to poly (A) reduced the rate of deadenylation (PAB1-F170V), we subsequently examined the model that PAB1 integrity and specifically its contacts to the poly (A) tail are being communicated to the CCR4-NOT complex. One mechanism for this may be that PAB1-RNA contacts establish an mRNP structure that influences CCR4-NOT function. Although there may be several ways that this communication may be evinced, we initially tested this model by determining whether there was any effect of PAB1 defects on the formation of the CCR4-NOT complex. To this end, in collaboration with Yueh-Chin Chiang, we analyzed whether the various PAB1 deletions that we constructed affected the CCR4-NOT complex by immunoprecipitating the complex with antibody directed against CAF40 (Chen et al 2001). We found that deletion of RRM2, but not other regions of PAB1 (Figure 9C, ΔRRM2, lane 3, as compared to wild-type, lane 1, ΔRRM1, lane 2, or the other PAB1 deletions, Y.-C. Chiang, pers. comm.) resulted in diminished quantities of the CCR4, CAF1, NOT4, and NOT1 components and therefore the whole CCR4-NOT complex in the cell as ascertained by immunoprecipitation of CAF40. RRM2 contact to RNA was required for this phenotype as PAB1-F170V also reduced the amount of the complex in the cell (Figure 9C, lane 4) whereas abrogating RRM2 contacts to eIF4G (PAB1-184) or other RRM2 defects (PAB1-134) had little impact on the CCR4-NOT complex (lanes 5 and 6). Three additional observations suggest that RRM2 contacts to the poly (A) tail are

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required for stabilizing the CCR4-NOT complex. First, the abundance of the CCR4-NOT components but not that of other non-specific proteins is reduced in crude extracts in the strains carrying the RRM2 RNA binding defect or RRM2 deletion (Figure 9A; Y.-C. Chiang, pers. comm.). Because in this strain background, CCR4 could not readily be detected in crude extracts we also conducted immunoprecipitations with CCR4 antibody to verify its presence. We found that in PAB1-ΔRRM2 and PAB1-F170V backgrounds that CCR4 protein concentration was also less than in the controls (Figure 9B). CAF40 protein abundance appears to be unaffected by PAB1-ΔRRM2 because its concentration in the cell is about 6-10-fold greater than that of each of the other CCR4-NOT components. The fact that CAF40 abundance is not affected by PAB1 defects indicates that only the CCR4-NOT complex is being affected. Second, the rate of protein translation was quantitated in each of these strains carrying the PAB1 deletion variants and PAB1-F170V and what was found not to significantly affect the rate of incorporation of [^{35}S]-methionine into newly synthesized proteins as compared to that observed with wild-type PAB1 (D. Lee, pers. comm.). It is unlikely, therefore, that the specific loss of CCR4-NOT complex components observed with PAB1-F170V and PAB1-ΔRRM2 are the result of general effects on protein translation. Third, mRNA levels of CCR4, CAF1, and NOT4 were not affected by these RRM2 defects as analyzed by S1 analysis (D. Lee, pers. comm.). These observations suggest that the CCR4-NOT complex associates with a PAB1-mRNP in such a way that it is protected from proteolytic degradation. These data support an additional positive role for the PAB1 mRNP structure in

signaling deadenylation and a specific role for RRM2 in deadenylation separate from that of RRM1.

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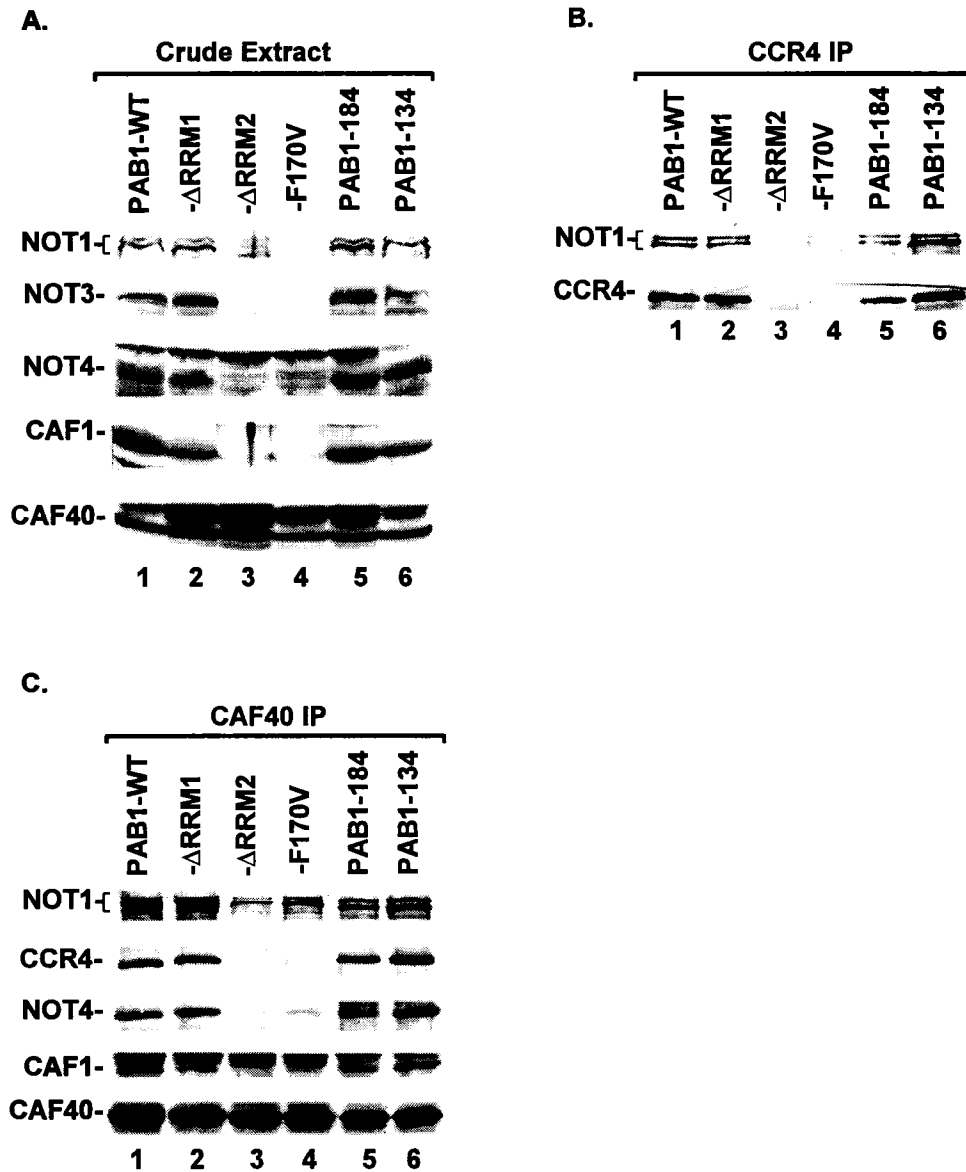
Figure 9. PAB1 RRM2 defects affect the CCR4-NOT complex. Antibody directed against CAF40 or CCR4 was used to immunoprecipitate the CCR4-NOT complex from strains carrying the different PAB1 variants from strain AS319 as indicated. Western analysis was conducted with the indicated antibodies. Other experiments indicate that deletion of RRM3, RRM4, or C-terminal regions of PAB1 did not affect CCR4-NOT complex formation (data not shown). A- Crude extracts; B- CCR4 immunoprecipitation; and C- CAF40 immunoprecipitation.

The RRM3 domain of PAB1 plays multiple roles in inhibiting deadenylation and decapping

a. RRM3 domain of PAB1 inhibits the deadenylation activity of both CCR4 and PAN2/3

In contrast to deleting RRM1 and RRM2 and portions of the C-terminus, deleting RRM3 resulted in augmentation of several different aspects of the deadenylation process. First, as shown in Figure 7A, B and Table 1, PAB1-ΔRRM3 accelerated the rate of deadenylation of *GAL1-L*, *GAL1-S*, and *MFA2pG* by 1.6- to 1.7-fold. The RNA binding mutation in RRM3 (F263V), in contrast, slowed the deadenylation rate, implying it was RRM3 contacts to other proteins that inhibited the rate of deadenylation (Table 1). As replacing RRM3,4 with RRM1,2 also augmented the rate of deadenylation for the *GAL1-S* mRNA (Figure 4; Table 1), RRM3 appears to be playing an inhibitory role in CCR4 deadenylation.

In order to study the mechanism by which the RRM3 region of PAB1 inhibits deadenylation, I examined whether RRM3 could inhibit CCR4 or PA2/3 or both. Using a *ccr4* strain in which PAN2/3 is the only yeast deadenylase, I showed that the rate of PAN2/3 deadenylation of *GAL1* was augmented with a RRM3 deletion (from 1.3 A's/min to 2.1 A's/min); on the other hand, in a *pan3* strain, when CCR4 is the only deadenylase, deleting RRM3 also accelerates the deadenylation rate of *GAL1* (from 1.6 A's/min to 2.1 A's/min). These results indicate that RRM3 inhibition of deadenylation is most likely affecting some general feature of the

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mRNP structure and is not specifically targeting the CCR4-NOT complex or PAN2/3 alone.

b. RRM3 affects the CCR4-NOT deadenylation endpoint

Second, an RRM3 deletion caused *GAL1* steady state poly (A) lengths to be shorter (0-6 A's) (Figure 6A, lane 4) than that found in wild-type PAB1 (lane 1) (8-12 A's). Similar effects were also observed on *MFA2pG* poly (A) lengths (Figure 6B, lane 5 as compared to lane 1). Mutating the RRM3 contact to RNA (PAB1-F263V) did not result in this phenotype (Figure 6A, lane 8; data not shown), again suggesting that RRM3 is making other critical protein contacts. Deleting RRM4 also tended to result in shorter steady state lengths for the poly (A) (Figure 6A, lane 5). As the analysis of a number of different mRNA poly (A) lengths (Decker and Parker 1993; Viswanathan et al 2004) has indicated that CCR4-NOT deadenylation stops at 8-12 A's, these results implicate RRM3, and to a lesser extent RRM4, in establishing a terminal mRNP structure that resists CCR4-NOT deadenylation. Since it is predicted that RRM3,4 would bind about 12 nt of RNA in a manner similar to that observed for RRM1,2 (Deo et al 1999), our data suggest that RRM3 and RRM4 are being retained on the poly (A) tail when CCR4-NOT ceases deadenylation (see Discussion, Figure 12, step 4).

c. RRM3 of PAB1 is required for formation of a terminal PAB1-mRNP structure that inhibits PAN2/3

PAN2/3 by itself can not deadenylate past 24 nt when CCR4 is absent (Tucker et al 2001), suggesting that PAN2/3 are blocked by a terminal PAB1–mRNP structure. If this were true, I hypothesized that PAB1 deletions might affect this structure and allow further deadenylation in a *ccr4* background. I found that ΔRRM3 but not other PAB1 deletions allowed PAN2/3 to deadenylate down to about 13-14 A's (Figure 6A, lane 10 as compared to lane 9). These results suggest that the RRM3 domain of PAB1 is also critical to making contacts that establishes a particular terminal mRNP structure that CCR4 can partially deadenylate through (to about 10 nt) but which blocks PAN2/3 (24 nt).

d. RRM3 of PAB1 inhibits decapping

As defects in decapping factors also result in deadenylation to proceed to about 0 A's (Tharun et al 2000; Schwarz and Parker 2000; Dunckley and Parker 1999)), it may be that RRM3 of PAB1 is involved with decapping factors at this stage of deadenylation in the transition and signaling from deadenylation to decapping. We consequently examined if any of the PAB1 variants increased the conversion of full-length *MFA2pG* mRNA to that of the *pG* fragment, a typical assay used to quantitate the extent of decapping (Coller et al 2001). As shown in Figure 10, PAB1-ΔRRM3 resulted in increased levels of the *pG* fragment relative to that of the full-length mRNA (lane 3), suggesting that it was accelerating the rate of decapping. This result would be consistent with an inhibitory role for

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PAB1 in decapping. A *dhh1* control is displayed in lane 1 in which the decapping process is severely compromised.

Significantly, we observed that swapping of the RRM1,2 cassette for that of RRM3,4 in PAB1-1,2,1,2,P,C caused a substantial augmentation (2.2-fold) in the formation of the *pG* fragment from *MFA2pG* relative to that of the full-length mRNA (Figure 10, lane 8 compared to lane 7). Moreover, PAB1-1,2,1,2,ΔP, which displayed as slow of a rate of deadenylation of the full-length *MFA2pG* transcript as did PAB1-ΔP (data not shown), still displayed a large increase (lane 10) in the formation of the *pG* fragment as compared to PAB1-ΔP (lane 6). These results implicate PAB1, specifically the RRM3 domain, in playing a critical role in inhibiting the decapping process.

We subsequently tested whether there was any genetic linkage between RRM3 and decapping factors by ascertaining if any of the PAB1 variants genetically interacted with loss of the decapping regulators DHH1 or DCP1. A *dhh1* or *dcp1* deletion results in temperature sensitive growth. Deletion of either RRM3 or RRM4, but not of any other PAB1 region, was found to suppress *dhh1* or *dcp1* temperature sensitive lethality (Table 1). Also, PAB1-1,2,1,2,P,C was capable of suppressing the *dhh1* or *dcp1* lethality. These data support a functional connection between RRM3 and RRM4 and that of the decapping process.

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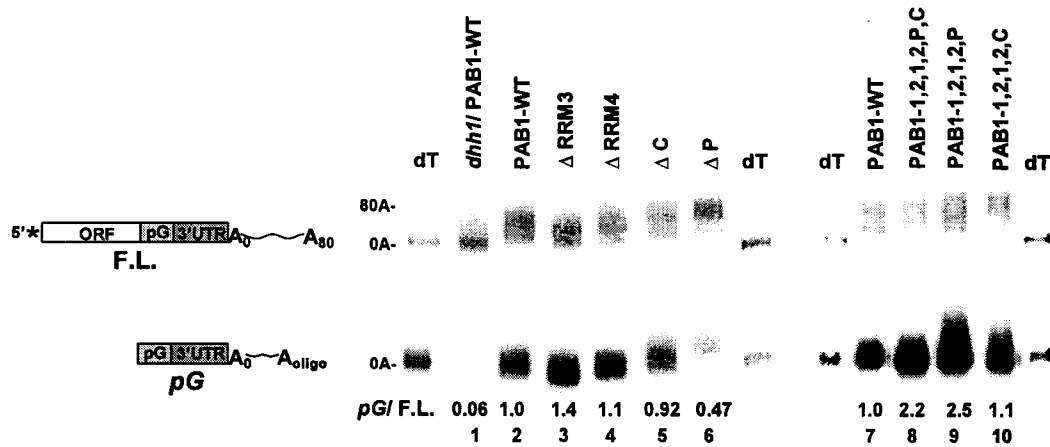
FIGURE 10. Formation of the pG fragment from MFA2pG is accelerated by PAB1 alterations. Steady state levels of the MFA2pG mRNA were obtained following a 3 hr growth under galactose conditions using the PAB1 variants in strain AS319. The pG/F.L. ratio refers to the abundance of the pG fragment relative to that of the full-length mRNA. The wild-type ratio was set 1.0. Values represent the average of two separate experiments. The SEM values were less than 15%.

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Translation termination factor eRF3 contacts to the C-terminus of PAB1 are required for CCR4 deadenylation

As shown in Table 1, deleting the globular C-terminal region of PAB1 slowed CCR4-NOT deadenylation. Contacts made by the C domain include those made to PAN2/3 (Mangus et al 2004a) or to translation termination factor eRF3. Of these two known contacts, the most likely factor that would be required for CCR4-NOT deadenylation would be eRF3 since deletion of either PAN2 or PAN3 does not affect the deadenylation rate. As translation termination may be a signal that initiates deadenylation, I decided to test whether the slowing of deadenylation that was observed in PAB1-ΔC was due to loss of the ability of PAB1 to bind eRF3.

To test this hypothesis, I overexpressed in yeast either the N-terminus of eRF3 (N-eRF3) that abrogates wild-type eRF3 contacts to the C-terminal region of PAB1 or the C-terminus of eRF3 that does not interfere with any interactions (Hoshino et al 2004). After a 1.5 hr induction of eRF3 expression and of *GAL1* mRNA with galactose, transcription was shut off with the addition of glucose. Because this methodology does not produce a pulse of *GAL1* synthesis, the deadenylation of the *GAL1* poly (A) tail can best be followed by observing the shortening of the longest length poly (A) tails (Figure 11A). Overexpression of N-eRF3 as compared to C-eRF3 slowed the rate of deadenylation of *GAL1* mRNA. In the C-eRF3 the majority of the poly (A) tails are shortened to an oligo (A) size within 10-15 mm (deadenylation rate of 3.7 A's/mm) whereas with

overexpression of N-eRF3 deadenylation was not complete until about 30 mm (deadenylation rate of 2.0 A's/mm). The same results were obtained when overexpression of N-eRF3 was compared to a control plasmid that did not express either segment of eRF3. Moreover, overexpression of N-eRF3 in conjunction with several different PAB1 RNA binding defects severely slowed CCR4 deadenylation (Figure 11A, for PAB1-ΔRRM1: deadenylation rate of 2.4's/min with C-eRF3 and 1.4A's/min with N-eRF3).

We subsequently examined whether other mutants that interfered with translation termination also reduced deadenylation. Using an eRF3 variants (*gst1-N106I*) that fails to bind well to eRF1 (Hosoda et al 2003), we found that both *GAL1-L* deadenylation was severely compromised (Figure 11B, C: 6.5 A's/min for *GST1* and 4.2 A's for *gst1-N106I*). Similar results were found for *GAL1-S* (4.9 A's/min for *GST1* and 4.0 A's for *gst1-N106I*). Because *gst1-N106I* interferes with translation termination (Hosoda et al 2003), our results support the model that translation termination is one signal required for the onset of CCR4-NOT deadenylation.

PAB1-WT/ C-eRF3

dT 0 2 5 10 15 20 30 45 60

80A-

0A-

PAB1-ΔRRM1/ C-eRF3

dT 0 2 5 10 15 20 30 45 60 dT

80A-

0A-

PAB1-WT/ N-eRF3

dT 0 2 5 10 15 20 30 45 60

80A-

0A-

PAB1-ΔRRM1/ N-eRF3
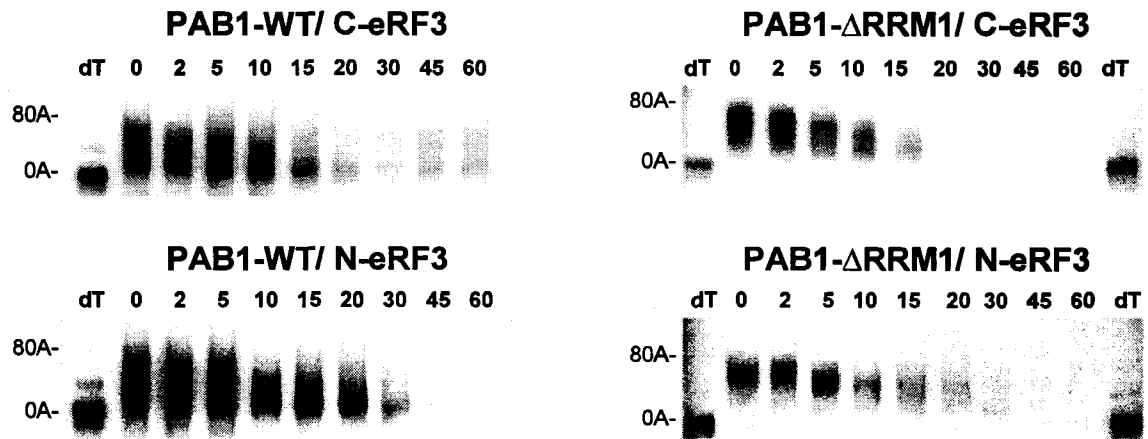
dT 0 2 5 10 15 20 30 45 60 dT

80A-

0A-

FIGURE 11A: Blocking PAB1-eRF3 interactions slows deadenylation. Time course of *GAL1-L* poly (A) lengths as determined in Figure 4. Yeast (strain 1729-3 with the indicated PAB1 variant) were grown for 1.5 hr in galactose and shut off at time zero with the addition of glucose. Effect of overexpression of the N-terminus of eRF3 on CCR4 deadenylation. C-eRF3 and N-eRF3 represent the carboxy and amino terminal halves of the eRF3 protein expressed from a *GAL1* promoter (Hoshino et al 2004).

B:

C:

Minutes after inhibition of transcription
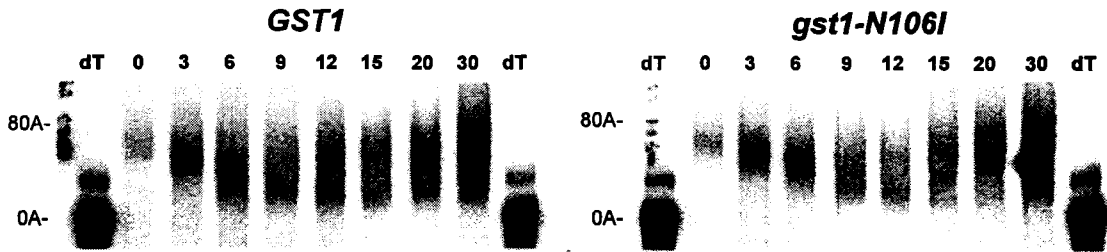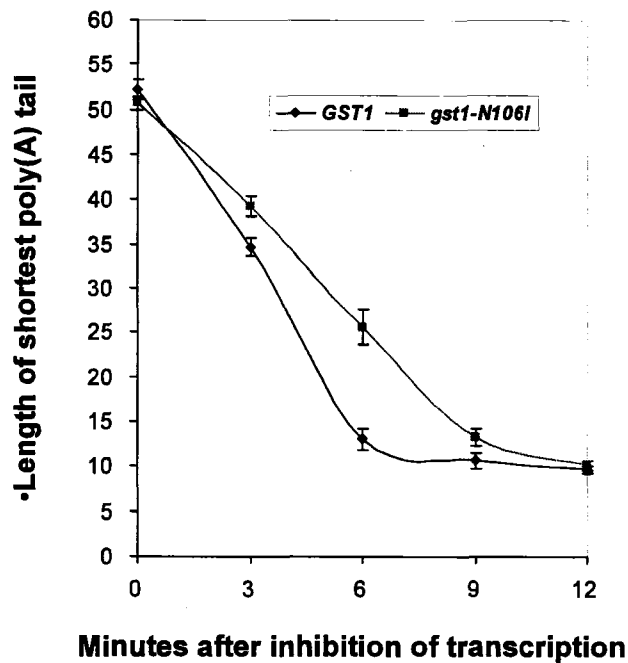
FIGURE 11B, C: Blocking eRF1-eRF3 interactions slows deadenylation. The eRF3-eRF1 contact was blocked with allele *gst1-N106I*; wild-type: *GST1*. B-Time course of *GAL1-L* poly (A) lengths was determined as described in Figure 4. C- Average poly (A) tail length as a function of time.

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CHAPTER 4

DISCUSSION

PAB1 plays multiple roles in the mRNA degradative process

The results presented herein demonstrate that PAB1 plays a number of key roles in integrating the mRNA turnover process. It conjoins and links translation initiation, termination, deadenylation, and decapping. Our results establish that PAB1 does not simply inhibit deadenylation, although the RRM3 domain plays an inhibitory role. Instead, we have identified four critical mechanisms by which regions of PAB1 are required for the deadenylation process: proper PAB1 binding to the poly (A) tail, PAB1-PAB1 protein interactions, contacts to translation termination factors, and stabilization of the CCR4-NOT deadenylase complex.

Several pieces of evidence indicate that PAB1 must be bound to the poly (A) tail in order for CCR4-NOT to deadenylate. Defects in RRM1 and RRM2 that reduce PAB1 ability to bind RNA (Deardorff and Sachs 1997; Kessler and Sachs 1998) significantly reduced CCR4-NOT deadenylation. These effects occurred for both rapidly deadenylated mRNA (*MFA2pG*) and for slowly deadenylated mRNA (*GAL1-S*). Other mutations in RRM2 such as PAB1-134 or PAB1- 184 that do not affect RNA binding but do affect other translation related functions of PAB1 (Otero et al 1999) had no effect on deadenylation. Importantly, PAB1-

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1,2,1,2,P,C, that could bind 6-fold better in vitro to poly (A) than wild-type PAB1, did not reduce deadenylation and instead resulted in faster rates of deadenylation for *GAL1-S* mRNA. PAB1 clearly does not simply play an obstructionist, inhibitory role in regards to CCR4-NOT deadenylation in vivo.

PAB1-PAB1 interactions are required for the distributive to processive transition during deadenylation

Our results (Figure 4, Figure 5 and Figure 7) and those of others (Decker and Parker 1993) indicate that the CCR4-NOT complex undergoes a switch from a primarily distributive to a processive state once the poly (A) tail has reached a length of about 35-50 A's for rapidly deadenylated mRNA (see, for example, Figure 7B, 1-2 min for *MFA2pG,* and 7A, 6-8 min for *GAL1-L,* when both long and short poly (A) tails are present indicative of a processive deadenylation reaction). This switch not only significantly accelerates deadenylation of a particular mRNA and hence the degradation of the mRNA but also can be envisaged as a major site for controlling the vastly different rates at which mRNA are degraded. For instance, *PGK1* and *GAL1-S* are primarily deadenylated distributively, resulting in a slower turnover of the mRNA (Decker and Parker 1993; Viswanathan et al 2004) whereas processively deadenylated mRNA turnover more rapidly (*MFA2, STE3, COX17, GAL1-L*) (Decker and Parker 1993; Viswanathan et al 2004; Olivas and Parker 2000).

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We have identified parts of PAB1 that are important to this transition: the P domain and the RRM1 and RRM2 domains. Deletions or defects within these regions produced PAB1 proteins that failed to transition well from the distributive to processive phase for both *MFA2pG* and *GAL1-L* mRNA. These observations suggest that the mRNP undergoes a significant PAB1-dependent transition during deadenylation at about the 40 A stage in regards to altering the ability of CCR4-NOT to switch from a primarily distributive to a processive mode of action. As we have shown that CCR4 can itself move from the distributive to the processive mode as it sees greater lengths of the RNA substrate (at least 30 nt) (Viswanathan et al 2003, 2004), the rearrangement of PAB1 on a long stretch of RNA might aid CCR4 in becoming a processive enzyme. Our observations and those made previously that the P domain of PAB1 functions to promote cooperative PAB1-PAB1 intermolecular interactions (Melo et al 2003; Kuhn and Pieler 1996) support a role for the P domain in this transition. An attractive model for this transition would be that PAB1-PAB1 interactions enhance the sequential removal (unzipping) or rearrangement of PAB1 on the poly (A) tail allowing CCR4-NOT to switch from the distributive to processive mode (see Figure 12, step 3).

The model that PAB1-PAB1 interactions are critical to the distributive to processive transition is supported by our demonstration that both PAB1-ΔP and PAB1-ΔRRM1 were severely deficient in their ability to multimerize. The P region, based on its sequence, is an apparently unstructured domain that is known to

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make two types of protein contacts: to PBP1, an inhibitor of PAN2/3 (Mangus et al 2004), and to the P region in other PAB1 molecules. It is very unlikely that PBP1 is required for CCR4-NOT function, as defects in PAN2/3 do not affect the deadenylation rate (Tucker et al 2001; unpublished observations). We hypothesize, therefore, that it is the inability of the P region to interact with other PAB1 proteins in PAB1- P that causes the severe defect in CCR4-NOT deadenylation. RRM1, located as it is, at one end of the PAB1 molecule, may help stabilize adjacent PAB1-PAB1 protein contacts (see Figure 12).

The extensive PAB1-PAB1 interactions that we observed may also influence a number of other aspects of mRNA metabolism. For example, the P domain restricted PAB1 association with the RNA (Table 1), suggesting that PAB1 multimerization may be inhibitory or regulatory for PAB1-poly (A) associations. Also, intracellular localization of mRNA is critical to a number of key cellular processes (Shepard et al 2003; Gu et al 2004). Proper association of the mRNA at particular sites may be influenced by PAB1-PAB1 self-association as governed by other particular RNA binding factors (Gu et al 2004).

In contrast to deletion of RRM1 or the P domain, we observed that deletion of RRM2 did not affect PAB1 multimerization, although it did slow the rate of deadenylation and the distributive to procession transition. In this case, however, deleting RRM2 or altering its ability to bind RNA (PAB1-F170V) resulted in destabilization and reduced levels of the CCR4-NOT complex. Other mutations in

RRM2, such as evidenced in the PAB1-184 or –134 proteins, did not affect the formation of the CCR4-NOT complex. It appears, therefore, that it is the contact to poly (A) that affects the CCR4-NOT complex abundance in the cell, thereby explaining why such RRM2 defects would be defective in deadenylation. One model in agreement with these observations would be that the integrity of the mRNP structure involving PAB1 bound to the poly (A) tail is 'read by' or influences the state of the CCR4-NOT complex. The RRM2 domain contact to the RNA must be critical for the formation of this mRNP structure. In this regard, it may be significant that the deadenylase domain of CCR4 is known to make contact to eIF4G (Y.-C. Chiang, pers. comm.), one of the factors that would be expected to be within this mRNP structure.

Deletion of the terminal C globular domain of PAB1 also slowed the rate of CCR4-NOT deadenylation, and we demonstrated that contacts to the translation termination factor eRF3 were likely to mediate this process. Interference with the eRF3 contact to eRF1, a defect that is known to block translation termination (Hosoda et al 2003), also slowed deadenylation as did mutations that interfered with the GTPase activity of eRF3 and hence its ability to terminate translation (Salas-Marco and Bedwell 2005). Proper protein translation termination is, therefore, one of the steps that ensure complete CCR4-NOT deadenylation of the mRNA. However, loss of the P region of PAB1 and interfering with PAB1 RRM1 contact to the RNA resulted in much more significant declines in mRNA deadenylation than did deleting the C region. Translation termination appears to

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play a more minor role in initiating deadenylation. The exact role of translation termination in deadenylation is also made problematic given that for the average yeast mRNA there are a very large number of translation termination events per mRNA. While it is unlikely that each of these specific transversals of the mRNA by the ribosome directly affects deadenylation, there may be some type of requirement or interplay between the presence of translation termination factors, the reinitiation of translation, and the deadenylation process.

RRM3 of PAB1 inhibits deadenylation and decapping and affects the mRNP structure of the terminal PAB1 bound to poly (A)

Deletion of RRM3 of PAB1 accelerated deadenylation of each of the mRNA that we analyzed. In that deleting RRM3 also resulted in increased abundance of the *pG* fragment relative to full-length *MFA2pG*, a sign of an increased decapping rate (Coller et al 2001), RRM3 appears to be critical for restricting both deadenylation and decapping. Our observation that a defect in RRM3 binding to RNA did not elicit the same phenotypes as that of deleting RRM3 argues for a critical protein contact made by RRM3 in these processes. Previously, it had been shown that defects in translation initiation factors also enhance both the deadenylation rate and the decapping process (Schwarz and Parker 1999). It is hence possible that RRM3 exerts its effect on deadenylation/decapping by interacting with or affecting some aspect of the translation initiation process. Alternatively, RRM3 may be required to form a general mRNP structure required for both processes. It is also possible that ΔRRM3 accelerates decapping by

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accelerating deadenyltion, although PAB1-1,2,1,2,P,C accelerated decapping without affecting deadenylation.

Another novel role that was observed for RRM3 was that its deletion resulted in significantly shorter poly (A) tails (0-6 A's) at the end of the deadenylation process than were observed with wild-type PAB1 (8-12 A's) or other PAB1 defects. Deletion of RRM4 also elicited a similar phenotype. These results are consistent with the model that RRM3 and RRM4 are actually retained on the poly (A) tail at the cessation of CCR4-NOT deadenylation, even though RRM2 and RRM1 are presumably no longer making contact with the poly (A) (see Figure 12, step 4). The size of the poly (A) tail at this juncture would be consistent with the expected occlusion size of RRM3 and RRM4 (8-12 A's) (Deo et al 1999). These observations would suggest that the terminal PAB1 does not come off the poly (A) tail at the end of deadenylation and is instead bound to the poly (A) tail in a terminal mRNP structure as mediated by RRM3 and RRM4. The inhibition of decapping that we observed with RRM3 is consistent with it playing a role in the mRNP structure that restricts decapping. Substitution of RRM1,2 domains (as in PAB1-1,2,1,2,P,C) for that of RRM3,4 also caused a substantial increase in decapping, suggesting that RRM3,4 are inhibitors of this process.
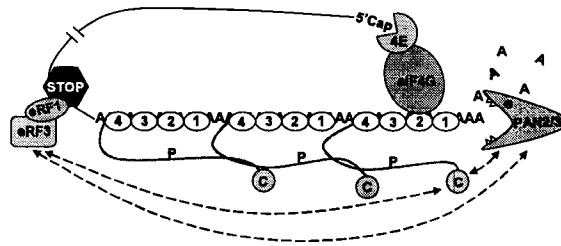
Model for the deadenylation process

Our data and that of others suggest the following model for the deadenylation process. Following the initial trimming of the poly (A) tail by

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PAN2/PAN3 (Brown and Sachs 1998; Tucker et al 2001) (Figure 12, step 1), the major process of deadenylation requires CCR4 as it shortens the poly (A) tail down to an end-point size of about 8-12 nt (Decker and Parker 1993) (Figure 12, steps 2 and 3). Dissociation of the first PAB1 may promote in a cooperative manner at the 40-50 A stage, through intermolecular P domain contacts as mediated by RRM1, the subsequent rearrangement of the next PAB1 and the shift of CCR4-NOT to a processive mode of action (step 3). CCR4 deadenylation is finally blocked at the 8-12 nt stage by a terminal PAB1 that is stabilized by other proteins, possibly decapping factors (step 4). These 8-12 nt of poly (A) may still be associated with RRM3 and 4 domains of PAB1.

Specific mRNA binding proteins that are known to regulate mRNA turnover (Olivas and Parker 2000; Lykke-Andersen and Wagner 2005; Semotok et al 2005; Tran et al 2004) may also be envisaged as controlling PAB1 interactions with the poly (A), with itself, with decapping proteins, with translation initiation factors, and with the CCR4-NOT deadenylase. Any alteration in the mRNP structures involving PAB1 could be used as a potent method for selectively controlling deadenylation, translation, and degradation of the mRNA. Our studies indicate that PAB1, far from being a simple block to deadenylation, integrates various signals to control the fate of the poly (A) tail and hence the expression and destiny of the mRNA. Importantly, they indicate that, although RRM1 and RRM2 domains of PAB1 are clearly needed for RNA binding (Deo et al 1999), multiple regions of PAB1 are integral to the control of several critical aspects of mRNA

metabolism. Future studies will be directed towards understanding the regulation of PAB1 protein and RNA contacts in the merging and transmission of these signals and interactions.
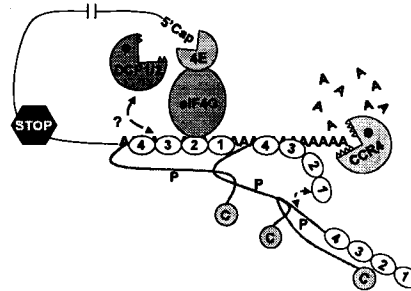
Step 1: PAN2/3 trims poly (A) tail to 60-80A's.

Step 2: CCR4 replaces PAN2/3 and shortens the poly (A) tail distributively.
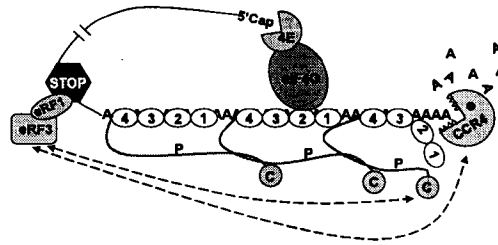
Step 3: At about 35-50A's CCR4 switches to processive deadenylation, possibly mediated by P domain interactions.
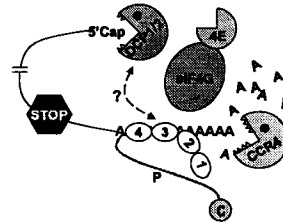
Step 4: CCR4 stops at 8-12A's.

FIGURE 12. Model for the deadenylation process in yeast as described in the text. The binding of eIF4G to the rightmost PAB1 is conjectural and it may bind to other PAB1s. Three PAB1 molecules are assumed to bind each poly (A) tail, in an anti-parallel fashion, as each PAB1 is expected to occlude about 25 nt (Deo et al 1999). The P domain of PAB1 is about 100 residues long and based on its sequence would be unlikely to form the most common secondary elements. Depending on the form it would take, it could extend at least 150-350 angstroms, sufficient to span the predicted length of four contiguous RRM domains (100 angstroms, Deo et al 1999). The P domain of one PAB1 would therefore be able to interact with an adjacent P domain.

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Table 1. PAB1 effects on Poly (A) Binding, Deadenylation and Yeast lethality

PAB1 variant	poly (A) binding K_D (nM)	Deadenylation rate, MFA2pG, A's/min	Deadenylation rate, GAL1-L, A's/min	Deadenylation rate, GAL1-S, A's/min	dhh1 or dcp1 suppression
PAB1 (wt)	11	11	3.7	2.8	-
PAB1-1,2,1,2,P,C	1.9	11	4.1	3.3	+
PAB1-Y83V	220	2.6	2.0	1.8	-
PAB1-F170V	270	3.0	2.3	2.1	-
PAB1-F263V	99	4.6	ND	ND	-
PAB1-F366V	39	7.9	ND	ND	-
PAB1-ΔRRM1	29	3.4	2.0	2.0	-
PAB1-ΔRRM2	35	4.2	3.2	2.4	-
PAB1-ΔRRM3	15	18	6.3	4.6	+
PAB1-ΔRRM4	22	11	3.9	2.9	+
PAB1-ΔP	7.4	2.1	1.5	1.1	-
PAB1-ΔC	18	7.5	3.2	2.1	-
PAB1-ΔP,C	6.0	ND	ND	ND	
PAB1-184	12	9.7	ND	ND	-
PAB1-134	18	9.5	ND	ND	+

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Table 1: PAB1 effects on Poly (A) Binding, Deadenylation and Yeast lethality PAB1. PAB1 variants are depicted as in Figure 1. Poly (A) binding was conducted in vitro using a gel shift assay with a 7N+23A radiolabeled RNA (Kessler and Sachs 1998). Our K_D values for PAB1 variants binding to poly (A) agreed very well with those of published values (Kessler and Sachs 1998; Deardorff et al 1997). Deadenylation rates were conducted in strain AS319 with the indicated PAB1 variant expressed from a pRS314 vector (*TRP1-PAB1*) replacing that of pAS77 by measuring the lengths of the shortest poly (A) tail as a function of time after transcriptional shut off. *GAL1-L* (long) and *GAL1-S* (short) refer to the two *GAL1* mRNA that result from differential poly (A) site usage and differ by 110 nt in their 3' UTR end (Cui and Denis 2003). Poly (A) binding assays are the average of 2-3 determinations with SEMs less than 20%. Deadenylation rates are the average of two to three determinations and the SEMs were less than 10% except for *MFA2pG* PAB1-ΔRRM4 it was 25%, PAB1-ΔP it was 21%, and PAB1-ΔC it was 19% and for *GAL1-L* PAB1-ΔRRM4 it was 12%, and for *GAL1-S* PAB1-ΔC it was 12%. The SEMs for the deadenylation rates for wild-type PAB1 were less than 4% for *MFA2pG* and *GAL1-L* and less than 2% for *GAL1-S*. Deadenylation rate values in bold differ significantly from wild-type. ND- not done. *dhh1* suppression was conducted in strain AS319-d1-uL and *dcp1* was conducted in strain 1716-1: '-': no growth at 37^0C; '+': growth at 37^0C.

CHAPTER 5

SUMMARY

The major objective of this study was to explore the function of poly (A) binding protein (PAB1) in mRNA degradation process in yeast and to determine the mechanism by which it regulates the mRNA degradation process.

First, we clarified the role PAB1 in the CCR4 deadenylation process. PAB1 does not simply inhibit the CCR4 deadenylation. In contrast, PAB1 protein must be properly bound to the poly (A) tail in order to allow CCR4 deadenylation. Increasing the PAB1 poly (A) binding by 6-fold better does not reduce the deadenylation rate in vivo, although defects in PAB1 poly (A) binding ability slow the CCR4 deadenylation rate for both rapidly deadenylated mRNA (*MFA2pG* and *GAL1-L*) and slow deadenylated mRNA (*GAL1-S)*.

Second, we established that the PAB1-PAB1 interaction exists in yeast as mediated by the RRM1 and P domains. In addition we found that the PAB1-PAB1 interaction is important for the CCR4 transition from the distributive deadenylation to the processive deadenylation. Previous research shows that the CCR4 deadenylation undergoes a switch from the distributive deadenylation to the processive deadenylation when the CCR4 shortens the poly (A) tail reach about 35-50 nt for the rapidly deadenylated mRNA (*MFA2pG* and *GAL1-L*). Our studies showed that deleting the PAB1 RRM1 or P domain, which displayed

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weakened PAB1-PAB1 interaction failed to either distributive deadenylation or to transition from the distributive to the processive deadenyltion state. We propose that the PAB1-PAB1 interaction is one factor required for the CCR4 deadenylation.

Third, deleting RRM2 or altering its ability to bind RNA (PAB1-F170V) resulted in destabilization and reduced levels of the CCR4-NOT complex. Other mutations in RRM2, such as evidenced in the PAB1-184 or –134 proteins, did not affect the formation of the CCR4-NOT complex. In addition the mRNA levels of CCR4, CAF1, and NOT4 were not affected by these RRM2 defects. These data explain why such RRM2 defects would be defective in deadenylation and support an additional positive role for the PAB1 mRNP structure in signaling deadenylation and a specific role for RRM2 in deadenylation separate from the other PAB1 domains.

Fourth, RRM3 of PAB1 inhibits deadenylation and decapping and affects the mRNP structure of the terminal PAB1 bound to poly (A). Deleting the PAB1 RRM2 accelerates the deadenylation rate for all RNA reporters which were used in this study. In contrast, PAB1-F263V which is specifically defective in Poly (A) binding slowed the deadenylation rate. It implies that a RRM3-protein interaction or the contribution of RRM3 to an important mRNP structure inhibits or regulates the mRNA deadenylation process. Deleing PAB1-RRM3 also causes the CCR4 deadenylation end point to reach 0-6 A's and PAN2/3 deadenylation end point to

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13-14A's. These results indicated that the terminal PAB1 is making a specific PAB1-mRNP structure which blocks the PAN2/3 deadenylation at 24 A's in the absence of CCR4 and also blocks the CCR4 deadenylation at 8-12 A's. In addition, after the poly (A) length reached the oligo A form, CCR4 stops deadenylating, and at this point this terminal PAB1 is still bound to the poly (A) tail through the RRM3 and RRM4 domains. From both biochemical and genetic analysis it seems that PAB1 RRM3 domain also play a role in the decapping process, which is consistent with PAB1 inhibitory function in decapping process.

Fifth, translation termination factor eRF3 contacts to the C-terminus of PAB1 are required for CCR4 deadenylation. Defects in PAB1 contacts to the translation termination factor eRF3 decreased the mRNA deadenylation rates. In addition, interference with the eRF3 contact to eRF1 and defects in the eRF3 GTPase activity which block translation termination also cause decreased deadenylation rates in vivo. These results indicate that proper protein translation termination is one of the steps that ensure complete CCR4-NOT deadenylation of the mRNA.

Although our work has made a significant advance in our understanding of the function of poly (A) binding protein in the mRNA degradation process in yeast, there are still many questions need to be answered. For example, what is the composition of the terminal PAB1-mRNP structure, which regulates the end of deadenylation? What kind of PAB1-protein contact through PAB1 RRM3 inhibits both the deadenylation and decapping processes? What specific protein contacts

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does PAB1 make through each individual domain? All of these questions will be

subjects of future study.

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