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Yeast Pab1 Interacts with Rna15 and Participates in the Control of the Poly(A) Tail Length In Vitro

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In *Saccharomyces cerevisiae*, the single poly(A) binding protein, Pab1, is the major ribonucleoprotein associated with the poly(A) tails of mRNAs in both the nucleus and the cytoplasm. We found that Pab1 interacts with Rna15 in two-hybrid assays and in coimmunoprecipitation experiments. Overexpression of *PAB1* partially but specifically suppressed the *rna15-2* mutation in vivo. *RNA15* codes for a component of the cleavage and polyadenylation factor CF I, one of the four factors needed for pre-mRNA 3′-end processing. We show that Pab1 and CF I copurify in anion-exchange chromatography. These data suggest that Pab1 is physically associated with CF I. Extracts from a thermosensitive *pab1* mutant and from a wild-type strain immunoneutralized for Pab1 showed normal cleavage activity but a large increase in poly(A) tail length. A normal tail length was restored by adding recombinant Pab1 to the mutant extract. The longer poly(A) tails were not due to an inhibition of exonuclease activities. Pab1 has previously been implicated in the regulation of translation initiation and in cytoplasmic mRNA stability. Our data indicate that Pab1 is also a part of the 3′-end RNA-processing complex and thus participates in the control of the poly(A) tail lengths during the polyadenylation reaction.

In eucaryotes, the 3′ end of mRNAs results from endonucleolytic cleavage followed by the polyadenylation of the upstream fragment. These processing mechanisms involve large protein complexes. Most of them have been characterized, and the genes for many of their components have been isolated (2, 11, 16–18, 25, 27, 29, 36, 41, 46). In vitro systems capable of processing 3′ RNA are generally used for their analysis (12, 38). The signal sequences in pre-mRNA and the transacting protein factors are different between yeast and higher eucaryotes, although overall the 3′-end-processing reactions are similar (19, 44).

In *Saccharomyces cerevisiae*, four complexes, first described as chromatographic fractions (12), are involved in mRNA 3′-end formation. CF I and CF II (cleavage factors I and II) are needed for the cleavage reaction. PF I (polyadenylation factor I), poly(A) polymerase (Pap), and CF I are required for polyadenylation. Thus, CF I is the only factor essential for both steps of processing. Poly(A) tails newly synthesized either in vitro or in vivo are 60 to 70 nucleotides long. Two proteins of CF I, Rna14 and Rna15, have been characterized (27). A new gene, *PCF11*, was recently identified and encodes a third protein of this complex (2). CF I has been purified and separated into two functional complexes, CF IA and CF IB (21). CF IA consists of four proteins, of 76 kDa (Rna14), 70 kDa (Pcf11) (20), 50 kDa, and 37 kDa (Rna15). CF IB is a single 73-kDa protein. Recently, one component of CF II, Cft1, was identified by its sequence similarity to the 160-kDa subunit of mammalian cleavage and polyadenylation specificity factor (CPSF) (36). PF I is composed of several polypeptides including Pap (30) and at least two other components encoded by *FIP1* and *YSHI/BRR5* (11, 18). Fip1 interacts with both Rna14 and Pap (29). Pap is also found in a complex with CF I (22). Thus, there appear to be multiple interactions between the various components of the polyadenylation complex.

Six factors are necessary to reconstitute mammalian 3′-end processing. Two, CPSF (16, 17) and Pap (31), are required for both steps. Three components are involved only in cleavage, CstF (cleavage-stimulatory factor) (26), CF Im, and CF IIm (32). The last two are probably unrelated to yeast CF I and CF II. The specific addition of poly(A) by Pap depends on two cofactors, CPSF and the poly(A) binding protein Pab II. The ternary complex bound to cleaved pre-mRNA enables the processive synthesis of full-length poly(A) tails of about 200 to 250 nucleotides both in vitro and in vivo (3, 43). Functionally, CPSF is analogous to yeast CF I, but there are no significant similarities among the three known CF I gene sequences and available CPSF gene sequences. The stimulation of Pap by Pab II is specific to higher eucaryotes and provides additional evidence that mechanisms of polyadenylation have diverged during evolution. The mammalian Pab II is located in the nucleus and is totally distinct from the poly(A) binding protein, Pab I, found in the cytoplasm (43). Pab I is highly conserved and is homologous to the single Pab1 of yeast, which is associated with poly(A) tails in both the nucleus and the cytoplasm (33).

In *S. cerevisiae*, the Pab1 associated with poly(A) tails stimulates the initiation of translation (15, 39) and regulates mRNA stability. Most mRNAs are degraded by a deadenylation-dependent pathway. The first step is shortening the poly(A) tail followed by decapping and RNA core degradation. Pab1 participates in the regulation of both mRNA decapping and deadenylation (9, 10). Poly(A) tails are longer in *pab1* mutants than in the wild type in vivo (34). Similarly, Pab1-dependent poly(A) nuclease (Pan) mutants also have longer poly(A) tails. Pan specifically degrades poly(A) bound by Pab1 (4, 7), and thus the long poly(A) tails may result from inefficient cytoplasmic deadenylation. We report the association of Pab1 with the polyadenylation complex. Pab1 was specifically associated with partially purified CF I and interacted

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TABLE 1. Yeast strains used in this study

Strain	Genotype and description	Source
W303-1B	*MAT***a** *ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15*	R. Rothstein
rna15-2 W[a]	*MAT***a** *ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15 rna15-2*	This study
rna14-1 W[a]	*MAT***a** *ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15 rna14-1*	This study
NA65[a]	*MAT***a** *ura3-1 trp1Δ ade2-1 leu2-3,112 his3-11,15 pcf11-2*	This study
YAS 120	*MAT***a** *PAB1::HIS3 ura3 trp1 ade2 leu2 his3/pAS86* (*pab1 TRP1*)	A. Sachs (34)
J 692	*MAT***a** *trp1-1 ade2-1 leu2-3,112 his3-11,15 URA3::UAS GAL1-lacZ gal4Δ gal80Δ LYS2::UASGAL1-HIS3*; *cych2*^{r}	R. Rothstein
J 693	*MAT*α *trp1-1 ade2-1 leu2-3,112 his3-11,15 URA3::UAS GAL1-lacZ gal4Δ gal80Δ LYS2::UASGAL1-HIS3*; *cych2*^{r}	R. Rothstein

[a] Strains isogenic to W303-1B.

with the Rna15 component both in vivo and in vitro. In vitro studies indicate the involvement of Pab1 in determining the length of poly(A) tails and suggest that this control is different in yeast and higher eucaryotes.

MATERIALS AND METHODS

Strains, media, and genetic techniques. Yeast strains (Table 1) were maintained on YPD (1% yeast extract, 2% peptone, 2% glucose) or on selective YNB medium (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with the appropriate nutritional complements (35). *Escherichia coli* strains were grown on Luria-Bertani medium supplemented with ampicillin (50 mg/ml). Yeast cells were transformed by treatment with lithium acetate and polyethylene glycol (14).

Two-hybrid assay. Open reading frames (ORFs) were amplified by PCR with oligonucleotides which introduced restriction sites (Table 2). PCR products were cloned by fusing the ORFs in frame with the *GAL4* DNA binding domain (GBD) into the pAS2 or pGBT9 plasmid and those in frame with the *GAL4* activating domain (GAD) into the pACTII plasmid (40). *RNA14* and *RNA15* have been cloned previously (2). The yeast reporter strains are described in Table 1. J 692 (*Mat***a**) was transformed with the plasmids containing the GAD-ORF fusions, and J 693 (*Mat*α) was transformed with the plasmids containing the GBD-ORF fusions; the transformants were screened for Leu^+ and Trp^+ colonies, respectively. Production of the fusion proteins was verified by Western blotting with the anti-Gal4 antibodies (Clontech Corp.) and the enhanced chemiluminescence detection kit (Amersham Corp.). Diploid strains bearing the two types of plasmids were selected by complementation on medium lacking tryptophan and leucine and tested for *lacZ* expression by a filter lift assay (40). β-Galactosidase activity was determined as described previously (23).

In vitro 3′-end processing assay. 3′-End processing was assayed in whole-cell extracts fractionated by ammonium sulfate precipitation at 40% saturation as described by Lin et al. (24) and modified by Chen and Moore (12). The processing reaction was carried out at 23°C in a volume of 25 μl, containing 2 μl of extract. Full-length and precleaved ^{32}P-labeled *CYC1* precursors were used for cleavage and polyadenylation reactions, respectively, as previously described (1). For labeling of poly(A), the polyadenylation reaction was performed with unlabeled or labeled precleaved *CYC1* precursor, as indicated below, in the presence of 5 μCi of [α-^{32}P]ATP and 0.2 mM ATP per assay. The products were analyzed on 6% polyacrylamide–7 M urea gels.

Production of recombinant Pab1 protein and antibodies. The *PAB1* and *RNA15* ORFs were amplified by PCR (the oligonucleotides are listed in Table 2) and fused in frame into the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible $(His)_6$-tagged vector pET-22b (Novagen Corp.). Proteins were produced as translational fusions with C-terminal polyhistidine in *E. coli* BL21(DE3). The Pab1 and Rna15 fusion proteins were insoluble in inclusion bodies after a 3-h induction at 37°C with 1 mM IPTG. Denatured proteins were purified from 500 ml of bacterial culture (4 mg of Pab1 and 1.2 mg of Rna15) and used to produce antibodies. The Pab1 fusion protein was partially soluble after a 1.5-h induction at 30°C with 1 mM IPTG, and active protein (0.6 mg) was purified from 500 ml of culture. Soluble or insoluble proteins were extracted and purified on a nickel-agarose column in the presence of imidazole as described by the supplier of the QIA expressionist kit (Qiagen). Rabbits were given injections of 1 mg of the protein three times over a 9-week period, and serum was collected. To purify antibodies, 3 mg of purified protein was immobilized on 2 ml of Aminolink agarose, 3 ml of crude serum was applied on the resin, and specific antibodies were eluted as specified in the protocol supplied with the ImmunoPure Antigen/Antibodies Immobilization kit (Pierce). To purify antibodies from preimmune serum, 0.5 ml of crude serum was applied to protein A-Sepharose (Sigma Corp.) and resin was washed with 100 mM sodium phosphate buffer (pH 7). Antibodies were eluted with 100 mM glycine (pH 2.8) and neutralized with 1 M Tris (pH 10.2).

For antibody neutralization studies, 0.1 to 0.5 μl of the purified anti-Pab1 antibodies was incubated with 2 μl of wild-type extract in a standard processing reaction assay. The mixtures were incubated for 45 min at 4°C, and the RNA precursor was added. A 2-μl volume of extract without antibody was treated under the same conditions.

Protein procedures. The *RNA15* ORF cloned into the vector pGEM3 was used to perform in vitro transcription and synthesis of ^{35}S-labeled protein with the rabbit reticulocyte lysate system (Promega Corp.) (5). For coimmunoprecipitation, we first used cell extract prepared as described for RNA processing and then also used translated and labeled Rna15 with recombinant Pab1 for direct immunoprecipitation. Purified antibodies were mixed with protein A-Sepharose (Sigma Corp.) in 20 mM Tris-HCl (pH 8)–0.2 mM EDTA–0.5 mM dithiothreitol–50 mM KCl–20% glycerol–1 mM phenylmethylsulfonyl fluoride PMSF–0.01% Nonidet P-40 for the cell extract or in 20 mM Tris-HCl (pH 8)–150 mM KCl–5% glycerol–0.01% Nonidet P-40 for the direct Rna15-Pab1 immunoprecipitation. After overnight incubation at 4°C on an end-over-end shaker, the resin was washed and added to 10 μl of cell extract or to 300 μl of buffer containing a mixture of 0.6 μg of recombinant Pab1 and 8 μl of [^{35}S]Rna15 incubated for 15 min at 4°C before resin addition. After 2 h at 4°C, the protein A-Sepharose-antibody-protein complexes were pelleted, washed five times, directly boiled in loading buffer, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). When indicated, cell extracts were treated with S1 nuclease (1 U in 1 μl; Pharmacia Corp.) for 1 h at 4°C in the specific buffer purchased from the manufacturer. Under these conditions, luciferase mRNA, used as a control, was totally degraded.

Cleavage and polyadenylation factors were separated by chromatography on a Mono Q HR 5/5 fast protein liquid chromatography column (Pharmacia Corp.) with an extract from W303 strain, as described previously (12). Pab1 and Rna15 were detected in the fractions by immunoblot analysis with the ECL detection kit.

TABLE 2. Oligonucleotides used for PCR amplification

Gene	Oligonucleotide	Sequence	Site	Plasmid
PAB1	2735	5′-GCAAGATCTTTATGGCTGATATTACTG-3′	*Bgl*II	pAS2/pACTII
	2736	5′-CTCAGATCTTTAAGCTTGCTCAGTTTG-3′	*Bgl*II	pAS2/pACTII
	6258	5′-GCCTCGGATCCGGCTGATATTACTGATAAG-3′	*Bam*HI	pET 22
	6257	5′-GGCCGCAAGCTTAGCTTGCTCAGTTTGTTG-3′	*Not*I	pET 22
PAP1	5267	5′-CGGGATCCCGAGCTCTCAAAAGGTTTTTGG-3′	*Bam*HI	pAS2/pACTII
	5266	5′-CGGGATCCCGTTAGTTTACGTCAACAGCTGC-3′	*Bam*HI	pAS2/pACTII
PCF11	6886	5′-CATGCCATGGATCACGACACAGAAGTTATAG-3′	*Nco*I	pAS2/pACTII
	6885	5′-CGGGATCCCGTTATTTTGTGACCAATTTCTTTAAG-3′	*Bam*HI	pAS2/pACTII
RNA15	6133	5′-GCCCCGAATTCGAATAGGCAGGCGGTGTG-3′	*Eco*RI	pET 22
	6135	5′-GGCCGCAAGCTTAAATGCACCAAATTCTCC-3′	*Hin*dIII	pET 22

TABLE 3. Double-hybrid interactions[a]

GAD fusion	Colony color in GBD fusion[b] with:				
	RNA14	*RNA15*	*PAP*	*PAB1*	*PCF11*
RNA14	++	+++	++	−	±
RNA15	+++	+	−	−	±
PAP	−	−	−	−	−
PAB1	−	+	−	−	−
PCF11	±	+	−	−	+

[a] Interactions between Rna14, Rna15, Pap, Pab1, and Pcf11 in the double-hybrid system. The corresponding ORFs were fused to GBD or GAD as indicated. Colony color was monitored by the filter lift assay to assess *lacZ* expression.

[b] +++, dark blue colony; ++, blue colony; +, light blue colony; ±, grey-blue colony; −, white colony.

RESULTS

Pab1 associates with Rna15 in vivo and in vitro. (i) Protein interactions in the *GAL4* two-hybrid system. Interactions between various proteins implicated in the metabolism of the poly(A) tail were monitored by using the double-hybrid system (for a review, see reference 13). *RNA14*, *RNA15*, *PAP*, *PAB1*, and *PCF11* (2) ORFs were fused in frame to sequences encoding the *GAL4*-activating or the *GAL4*-binding domains as described in Materials and Methods. The hybrids which reconstituted the *Gal4* complex were detected by expression of the *lacZ* reporter gene (Table 3). As already shown, Rna14 and Rna15 interact strongly (2). Interaction between the complete Pcf11 and Rna14 or Rna15 was weak or almost undetectable, while screening with Rna14 or Rna15 as bait previously revealed a strong association between these two proteins and a 100-amino-acid domain of Pcf11 in pACT-*PCF11*(271) (2). However, this result is certainly significant, as Pcf11 is the 70-kDa subunit which is recognized by anti-Pcf11 antibody in the CF IA complex (20). An interaction between Rna14 and Pap was detected only when Pap was fused to GBD and Rna14 was fused to GAD. Similar observations have already been reported (8, 29), in which one orientation of the hybrid activates the transcription of the reporter genes much more efficiently than the other.

One of the surprising features of our analysis was the interaction between Pab1 and Rna15. It was weak and was observed only for Rna15 fused to GBD and Pab1 fused to GAD. However, no association was detected between Pab1 and the three other baits used, suggesting that the Pab1-Rna15 interaction could be specific. β-Galactosidase activity was compared for various combinations of fusion proteins involving Pab1 and Rna15 and for complete and truncated Pcf11 used as a positive control (Table 4). The value of 20 obtained for the interaction between pACT-*PAB1* and pGBT-*RNA15* is clearly much higher than the background obtained with the two controls.

(ii) Suppression of the *rna15-2* mutation by multicopy *PAB1*. To investigate the apparent in vivo association of Pab1 and Rna15, we sought a possible genetic association between the *RNA15* and *PAB1* genes. We tested the effect of *PAB1* overexpression on the *rna15-2* mutant allele. A strain containing the thermosensitive *rna15-2* allele was transformed either with the *URA3*-marked multicopy plasmid pFL44 (6) or with plasmid pFL44-*PAB1*, expressing the *PAB1* gene. The corresponding transformants were analyzed for thermosensitivity. The strain which contained a multicopy wild-type *PAB1* plasmid was able to grow at 33°C, whereas the strain bearing pFL44 was unable to grow at this temperature. Protein extracts were prepared as described previously (1). A weak but significant overexpression of Pab1 has been demonstrated by Western blotting in an extract of the *rna15-2* strain transformed by the pFL44-*PAB1* multicopy plasmid compared to a strain transformed by the pFL44 plasmid as a control. Detection of a control ribosomal protein (Ssm1) showed no difference in protein concentrations between the two types of extracts. The pFL44-*PAB1* multicopy plasmid did not complement *rna14-1* and *pcf11* temperature-sensitive (Ts) alleles, showing the specificity of *rna15-2* suppression. This result is consistent with a functional interaction between Pab1 and Rna15.

(iii) Pab1 and Rna15 are associated in vitro. To confirm further the apparent in vivo association of Pab1 with Rna15, immunoprecipitation assays were performed with cell extracts prepared from a wild-type strain as described for the processing assay. Polyclonal antibodies were raised against Pab1 and Rna15 produced in and purified from bacteria (see Materials and Methods). Extracts were treated with either preimmune immunoglobulin G or affinity-purified anti-Pab1 and anti-Rna15 antibodies coupled to protein A-Sepharose beads. Blots of precipitated complexes were probed with anti-Pab1 and anti-Rna15 antibodies (Fig. 1A). The 68-kDa Pab1 protein was detected by anti-Pab1 antibodies in protein A–anti-Pab1 and protein A–anti-Rna15 pellets (Fig. 1A, lanes 2 and 3). The immunoprecipitation of Pab1 with the anti-Rna15 antibodies was specific: the preimmune serum did not bring down the Pab1 protein (lane 1). The reciprocal immunoprecipitation was also observed. Rna15 was detected in the pellets precipitated with either anti-Pab1 or anti-Rna15 beads (lanes 5 and 6). Rna15 was not present in immune complexes obtained with preimmune serum (lane 4). Since Pab1 and Rna15 are both RNA binding proteins, we verified that interaction between these proteins was direct and did not occur through an intermediate RNA. S1 nuclease treatment (see Materials and Methods) did not modify the coimmunoprecipitation between Pab1 and Rna15 (data not shown). To verify that the interaction between Pab1 and Rna15 did not involve another protein, we performed a coimmunoprecipitation experiment with purified recombinant Pab1 and translated and labeled Rna15 proteins. In vitro proteins were immunoprecipitated and analyzed as described above (Fig. 1B). Pab1 was directly precipitated by anti-Pab1 antibodies and not by the preimmune serum (Fig. 1B, lanes 1 and 2). It was specifically coprecipitated in complexes with Rna15 by anti-Rna15 antibodies (lane 3). Radiolabeled Rna15 protein was not detected with anti-Rna15 antibodies because of its low concentration, but it was detected in protein A–anti-Rna15 antibody pellets by direct autoradiography of the blots (data not shown). This confirmed the direct Pab1-Rna15 interaction in vitro.

Thus, like Rna15, Pab1 may be a part of the polyadenylation complex.

Copurification of Pab1 within partially purified CF I. We investigated the possibility that Pab1 is involved in mRNA

TABLE 4. β-Galactosidase activities[a]

GAD-expressing plasmid	GBD-expressing plasmid	β-Galactosidase activity[b]
pACT-*PAB1*	pGBT-*RNA15*	20
pACT-*PCF11*(271)	pGBT-*RNA15*	40
pACT-*PCF11*	pGBT-*RNA15*	15
pACTII	pGBT-*RNA15*	1.9
pACT-*PAB1*	pGBT9	1

[a] Quantitative β-galactosidase assays were performed on diploid strains expressing the indicated constructs. Assays were as described previously (23).

[b] β-Galactosidase activity is expressed in Miller units. Values are the averages of two different mixture of transformants. Standard errors were <20%.

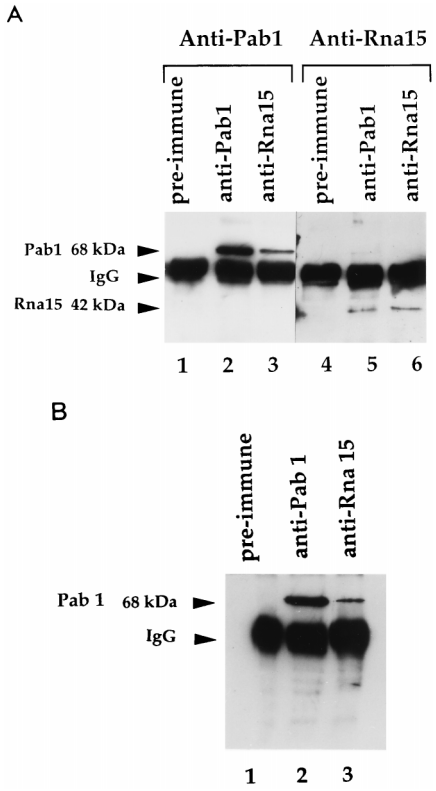

FIG. 1. Coimmunoprecipitation of Pab1 and Rna15. (A) A cell extract from a wild-type strain was immunoprecipitated with purified antibodies directed against Pab1 or Rna15 as described in Materials and Methods. The preimmune serum coupled to protein A-Sepharose beads was used as a control. Blots were probed with anti-Pab1 antibody or with anti-Rna15 antibody diluted 1/5,000. The migration positions of Pab1, Rna15 and immunoglobulin G (IgG) are shown on the left. (B) Formation of the Pab1-Rna15 protein complex in vitro. Recombinant Pab1 (0.6 μg) and translated and radiolabeled Rna15 (8 μl) were mixed and immunoprecipitated with preimmune serum as a control or with purified anti-Pab1 antibody or anti-Rna15 antibody. The blot was probed with anti-Pab1 antibody. As in panel A, the migration positions of proteins are indicated on the left.

3′-end processing, as suggested by its interaction with Rna15. We examined the presence of Pab1 in the CF I complex, because Rna15 protein belongs to this complex. Cell extract was applied to a Mono Q anion-exchange column, and the proteins were eluted with an increasing salt gradient. CF I was separated from the other factors, Pap, CF II, and PF I, as previously described (12). The fractions were analyzed by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with anti-Rna15 and anti-Pab1 antibodies. As shown in Fig. 2 (lanes 3 and 4), the Pab1 protein coeluted with Rna15: fractions 27 and 28 gave intense signals for the 68-kDa Pab1 and Rna15. A 53-kDa polypeptide was also present in the fractions that contained Pab1; this less intense band is specifically detected by anti-Pab1 antibody and is certainly the cleavage product of Pab1 (33). Pab1 was reproducibly eluted in the Mono Q column with Rna15 at an ionic strength of around 410 mM KCl, the same as that at which CF I is eluted (12). As previously shown (2), Rna14 and Pcf11, two other subunits of CF I, were also found in the same fractions (data not shown). No Pab1 was detected in any other fraction from the Mono Q column, including the wash volume. Thus, Pab1 copurified with CF I, suggesting that Pab1 may interact with Rna15 in the nucleus during poly(A) synthesis.

Mutations in *PAB1* affect poly(A) tail length in vitro. We tested whether the Ts allele of *pab1* was associated with a defect in the cleavage or the polyadenylation of pre-mRNA in vitro. Extracts from the wild-type strain and the mutant YAS 120 *pab1* Ts strain (34), grown at the permissive temperature (23°C), were tested for in vitro 3′-end processing. We first analyzed the cleavage of the labeled *CYC1* precursor under conditions that allowed only the cleavage reaction. The results are shown in Fig. 5A (see below). Both extracts cleaved the pre-mRNA with the same efficiency, generating 5′ and 3′ fragments (185 and 116 nucleotides, respectively) (see Fig. 5A, lanes 2 and 5). The polyadenylation reaction was performed first by addition of unlabeled AMP to the precleaved labeled precursor. As expected, an average of 50 to 70 adenosines were polymerized on the 5′ *CYC1* fragment by the wild-type extract (Fig. 3A, lane 1). However, polyadenylation was modified in the Ts *pab1* extract, longer poly(A) tails, 80 to 200 nucleotides long, were synthesized (lane 2). Under these conditions, only the RNA core was labeled and not the added poly(A), and the labeling was proportional to the number of RNA molecules irrespective of the length of the poly(A) tail. Thus, the mean poly(A) length was more than double in the *pab1* Ts strain than in wild-type extracts. In the *pab1* mutant extracts, only the second step of processing was modified; the polyadenylation was enhanced, whereas all mutants with mutations of CF I subunits, *rna14-1*, *rna15-2* (27), and *pcf11-2* (2), are deficient in cleavage and polyadenylation in vitro. Moreover, polyadenylation by the *pab1* mutant extract in vitro was entirely consistent with the results obtained in vivo. Indeed, Sachs and Davis (34) have shown that depletion of Pab1 at the restrictive temperature leads to the accumulation of long poly(A) tails (30 to 40 nucleotides longer) in the *pab1* mutant. In a second experiment, unlabeled precleaved precursor was polyadenylated in the presence of [α-^{32}P]ATP to label the poly(A) uniformly. Labeling was not proportional to the amounts of RNA but depended on the lengths of the poly(A) tails synthesized. The wild-type extract again gave poly(A) tails of homogeneous length, about 50 to 70 nucleotides (Fig. 3B, lane 1). Conversely, the mutant extract synthesized extremely long and heterogeneous poly(A) tails, several hundred to more than 1,000 nucleotides long (the limit of gel resolution) (lane 2). Such molecules were strongly labeled under these conditions and were too heterogeneous to be clearly observed in the first experiment. Thus, there was no apparent length limitation for poly(A) tails synthesized by Pap in the mutant extract, whereas the length seems to be strictly controlled in the wild-type extract.

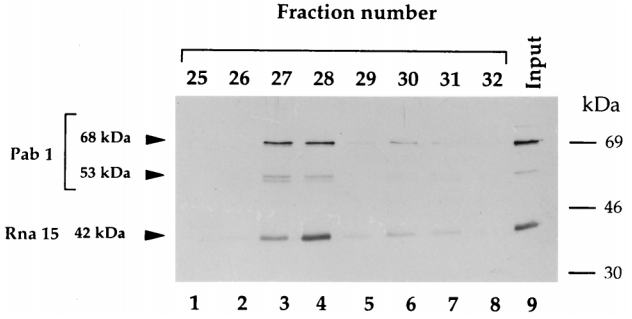

FIG. 2. Copurification of Pab1 and Rna15 with the CF I complex. Fractions from a Mono Q column obtained as described in Materials and Methods were loaded on an SDS–10% polyacrylamide gel. Pab1 and Rna15 were revealed by Western blotting with a 1/2,000 dilution of anti-Pab1 and anti-Rna15 antibodies. The migration positions of the full-length (68 kDa) and truncated (53 kDa) Pab1 protein and of the Rna15 protein are indicated on the right.

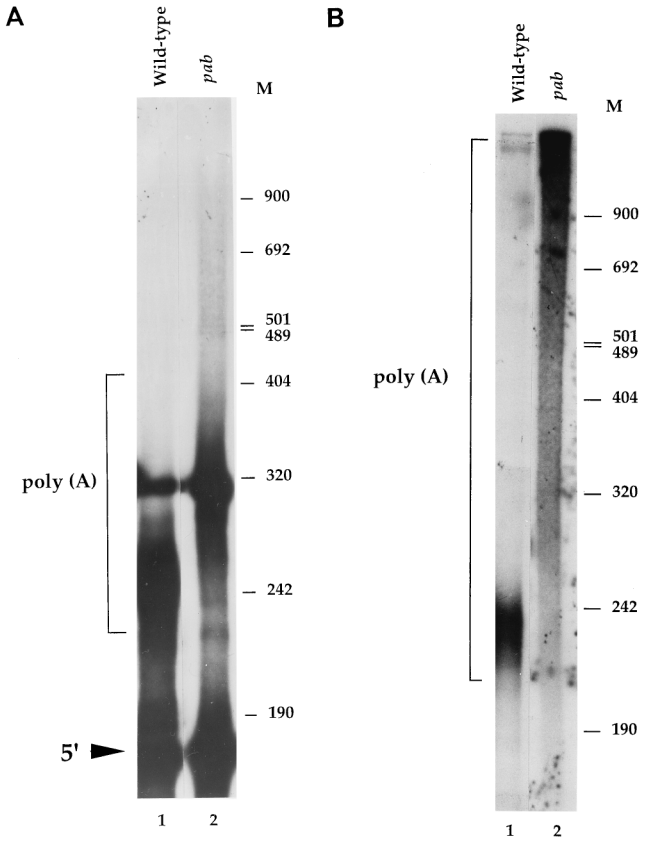

FIG. 3. In vitro polyadenylation of labeled and unlabeled precleaved *CYC1* in wild-type or *pab1* Ts mutant extract. (A) Labeled precleaved *CYC1* obtained as described in Materials and Methods was incubated for the polyadenylation reaction under standard conditions at 23°C in wild-type extract (lane 1) or *pab1* mutant extract (lane 2). The sizes of markers (M) are indicated in numbers of nucleotides on the right. (B) Unlabeled precleaved *CYC1* was used for polyadenylation in presence of 5 μCi of [α-^{32}P]ATP to label only the poly(A) tail as indicated in the text. In lane 1, the precursor was polyadenylated in the wild-type extract, and in lane 2 it was polyadenylated in the *pab1* mutant extract. The products were analyzed on a 6% polyacrylamide–7 M urea electrophoresis gel. As in panel A, the sizes of markers are indicated.

Pab1 is required to control poly(A) tail lengths in vitro. 3′-End RNA processing in vitro by Pab1-immunoneutralized wild-type extract was studied. We purified specific anti-Pab1 antibodies as described in Materials and Methods, so that protease and nuclease activities found in crude serum were eliminated. Various amounts of purified antibodies were added to the wild-type extract before addition of the precursor. RNA cleavage was not modified by the treatment (Fig. 4A, compare lane 2 to lanes 3, 4, and 5), but polyadenylation of the precleaved precursor (Fig. 4B, lane 2) was altered by the presence of specific antibodies (lanes 3 to 5). The poly(A) tails increased in length progressively until a limit (lane 5) maintained at higher antibody concentrations (data not shown). A 0.5-μl volume of nonspecific antibodies purified from preimmune serum had no effect on cleavage and polyadenylation reactions in wild-type extract (data not shown). Thus, Pab1 immunoneutralization had exactly the same effect as the *pab1* mutation: cleavage was preserved, and polyadenylation was enhanced.

We then tested whether exogenous Pab1 could give a normal length of poly(A) tail in the presence of the *pab1* Ts mutant extracts. Yeast *PAB1* was cloned in the inducible pET22 vector and encoded a histidine-tagged protein in *E. coli*. The induction was controlled to lower the synthesis rate so that the recombinant protein remained partly soluble in the transformed bacteria. Consequently, Pab1 could be purified without denaturation and the purified preparation was active (see below). Various amounts of purified Pab1 were added to wild-type and mutant extracts before RNA processing in vitro was assessed. Cleavage proceeded normally in both extracts (Fig. 5A, lanes 2 and 5) and was not modified by the addition of Pab1 (lanes 3 and 4 and lanes 6 and 7). In contrast, the abnormally long poly(A) tails were progressively shortened by the addition of exogenous Pab1 in the mutant extract (Fig. 5B, lanes 5 to 7) to the normal length found in the wild-type extract (compare lanes 2 and 7). Similar results were obtained with larger quantities of Pab1 (data not shown). In the wild-type extract, polyadenylation was unaffected by the amount of added protein (lanes 2 to 4). We conclude that exogenous Pab1 restores a normal rate of polyadenylation but does not have inhibitory effects when present in excess. This experiment confirmed the role of Pab1 in the regulation of poly(A) tail length.

Exonuclease activities are not involved in the control of poly(A) tail lengths in vitro. Poly(A) tails are longer than normal in vivo in the *Pab1* Ts mutant at restrictive temperatures (34) and in the *pan2* and *pan3* mutants of subunits of the Pab1-dependent poly(A) nuclease (Pan) (4, 7). Pan, an exonucleolytic enzyme specifically degrades poly(A) bound to Pab1. The poly(A) lengthening is presumably due in part to an inhibition of deadenylation in both *pan* and *pab1* mutants. We investigated whether exonucleolytic shortening contributes to the control of poly(A) lengths in the wild-type extract in vitro. The *pab1* Ts extract was used to elongate the labeled precleaved *CYC1* precursor with [α-^{32}P]ATP, when adenylation was apparently not limited. The resulting polyadenylated RNA was purified and used as a substrate to detect nuclease activities. This substrate was incubated with wild-type or *pab1* mutant extracts in the polyadenylation reaction assay without ATP to avoid polymerization. Active recombinant Pab1 was added to coat poly(A) chains and allow the detection of Pan-like nuclease activities. Poly(A) RNA was totally stable in both extracts during a 1-h incubation. Similar results were obtained in the absence of exogenous Pab1 (data not shown). To test a possible exonuclease activity requiring ongoing polyadenylation, another experiment was performed in presence of ATP. The precleaved precursor was used for a [α-^{32}P]ATP-labeled polyadenylation reaction with *pab1* mutant extract for 1 h. Then a 10-fold excess of ATP was added to strongly reduce the labelling, and the polyadenylation reaction was continued for another 1 h with or without added recombinant Pab1. The fate of the labeled long poly(A) tails was monitored. These poly(A) chains were stable under all conditions (data not shown). Thus, we conclude that exonucleases are not involved in vitro in the control of polyadenylation, which ensures the homogeneous poly(A) tail lengths obtained in the wild-type extract.

DISCUSSION

We describe a direct interaction between Pab1 and Rna15 in a double-hybrid assay. The interaction was further supported by coimmunoprecipitation of the two proteins in a complex from both cell extracts and in vitro mixtures of recombinant Pab1 and in vitro-expressed Rna15. This interaction is functional in vivo, since overexpression of the wild-type *PAB1* specifically suppressed the *rna15-2* mutation. The interaction with Rna15 is sufficiently strong to find Pab1 associated with CF I partially purified on a Mono Q column, a protocol which separates CF I from Pap and the two other cleavage and polyadenylation factors (12). Pap-Fip1 and Rna14-Fip1 interactions

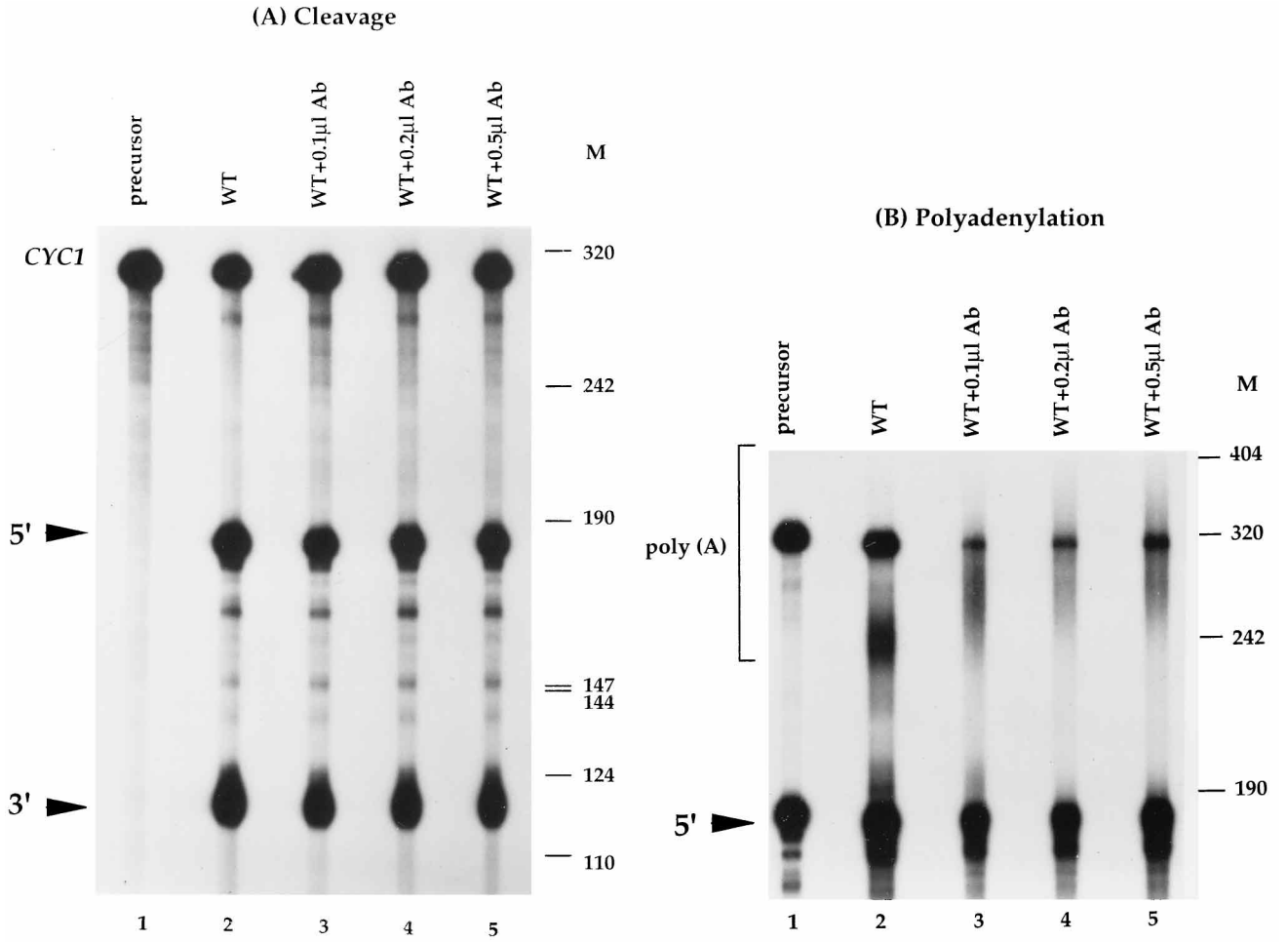

FIG. 4. Immunoneutralization of Pab1 in a wild-type (WT) extract. The extract was incubated with 0, 0.1, 0.2, or 0.5 μl of purified anti-Pab1 antibody (Ab) for 45 min at 4°C before being subjected to a processing assay. (A) Cleavage assay. Untreated (lane 2) or treated (lanes 3 to 5) samples were subjected to the cleavage assay under standard conditions. Lane 1 shows the precursor used in the assay. The migration positions of precursor *CYC1* and the two 5′ and 3′ fragments are shown on the left. (B) Polyadenylation assay. Immunoneutralization of Pab1 gives an accumulation of elongated poly(A) tails on precleaved *CYC1* (lanes 3 to 5). The untreated extract gives normal-sized poly(A) tails (lane 2).

are also dissociated under these conditions (29). Pab1, the most abundant protein associated with mRNA poly(A), may therefore be an additional constituent of the large RNA-protein complex responsible for specific polyadenylation.

A model for yeast polyadenylation has been previously proposed (44). CF I recognizes signal sequences upstream from the poly(A) site and fastens to pre-mRNA via Rna15, which contains an RNA binding domain and is the only subunit of CF I that can be cross-linked to mRNA (21). Rna14, Fip1, and Pap interactions indicate that PF I participates in the association between RNA-bound CF I and Pap (29). Our results suggest that Pab1 may be maintained near the polyadenylation site by an interaction with Rna15 so that it can bind the nascent poly(A) tails. Nevertheless, it cannot be excluded that Pab1 interacts with other components of CF I or PF I. This will certainly be tested soon as all these proteins are characterized.

The polyadenylation complex, consisting of CF I, PF I, Pap, and Pab1, is therefore the functional analog of the very well characterized CPSF-Pap-Pab II complex in higher eucaryotes (3, 43). However, Pap is the only 3′-end-processing component that is highly conserved in function and sequence between yeast and mammals (46). CPSF contains four subunits (160, 100, 73, and 30 kDa), the longest of which binds it to RNA (28). Like CF I, it recruits the complex near the polyadenylation site. However, the components of the two factors are not equivalent. Rna14 and Rna15 have similarities to the 77- and 64-kDa proteins of mammalian CstF involved only in cleavage (37). No homologs of the *PCF11* gene have yet been found in higher eucaryotes (2). The 73-kDa subunit of CPSF shows similarity to a new 87-kDa subunit of PF I encoded by the yeast *YSHI/BRR5* gene (11, 18). The Pab proteins found in the two complexes are also dissimilar. The single Pab1 encoded by yeast (33) is the homolog of the mammalian cytoplasmic Pab I, which shows no similarity in sequence or function to the nuclear Pab II involved in polyadenylation (45).

In the absence of cofactors, mammalian Pap is weakly active and not specific for precursor RNA (41). Pab II alone is able to stimulate Pap strongly (45). Two factors, CPSF and Pab II, are needed together with Pap and the substrate RNA for the elongation of full-length poly(A) tails. This complex is totally specific. The length is similar to that of the newly synthesized poly(A) tails in vivo (about 250 nucleotides) (3, 42). In contrast, cytoplasmic Pab I inhibits almost totally the specific addition of poly(A) in vitro when it is used in place of Pab II in the quaternary complex with CPSF, Pap, and pre-mRNA (45).

In *S. cerevisiae*, Pap efficiently polyadenylates any RNA in

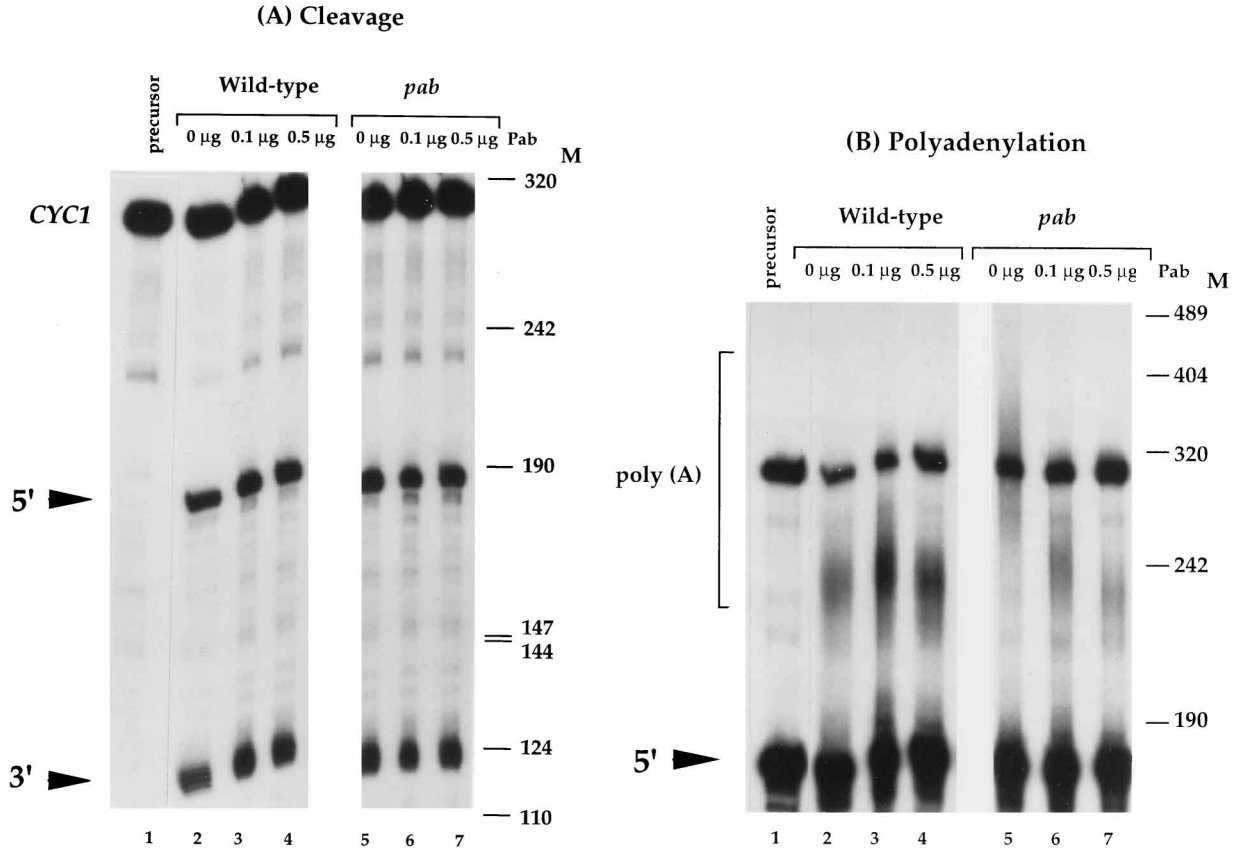

FIG. 5. Complementation of the *pab1* mutant extract by the recombinant Pab1. (A) The cleavage reaction in wild-type extract (lanes 2 to 4) or *pab1* mutant extract (lanes 5 to 7) was unaffected by addition of exogenous Pab1. The amounts of protein added are indicated. (B) Polyadenylation assay with wild-type extract without addition of exogenous Pab1 (lane 2) or following addition of 0.1 μg (lane 3) or 0.5 μg (lane 4) of recombinant Pab1. In the mutant extract, which gives the long poly(A) tail (lane 5), the addition of exogenous Pab1 restores the normal length of the poly(A) tails (lanes 6 and 7). No effect of recombinant Pab1 on the wild-type extract was observed (lanes 2 to 4).

the presence of manganese, yielding poly(A) tracts longer than 400 nucleotides (12). In such nonspecific assays, Pab1 strongly inhibits the elongation of the poly(A) tail, with Pap activity being reduced by more than 10-fold (25). Remarkably, the isolated Pap is practically inactive in the presence of magnesium, and PF I and CF I are needed for specific addition of adenosines. Under these conditions, the poly(A) tails synthesized by the wild-type extract are shorter and comparable in length to natural poly(A) tails in vivo (12). All previously studied mutants of CF I and PF I, i.e., *rna14-1*, *rna15-2*, *pcf11-2*, and *fip1-1*, (2, 27, 29), are totally deficient in polyadenylation. In the present study, we show that in an extract of the Ts *pab1* mutant, this step is again abnormal but in an inverse way: the mutation results in an increase in the length of the poly(A) tails. Moreover, in our tests, the length of the poly(A) tails was not homogeneous and some chains were several hundred nucleotides long. This was due to an inefficacy of the *Pab1* Ts protein, since the same effect was obtained by immunoneutralization of Pab1 in wild-type extract by specific anti-Pab1 antibodies. Conversely, recombinant Pab1 was able to restore normal polyadenylation to a mutant extract. The rescue was dependent on the amount of Pab1 added: intermediate lengths of poly(A) were detected for intermediate amounts of Pab1 added. A similar observation has previously been reported for polyadenylation reactions involving the four separate factors CF I, CF II, PF I, and Pap (12). The poly(A) tail obtained was about 24 nucleotides longer than that synthesized with the crude extract. Our results suggest that this was due to a partial deficiency in Pab1 after reconstitution.

In vivo, the inactivation of Pab1 in the *pab1* thermosensitive mutant grown at restrictive temperature led to an increase in the poly(A) tail length (34). However, the same result was obtained with mutants of either of the two subunits of the Pab1-dependent poly(A) nuclease (Pan). Pan is involved in the degradation of poly(A) bound by Pab1 (4, 7). Similarly, Caponigro and Parker (9) have detected long poly(A) tails for two messengers (PGK1 and MFA2) newly synthesized in a Δ*pab* mutant. The longer poly(A) detected in vivo in the *pab1* mutant may therefore result from inefficient deadenylation or synthesis of longer poly(A) tails, or both. We demonstrated that poly(A) was totally stable in both extracts in vitro. No degradation of the poly(A) tails, whether bound to Pab1 or not, was detected. Thus, we can conclude that the shorter poly(A) tails found in wild-type extract are not due to a deadenylation by a Pan-like activity. The nearly uniform poly(A) length found in this extract is presumably determined by control during poly(A) synthesis. Thus, the role of Pab1 in yeast may be to restrict the Pap activity and not to stimulate it as Pab II does in higher eucaryotes. Nevertheless, part of the process is common to yeast and mammals, since it seems that the stimulation by Pab II in mammals is followed by an inhibition once a length of 200 to 250 A residues is attained. In yeast, the inhibitory effect on polyadenylation may similarly appear only after the normal size is attained, since an excess of the recom-

binant protein added to wild-type extract did not modify the poly(A) tail length. It is Pab1 bound to polyadenylation complex and not the free Pab1 which regulates the length. However, it is possible that another Pab1-dependent catalytic process is involved in this control. We know that cofactors like CF I and PF I are essential for maximal Pap specificity and to control the polyadenylation rate. The details of the relative roles of CF I, PF I, Pap, and Pab1 in the control of the poly(A) tail synthesis will probably become clear only when all these factors are purified.

The poly(A) tail has at least two distinct cytoplasmic functions, one involving the initiation of translation (15) and the other involving mRNA degradation (10). The regulation of these functions depends on the rate of deadenylation. Thus, control of the length of poly(A) added to the mRNA in the nucleus is essential. This control occurs in yeast as in mammals but by mechanisms which seem at least partly different.

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