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David A. Mangus
University of Massachusetts Medical School

Et al.

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Pbp1p, a Factor Interacting with *Saccharomyces cerevisiae* Poly(A)-Binding Protein, Regulates Polyadenylation

DAVID A. MANGUS, NADIA AMRANI, AND ALLAN JACOBSON*

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655-0122

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The poly(A) tail of an mRNA is believed to influence the initiation of translation, and the rate at which the poly(A) tail is removed is thought to determine how fast an mRNA is degraded. One key factor associated with this 3′-end structure is the poly(A)-binding protein (Pab1p) encoded by the *PAB1* gene in *Saccharomyces cerevisiae*. In an effort to learn more about the functional role of this protein, we used a two-hybrid screen to determine the factor(s) with which it interacts. We identified five genes encoding factors that specifically interact with the carboxy terminus of Pab1p. Of a total of 44 specific clones identified, *PBP1* (for Pab1p-binding protein) was isolated 38 times. Of the putative interacting genes examined, *PBP1* promoted the highest level of resistance to 3-aminotriazole (>100 mM) in constructs in which *HIS3* was used as a reporter. We determined that a fraction of Pbp1p cosediments with polysomes in sucrose gradients and that its distribution is very similar to that of Pab1p. Disruption of *PBP1* showed that it is not essential for viability but can suppress the lethality associated with a *PAB1* deletion. The suppression of *pab1*Δ by *pbp1*Δ appears to be different from that mediated by other *pab1* suppressors, since disruption of *PBP1* does not alter translation rates, affect accumulation of ribosomal subunits, change mRNA poly(A) tail lengths, or result in a defect in mRNA decay. Rather, Pbp1p appears to function in the nucleus to promote proper polyadenylation. In the absence of Pbp1p, 3′ termini of pre-mRNAs are properly cleaved but lack full-length poly(A) tails. These effects suggest that Pbp1p may act to repress the ability of Pab1p to negatively regulate polyadenylation.

With rare exceptions, mRNAs whose synthesis originates within nuclei contain a 3′ poly(A) tail. Poly(A) tracts are not encoded within genes but are added to nascent pre-mRNAs in a processing reaction that involves site-specific cleavage and subsequent polyadenylation. Newly synthesized poly(A) tails of different transcripts are relatively homogeneous in length and encompass approximately 70 to 90 adenylate residues in *Saccharomyces cerevisiae*. After mRNA enters the cytoplasm, poly(A) tracts are shortened at mRNA-specific rates and, in some instances, may be completely removed. For some mRNAs, poly(A) shortening or removal is the rate-determining event in their decay, whereas for others, it may be an obligate event in their decay but is not the rate-determining step (34).

Poly(A) tracts are generally bound by the poly(A)-binding protein, a highly conserved protein with four RNA recognition motifs (RRMs) connected to a C-terminal domain with a predicted helical structure (39) via a proline- and methionine-rich segment (60). Association with poly(A) requires a minimal binding site of 12 adenosines, and multiple molecules can bind to the same poly(A) tract, spaced approximately 25 nucleotides (nt) apart (6, 7, 60, 63). In yeast, the poly(A)-binding protein (Pab1p) is encoded by the *PAB1* gene. The 70-kDa Pab1p is relatively abundant and is present in both the nucleus and the cytoplasm of the cell (60). *PAB1* is essential for growth on rich media, and depletion of Pab1p promotes misregulation of poly(A) addition, inhibits translation initiation and poly(A) shortening, and delays the onset of mRNA decay (4, 16, 17, 59, 61).

The effects of Pab1p depletion and *PAB1* mutations on mRNA poly(A) tail length are partially explained by the isolation of a poly(A) nuclease (PAN) that is dependent on Pab1p for its activity (45, 64). Yeast PAN is comprised of at least two polypeptides, and genes encoding the 135-kDa Pan2p and 76-kDa Pan3p subunits have been cloned and sequenced (13, 15). Deletion of either gene does not affect cell viability but does lead to the accumulation of longer mRNA poly(A) tracts in vivo. A role for Pab1p in the determination of mRNA poly(A) tail lengths is also suggested by experiments demonstrating that Pab1p copurifies with mRNA cleavage and polyadenylation factor CFI, specifically interacting with its Rna15p component (4, 37, 49), and by experiments demonstrating that extracts from *pab1* strains have normal pre-mRNA cleavage activity in vitro but promote large increases in poly(A) tail lengths (4).

A variety of experimental approaches have suggested that factors bound to the mRNA 5′ cap and the 3′ poly(A) tail interact to promote efficient translation initiation (34, 78). Evidence that Pab1p plays a prominent role in this process has been derived from experiments analyzing the in vivo and in vitro translational activities of *pab1* strains (61, 71), the extragenic suppressors of a temperature-sensitive *pab1* allele (61, 62), and the genetic and biochemical interactions between eukaryotic translation initiation factor 4G (eIF4G) and Pab1p (72, 73). Recent experiments suggest that, in yeast, eIF4G may bridge mRNA 5′ and 3′ ends by binding both to Pab1p and to the cap-binding protein, eIF4E (73). In metazoans, a similar function may be carried out by PAIP, a homolog of eIF4G shown to interact with both eIF4A and poly(A)-binding protein and to promote enhanced translation in vivo (19).

In order to gain new insights into the functions of Pab1p, we used a two-hybrid screen to identify factors with which it interacts. One factor identified in this screen, Pab1p-binding protein 1 (Pbp1p), interacts specifically with the C terminus of Pab1p. We determined that *PBP1* is not essential for viability but can suppress the lethality associated with a *PAB1* deletion. Whereas previously identified suppressors of *PAB1* mutations

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Ave. North, Worcester, MA 01655-0122. Phone: (508) 856-2442. Fax: (508) 856-5920. E-mail: ajacob@ummed.edu.

TABLE 1. Oligonucleotides

Name	Sequence (5′→3′)
UKN1.1	ATGAAGTTACGAAATTCAGGACTGATGTTGATATTTCTGGTTCTGGGGCCTCCTCTAGTACACTC
UKN1.2	GGCCTACAGAGTTCAATGTAGCTGAGATGTGGCATTGAAATACTGGCGCCTCGTTCAGAATGAC
UKN1.3	ACAAATATTGAAAAGGAAAGGG
YB83.1	CGTCCAGCGCGGCATTAAATAATCTTTCTGTAATACTCTTTAGCTCGGCCTCCTCTAGTACACTC
YB83.2	GTAGTTTCTGTATTTTTATTTTCTATGTGTTTTTATTGACTAGCAGGCGCCTCGTTCAGAATGAC
YB83.3	TACGCACCTAGTCGTTAGCCGC
YIM3.1	GTGTATATCTTAAATAAGATGTAGACTGGTTTGCATTTGGAAAGGGGCCTCCTCTAGTACACTC
YIM3.2	GGGGACCATAGTGATTGTGTGAGGTATAGGGGGTGAGATGTGTTCGCGCCTCGTTCAGAATGAC
YIM3.3	CAGTCAATAGAAGTTTCAGATC
HIS3.TEST	GCCTCATCCAAAGGCGC
64.2	GATAGCTCCACCAACTCAAG
36Rev.3	GTAGAGGTATCCGTGGAAAC
ADH1-5′	GATCTCTAGAGCTTGCATGCAACTTCTTTT
64Rev.2	GTCTTAGCCAAGTCATCCACAG
PBP1-ATG	CCCGCTCGAGGAATTCATGAAGGGAAACTTTAGGAAAAGAGATAGC
36Rev.4	TGCAATATGAATATTACCGG
PBP1-ATG	CCCGCTCGAGGAATTCATGAAGGGAAACTTTAGGAAAAGAGATAGC
PAB1.3	AAAACTGCAGAATTCATGGCTGATATTACTGATAAGACAGC
PAB1.4	CGCGGATCCAATTGGCTCAACAAATCCAAGCC
PAB1.5	CGCGGATCCAAGCCAACGATAACAACCAATTTTATC
PAB1.6	TTACGCGTCGACTTAAGCTTGCTCAGTTTGTTGTTC
PAB1.7	CGCGGATCCGTGACTCTCAATTGGAAGAGACTAAGGC
PAB1.8	CGCGGATCCAAAAGAAGAATGAACGTATGCATGTC

offset cytoplasmic defects in translation or mRNA decay (12, 16, 29, 61, 62), suppression by *pbp1*Δ is most likely attributable to nuclear effects. This conclusion follows from experiments demonstrating that deletion of *PBP1* has no effect on mRNA translation or decay but does lead to a substantial reduction in the ability of cell extracts to synthesize poly(A) tails.

MATERIALS AND METHODS

General methods. Preparation of standard yeast media and methods for cell culturing were as described previously (58). Transformation of yeast cells for library screens was done by the high-efficiency method (24); all other transformations were done by the rapid method (69). DNA manipulations were performed by standard techniques (66). All PCR amplifications were performed with *Taq* DNA polymerase (77) and confirmed, where appropriate, by DNA sequencing by the method of Sanger et al. (67) or by PCR sequencing at the Nucleic Acid Facility of the University of Massachusetts Medical School. Plasmid DNAs were propagated in *Escherichia coli* DH5α or NM522. Microscopy was performed on a Nikon Diaphot 300 inverted microscope. New gene names included here have been registered with the *Saccharomyces* Genome Database (SGD) and with the GenBank/EMBL/DDBJ databases.

Oligonucleotides. The oligonucleotides used in this study were prepared by Operon, Inc., and are listed in Table 1.

Yeast strains. The strains used in this study and their sources are shown in Table 2. Strains yDM128, yDM130, and yDM132 were constructed by PCR-based gene deletion as described previously (11). For deletion of *PBP1*, *PBP2*, and *PBP3*, oligonucleotide pairs UKN1.1-UKN1.2, YB83.1-YB83.2, and YIM3.1-YIM3.2, respectively, were used to amplify the *HIS3* marker from plasmid pJJ215 by PCR (36). The PCR product was recovered with a Geneclean kit (Bio 101, Inc.) and transformed into yeast strain yDM117. Colony PCR of individual transformants was performed to identify deletion mutations in the correct locus with gene-specific primers UKN1.3 (for *pbp1*Δ), YB83.3 (for *pbp2*Δ), and YIM3.3 (for *pbp3*Δ) in combination with an oligonucleotide specific for *HIS3* (HIS.TEST). Disruptions of *PBP1* with *LEU2* in strains yDM146 and yDM198 were constructed by transformation with pDM102 linearized by restriction digestion with *Sac*I and *Xho*I. Genomic DNA was isolated from individual transformants and used in PCRs with primers 64.2 and 36Rev.3. With these primers, wild-type strains produced a 1-kb band, while strains with disrupted *PBP1* alleles produced a 3-kb band. To create yDM206, strain yDM198 was grown on rich media for several generations, and cells which had lost the *PAB1-URA3-CEN* plasmid were selected on minimal media containing 5-fluoro-orotic acid. Strains yDM157, yDM227, and yDM214, containing the *TRP1::ADH1p-HA-PBP1* allele (*ADH1* promoter and HA epitope tag), were constructed by linearizing pDM110 with *Cla*I and transforming the DNA into strains yDM117, yDM119, and yDM120, respectively. Proper integration of the *TRP1::ADH1p-HA-PBP1* allele was confirmed by colony PCR of individual transformants with oligonucleo-

TABLE 2. Yeast strains

Strain	Genotype	Source
L40 (yDM61)	*MAT***a** *ade2 his3Δ200 leu2-3,112 trp1-901 LYS::*$(lexAop)_4$*−HIS3 URA3::*$(lexAop)_8$*−lacZ gal4 gal80*	Stanley Hollenberg
AMR70 (yDM62)	*MAT*α *ade2 his3Δ200 leu2-3,112 trp1-901 LYS::*$(lexAop)_4$*−HIS3 URA3::*$(lexAop)_8$*−lacZ gal4 gal80*	Stanley Hollenberg
BJ2168 (yDM33)	*MAT***a** *leu2 trp1 ura3-52 pep4-3 prb1-1122 prc1-407 gal2*	Elizabeth Jones
CY338 (yDM116)	*MAT*α *ade2-101 leu2Δ1 lys2-801 his3Δ200 ura3-52*	Craig Peterson
yDM128	*MAT*α *ade2-101 leu2Δ1 lys2-801 his3Δ200 ura3-52 pbp1::HIS3*	This study
yDM130	*MAT*α *ade2-101 leu2Δ1 lys2-801 his3Δ200 ura3-52 pbp2::HIS3*	This study
yDM132	*MAT*α *ade2-101 leu2Δ1 lys2-801 his3Δ200 ura3-52 pbp3::HIS3*	This study
yAS306 (yDM117)	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	Alan Sachs
yDM146	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 pbp1::LEU2*	This study
yAS392 (yDM119)	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 rpl46::LEU2*	Alan Sachs
yAS394 (yDM120)	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 rpl46::LEU2 pab1::HIS3*	Alan Sachs
yAS320 (yDM197)	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 pab1::HIS3 pPAB1-URA3-CEN*	Alan Sachs
yDM198	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 pab1::HIS3 pbp1::LEU2 pPAB1-URA3-CEN*	This study
yDM206	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 pab1::HIS3 pbp1::LEU2*	This study
yDM157	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 TRP1::ADH1p*-HA-*PBP1*	This study
yDM227	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 rpl46::LEU2 TRP1::ADH1p*-HA-*PBP1*	This study
yDM214	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 rpl46::LEU2 pab1::HIS3 TRP1::ADH1p*-HA-*PBP1*	This study

tides ADH1-5′ and 64Rev.2 and screening for amplification of an 0.8-kb DNA fragment.

Plasmid constructs (see Fig. 1 for nomenclature). (i) Plasmids for two-hybrid experiments. Plasmid pDM125, the *lexA*(DB)-*PAB1*-FL fusion, was constructed as follows. *Pst*I and *Eco*RI restriction sites were introduced just 5′ of the initiator ATG of *PAB1* by PCR with oligonucleotides PAB1.3 and PAB1.2, and the product was subcloned as a *Pst*I-*Eco*RV fragment into plasmid YPA3 (60). From the resulting plasmid, an *Eco*RI fragment carrying the entire gene was ligated into pBTM116 (9) (obtained from Stanley Hollenberg, Fred Hutchinson Cancer Research Center, Seattle, Wash.), and clones in the proper orientation were identified by restriction analysis. To create the other *lexA*(DB)-*PAB1* constructs, fragments of *PAB1* were amplified from plasmid YPA3 with oligonucleotide pairs PAB1.7-PAB1.6 [*lexA*(DB)-*PAB1* 3-H], PAB1.8-PAB1.6 [*lexA*(DB)-*PAB1* 4-H], PAB1.4-PAB1.6 [*lexA*(DB)-*PAB1* P-H], and PAB1.5-PAB1.6 [*lexA*(DB)-*PAB1* H]. The products were then digested with *Eco*RI and *Sal*I and subcloned into pBTM116. The *lexA*(DB)-*PAB1* P-h C-terminal truncation mutant carrying Pab1p amino acids 406 to 553 and the *lexA*(DB)-*pab1* P-H M(−14) and M(−74) point mutants were identified after random PCR mutagenesis (50) in a screen for *lexA*(DB)-*PAB1* P-H alleles that increased or decreased interactions with the *GAL4*(AD)-*PBP1* (198–722) construct. Plasmid pDM127, the *GAL4*(AD)-*PBP1*-FL fusion, was constructed as follows. *Xho*I and *Eco*RI restriction sites were introduced just 5′ of the initiator ATG of *PBP1* by PCR with oligonucleotides PBP1-ATG and 36Rev.4, and the product was subcloned as an *Xho*I-*Hin*dIII fragment into plasmid pDM63 to create pDM104. (pDM63 contains a 3.8-kb genomic *Eco*RI fragment of *PBP1* in the "reverse" orientation.) Next, a *Sal*I linker was inserted into the *Sma*I site of pDM104, creating pDM108. This step allowed an *Eco*RI-*Sal*I fragment carrying the entire *PBP1* gene to be ligated subsequently into pGAD424 (Clontech).

(ii) Plasmids for analysis of *PBP1*. Plasmid pDM102, which was used to create *LEU2* disruptions of *PBP1*, was generated in two steps. First, a 0.7-kb *Cla*I-*Bam*HI fragment of *PBP1* was subcloned from pDM64 into pBluescript SK(+) to create pDM98, and then a *Hin*dIII-*Sma*I fragment of pJJ250 (36) containing the *LEU2* gene was transferred into the *Hin*dIII-*Eco*RV site of pDM98. Plasmid pDM110, used for the integration of *TRP1::ADH1p*-HA-*PBP1* alleles, was generated in a single step as a three-piece ligation of the following DNA molecules: an *Xba*I-*Xho*I fragment, containing the *ADH1p*-HA sequences from pHF1083; an *Xho*I-*Hin*dIII fragment, containing the 5′ end of *PBP1* from pDM104; and YIplac204 (25) digested with *Xba*I and *Hin*dIII.

Two-hybrid screening. Yeast strain L40 (31) (Table 2) harboring the *lexA*(DB)-*PAB1* P-H plasmid (pDM79) was transformed with each of the two-hybrid *GAL4*(AD) yeast genomic DNA libraries (35) (generously provided by Philip James and Elizabeth Craig, University of Wisconsin Medical School, Madison) and plated on synthetic complete (SC) medium without His, Leu, and Trp but with 5 mM 3-aminotriazole (3-AT). The addition of 5 mM 3-AT to the plates suppressed the growth of noninteracting transformants resulting from weak transcriptional activation by the *lexA*(DB)-*PAB1* P-H construct alone. After 4 to 5 days of growth at 30°C, *lacZ* expression levels were assayed by filter lifting colonies (14). Positive clones were then grown in rich medium, and cells which had lost the "bait" plasmid were identified by plating on SC medium lacking Leu and then replica plating on SC medium lacking Trp. Clones which "self-activated" in this test, i.e., retained β-galactosidase activity in the filter-lifting assay, were discarded, whereas negative (white) clones were retained. To confirm that transcriptional activation was dependent on the presence of both gene fusions, the remaining clones were mated to strains AMR70 (31) bearing *lexA*(DB) alone or fused to *PAB1* FL, *PAB1* P-H, *PAB1* H, Lamin, *MTF1*, *MOT1*, or *PAF1* and plated on SC medium lacking Leu and Trp (heterologous baits were the generous gifts of Judith Jaehning, University of Colorado Health Sciences Center, Denver, and Stanley Fields, University of Washington, Seattle). These strains were again assayed for β-galactosidase activity, and clones positive for interaction with the *lexA*(DB)-*PAB1* P-H plasmid but negative for interactions with the other plasmids were retained. Total nucleic acid was isolated from each strain (32) and electroporated into *E. coli* JF1754 (26). The activation domain plasmids were selected by the ability of the *LEU2* gene to complement the *E. coli leuB* mutation when cells were replica plated on E medium lacking Leu but containing ampicillin (26, 74). Isolated plasmids were further characterized by restriction mapping, Southern blotting, and DNA sequence analysis. Nucleotide sequences were compared to existing sequence databases by use of the BLAST programs (1, 2). Activation domain plasmids representing each gene identified were then retransformed with the *lexA*(DB)-*PAB1* FL, -*PAB1* P-H, or -*PAB1* H plasmids, and β-galactosidase activity and 3-AT resistance were reconfirmed.

Cloning of *PBP1*. To identify genomic clones of *PBP1*, approximately 5,000 *E. coli* cells containing a yeast genomic YCp50 library (58) (pool A3, from Duane Jenness) were plated on Luria broth-ampicillin plates, and colony hybridization was performed (66). A radiolabeled probe for *PBP1* was generated by random priming (21) of an *Eco*RI fragment from the two-hybrid clone *GAL4*(AD)-*PBP1* (198–722). A total of six clones were isolated and restriction mapped. Oligonucleotide primers were generated to sequence the 3.8-kb *Eco*RI fragment that contained the entire gene. *PBP1* was sequenced prior to the completion of the SGD, and its chromosome assignment was determined by hybridization to blots of cosmid and lambda phage clones of yeast genomic DNA (purchased from the American Type Culture Collection, Rockville, Md.).

Polyribosome analysis. Whole-cell extracts from 200 ml of cells were prepared by glass bead lysis in the presence of cycloheximide, and 40 A_{260} units were fractionated on 15 to 50% sucrose gradients as described previously (8, 53). Gradients were centrifuged in a Beckman SW41 rotor at 35,000 rpm for 165 min at 4°C and analyzed by continuous monitoring of A_{260} (46). Each fraction from the gradient was precipitated with 6% trichloroacetic acid–0.015% sodium deoxycholate, washed with cold 80% acetone, and resuspended in 1× protein gel sample buffer.

Preparation of purified yeast nuclei. Yeast nuclei were isolated by osmotic lysis of spheroplasts, followed by banding two times on Ficoll gradients (26). The purity of the nuclei was monitored by Western blotting with, as criteria, enrichment for a nucleus-associated protein (Rpo21p) and the loss of a cytoplasmic protein (Pgk1p).

Protein gels, Western blots, and antibodies. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (42). Gels were electroblotted to Immobilon-P membranes (Millipore) under conditions recommended by the manufacturer. Binding conditions for antibodies were as described by Harlow and Lane (28). Detection was by enhanced chemiluminescence with an ECL kit from Amersham Corp. Blots were stripped and reprobed in accordance with instructions from the membrane manufacturer. Polyclonal antibodies specific for Pab1p and Pbp1p were generated by repeated injection of antigen into New Zealand White rabbits (28). For anti-Pab1p antibodies, recombinant Pab1p purified from *E. coli* (a generous gift from Alan Sachs, University of California, Berkeley) was injected. Sera were purified by ammonium sulfate fractionation and chromatography on DEAE-cellulose and carboxymethyl cellulose (28). For anti-Pbp1p antibodies, peptides corresponding to amino acids 505 to 524 and 702 to 721 were synthesized (at the Peptide Synthesis Core Facility of the University of Massachusetts Medical School), coupled to keyhole limpet hemocyanin, and injected. Antihemagglutinin (HA) antibody (12CA5) was from Boehringer Mannheim Biochemicals. Anti-Tcm1p, anti-Pgk1p, and anti-Rpo21p antibodies were generous gifts from Jonathan Warner, Duane Jenness, and Judith Jaehning, respectively.

RNA isolation, poly(A) selection, and analysis of mRNA poly(A) tail lengths. Total yeast RNA was isolated by the hot phenol method (30). Poly(A)$^+$ mRNA was isolated by binding to oligo(dT)-cellulose as described previously (33), except that the RNA was bound, washed twice with binding buffer and twice with wash buffer, and eluted in batches. Poly(A) tails were analyzed by end labeling with ^{32}pCp (Amersham Corp.) and RNA ligase, followed by digestion of the RNA with RNase A and subsequent fractionation on denaturing polyacrylamide gels (61, 70). Autoradiographs of poly(A) tail lengths were scanned with a Molecular Dynamics SI personal densitometer and displayed graphically.

In vitro 3′-end-processing assays. Whole-cell yeast extracts were prepared from logarithmic-phase (A_{600}, ~0.7) and stationary-phase (A_{600}, ~4.0) cells as described previously (18, 44). Substrates for cleavage assays used full-length *CYC1* precursors transcribed in vitro by T7 RNA polymerase (3). For polyadenylation assays, precleaved *CYC1* precursors were generated by incubation of full-length precursors with wild-type extracts and purified prior to use (3). All reactions were performed for 60 min at 30°C with 2 μl of extract in a final volume of 25 μl. Resulting products were analyzed on 6% polyacrylamide–7 M urea gels and visualized by autoradiography.

RESULTS

Identification of factors that interact with Pab1p. A two-hybrid screen (9, 10) was conducted to identify factors that interact with yeast Pab1p. As "bait," a *lexA* fusion which included only the proline- and methionine-rich domain and the C-terminal helical region of *PAB1* was created (*PAB1* P-H; Fig. 1C and 2B). We screened approximately 3,000,000 transformants and identified 75 clones that were resistant to 5 mM 3-AT and demonstrated significant β-galactosidase activity. These clones passed several tests for specificity, including the failure to activate transcription of the reporter constructs in the absence of the *lexA-PAB1* P-H construct and a lack of interaction with the *lexA*(DB) vector alone or fused to the heterologous baits Lamin, *MTF1*, *MOT1*, or *PAF1* (Fig. 1A). This same screen was performed with the *lexA-PAB1* F-L and *lexA-PAB1* H constructs (Fig. 1C) but failed to identify any interacting clones. Clones which interacted with the *lexA-PAB1* P-H construct were tested to determine if they could interact with other *PAB1* constructs (Fig. 1A and C). As more *PAB1* RRMs were included in the *lexA*(DB) constructs, we observed less interaction with the putative interacting clones, suggesting that the presence of the RNA-binding domains inhibited the two-hybrid assay. The construct containing only the *PAB1* C-terminal helical region also failed to interact, presumably because it was

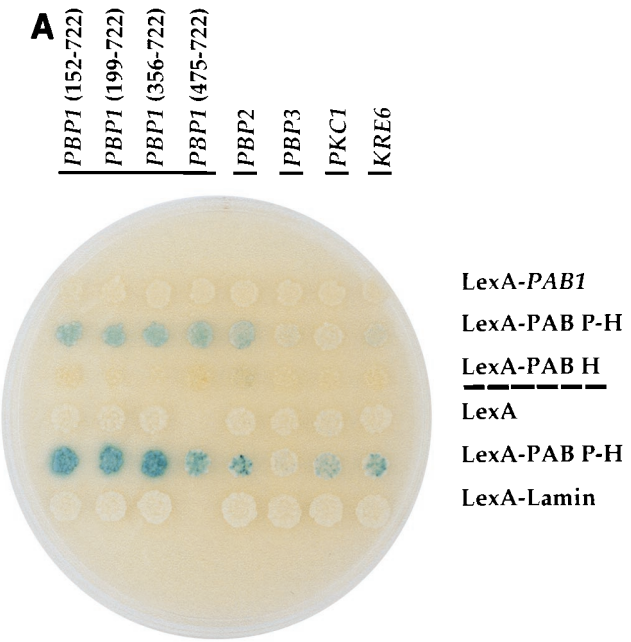

FIG. 1. Specificity tests of clones interacting with *lexA*(DB)-*PAB1* P-H and mapping of Pab1p-Pbp1p-interacting domains. All cells harbored a $(lexAop)_8$-*lacZ* reporter and were spotted on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates to monitor protein-protein interactions. (A) Top three rows: positively interacting *GAL4*(AD) clones identified in the screen (see Table 3 and below) were cotransformed into yeast strain L40 with *lexA*(DB) fusions that included full-length *PAB1*, *PAB1* P-H, or *PAB1* H. Bottom three rows: L40 cells harboring the same set of positively interacting *GAL4*(AD) plasmids were mated to AMR70 cells containing *lexA*(DB), *lexA*(DB)-*PAB1* P-H, or *lexA*(DB)-Lamin, and the resulting diploid strains were assayed for interactions. Matings of strains containing *PBP1* (475–722) and *lexA*(DB) or *lexA*(DB)-Lamin were not performed. (B) *GAL4*(AD)-*PBP1* construct interaction with *lexA*(DB)-*PAB1* P-H assayed by an X-Gal filter-lifting assay (for β-galactosidase [β-Gal]) and by the extent of resistance to 3-AT. (C) *lexA*(DB)-*PAB1* constructs that included *PAB1* FL (full length), *PAB1* 3-H (RRM 3 to C terminus), *PAB1* 4-H (RRM4 to C terminus), *PAB1* P-H (proline- and methionine-rich region to C terminus), *PAB1* P-h (proline- and methionine-rich region to C terminus with a short truncation), and *PAB1* H (C-terminal helical region only) were assayed for interaction with *GAL4*(AD)-*PBP1* (198–722) by an X-Gal filter-lifting assay and by the extent of resistance to 3-AT. Symbols for β-galactosidase assay: −, no interaction; +/−, barely detectable interaction; +, weak interaction; +++, strong interaction (as in panel A); +++++, very strong interaction. For the 3-AT assay, the highest concentration of 3-AT (on plates of SC medium lacking His, Leu, and Trp) that still allowed substantial cellular growth is noted; − his, cells could grow in the absence of histidine but were unable to grow in the presence of 5 mM 3-AT; no growth, cells could not grow in the absence of histidine.

B.

Allele	Pab1p Interaction: ß-Gal Activity	Pab1p Interaction: 3-AT Resistance
PBP1-FL (1–722)	+	10 mM
PBP1 (152-722)	+++	100 mM
PBP1 (198-722)	+++	100 mM
PBP1 (199-722)	+++	100 mM
PBP1 (339-722)	+++	100 mM
PBP1 (357-722)	+++	100 mM
PBP1 (475-722)	+	10 mM

C.

Construct	Pbp1p Interaction: ß-Gal Activity	Pbp1p Interaction: 3-AT Resistance
PAB1 FL (1–577)	-	no growth
PAB1 3-H (207–577)	+/ -	- his
PAB1 4-H (295–577)	+	80 mM
PAB1 P-H (406–577)	+++	100 mM
PAB1 P-h (406–553)	+++++	>100 mM
PAB1 H (494–577)	-	no growth

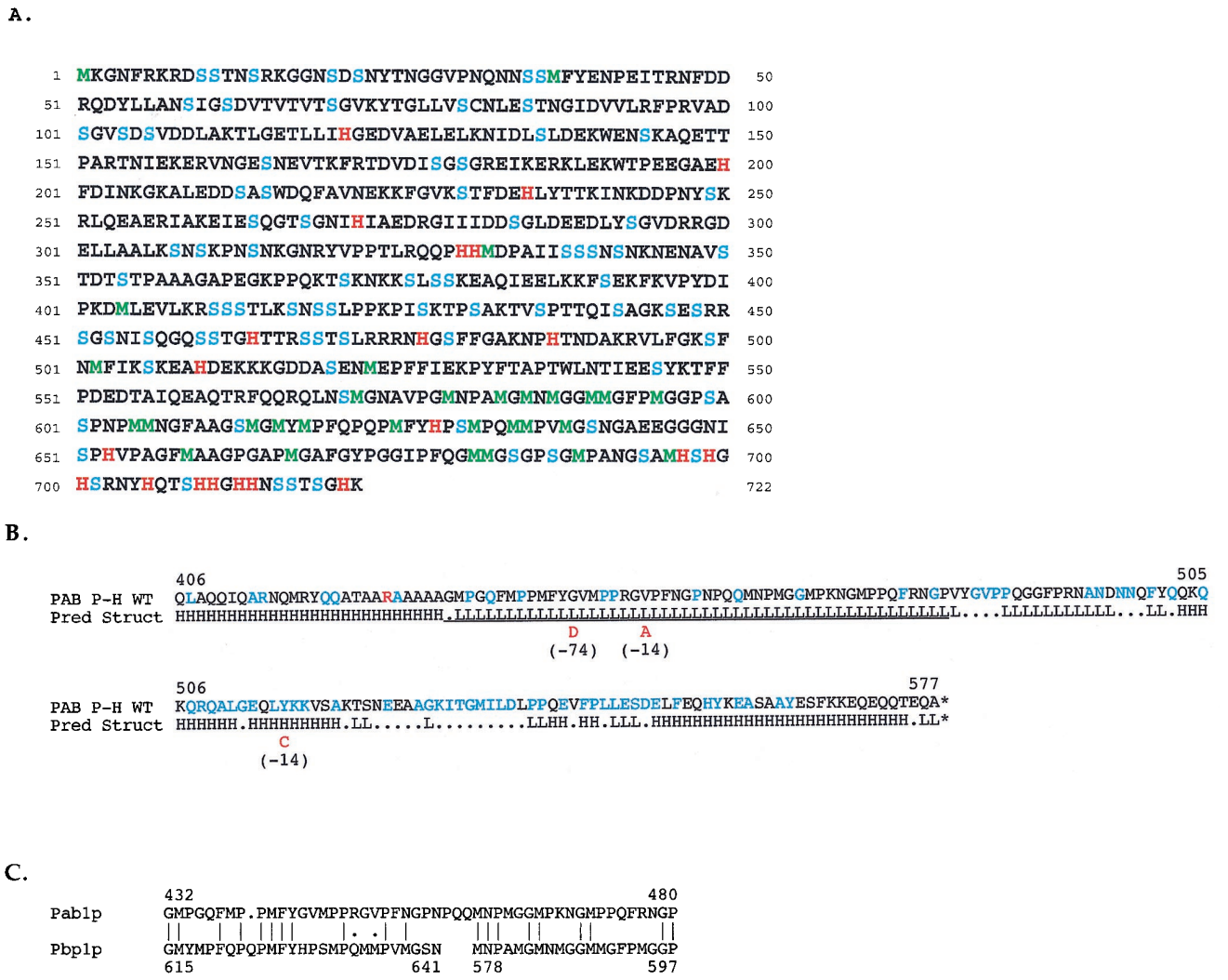

FIG. 2. Structural features of Pbp1p and Pab1p. (A) Amino acid sequence of Pbp1p deduced from the sequence of the *PBP1* gene. Histidine (H) is in red, methionine (M) is in green, and serine (S) is in blue. (B) Structural features of the Pab1p bait fragment. Pred Struct, predicted structure. H and L denote helical and loop regions, respectively, as predicted by the nnpredict program (39). Dots indicate residues for which no prediction was made. WT, wild type. Amino acids in blue are those with a strong evolutionary conservation among Pab1p homologs of eight different species (45a). The underlined segment corresponds to the region of Pbp1p homology shown in panel C. Amino acids in red denote substitutions found in two *lexA*(DB)-*pab1* P-H alleles that were incapable of promoting a detectable two-hybrid interaction with *GAL4*(AD)-*PBP1* (198–722). Mutant M(−74) has a single G→D substitution, and mutant M(−14) has both V→A and Y→C substitutions. The red R at position 426 denotes an A→R substitution in the bait fragment relative to the published sequence of *PAB1* (61). (C) Alignment of homologous C-terminal regions of Pab1p and Pbp1p. Vertical lines indicate identity; dots denote similarity.

too small and did not include the protein interaction domain. In contrast, the construct containing a short C-terminal truncation (*lexA-PAB1* P-h) showed a significantly stronger interaction than the *lexA-PAB1* P-H construct. After restriction mapping, Southern blotting, and DNA sequencing, a total of five genes encoding proteins that interacted with the C terminus of Pab1p were identified (Table 3). These included three previously uncharacterized genes (named *PBP1* to *PBP3*) and two known genes (*PKC1* and *KRE6*).

Genes encoding Pab1p-interacting factors. Of the putative Pab1p-interacting proteins identified, the product of the *PBP1* gene promoted the strongest interaction. With the expression

TABLE 3. Genes encoding putative Pab1p-interacting proteins

Gene	Homology or function	3-AT resistance (mM)	Molecular mass (kDa)	Gene disruption	Chromosomal location	No. of times isolated
PBP1	None	>100	79	Nonessential	VII	38
PBP2	hnRNPk	10	46	Nonessential	II	2
PBP3	Serine-rich family	10	48	Nonessential	IX	1
PKC1	Protein kinase C	10	145/150	Essential	II	2
KRE6	Required for (1→6)-β-glucan synthesis	10	80	Nonessential	XVI	1

of (*lexAop*)$_4$-*HIS3* as an indicator of interaction, cells harboring most *GAL4*(AD)-*PBP1* fusions were able to grow in the presence of 100 mM 3-AT. *PBP1* was isolated 38 times from the 44 independent clones recovered, and the six distinct N-terminal fusions to *GAL4*(AD) that were obtained mapped the putative Pab1p-interacting domain to the C-terminal third of the protein (Fig. 1B). *PBP1* was not present in any database when it was identified in our screen. Therefore, we mapped the gene to chromosome VII (using blots of cosmid and lambda phage clones of yeast genomic DNA) and cloned it from a yeast library by using probes from the fragments recovered in the two-hybrid screen. Sequencing of a 3.8-kb *Eco*RI fragment (corresponding to the *S. cerevisiae* Genome Project gene yGR178c and GenBank accession no. Z72963) which contained the entire gene identified an open reading frame of 2,166 nt. This open reading frame encoded a 722-amino-acid polypeptide (79 kDa) which was serine rich overall (83 amino acids) and had a proline- and methionine-rich segment (24 of 125 amino acids) as well as a histidine-rich C terminus (9 of 27 amino acids) (Fig. 2A). Interestingly, two segments from the C-terminal region of Pbp1p (amino acids 578 to 597 and 615 to 641) had a high degree of identity (45 and 46%, respectively) to a predicted loop region near the C terminus of Pab1p (Fig. 2B and C). Mutations in *lexA-PAB1* P-H that inactivated the Pab1p-Pbp1p interaction were localized to this same region (Fig. 2B), indicating that the proline- and methionine-rich segment was essential for protein-protein interactions.

Comparisons of the entire Pbp1p sequence to those in the available databases identified weak homologies with the protein encoded by the human gene responsible for spinocerebellar ataxia type 2 (*SCA2*) (1, 2, 55) and with human cell proliferation antigen Ki-67 (23, 68). However, the functions of these related genes are unknown. Disruption of the *PBP1* gene in yeast demonstrated that it is not essential for cell viability or for the maintenance of wild-type mRNA levels (data not shown). Experiments suggesting that Pbp1p is a bona fide Pab1p-interacting protein are described below.

The four other genes that were isolated (*PBP2*, *PBP3*, *PKC1*, and *KRE6*) interacted weakly with the *lexA-PAB1* P-H construct (cotransformants resistant to 10 mM 3-AT; Table 3). *PBP2* (yBR233w) has substantial homology to the gene encoding the human hnRNP K protein, including both KH domains, while *PBP3* (yIL123w) encodes one of a four-member family of serine-rich proteins. Disruption of these two genes demonstrated that they are not essential for cell viability or for the maintenance of wild-type mRNA levels or translation rates or steady-state poly(A) lengths of total cellular mRNA (data not shown).

PKC1 is believed to encode the yeast homolog of metazoan protein kinase C. It was originally cloned on the basis of homology to isozymes of rat protein kinase C, and Pkc1p has enzymatic properties similar to those of the mammalian enzymes (5, 43, 76). *PKC1* is essential for yeast cell viability; however, deletion of this gene can be suppressed by growth in the presence of 10% sorbitol (43). *KRE6* is thought to encode a membrane-associated factor required for (1→6)-β-glucan synthesis (56). Interestingly, *KRE6* acts as a high-copy suppressor of a *pkc1*Δ mutation, presumably due to an alteration in cell wall metabolism (57). In this report, we have focused on the *PBP1* gene; characterization of the other genes will be presented elsewhere.

Disruption of *PBP1* suppresses a *PAB1* deletion. The significance of the identification of *PBP1* in our screen was evaluated by determining whether there were any other genetic interactions between *PBP1* and *PAB1*. Mutations that alter the 60S subunit of the ribosome, as well as those that inhibit mRNA decay, act as suppressors of *PAB1* deletions (12, 16, 29, 61, 62). To determine whether a deletion of the *PBP1* gene acted in a similar fashion, *PBP1* was disrupted in a strain bearing both a chromosomal deletion of *PAB1* and a copy of *PAB1* on a *URA3* plasmid. Deletion of *PBP1* was shown to

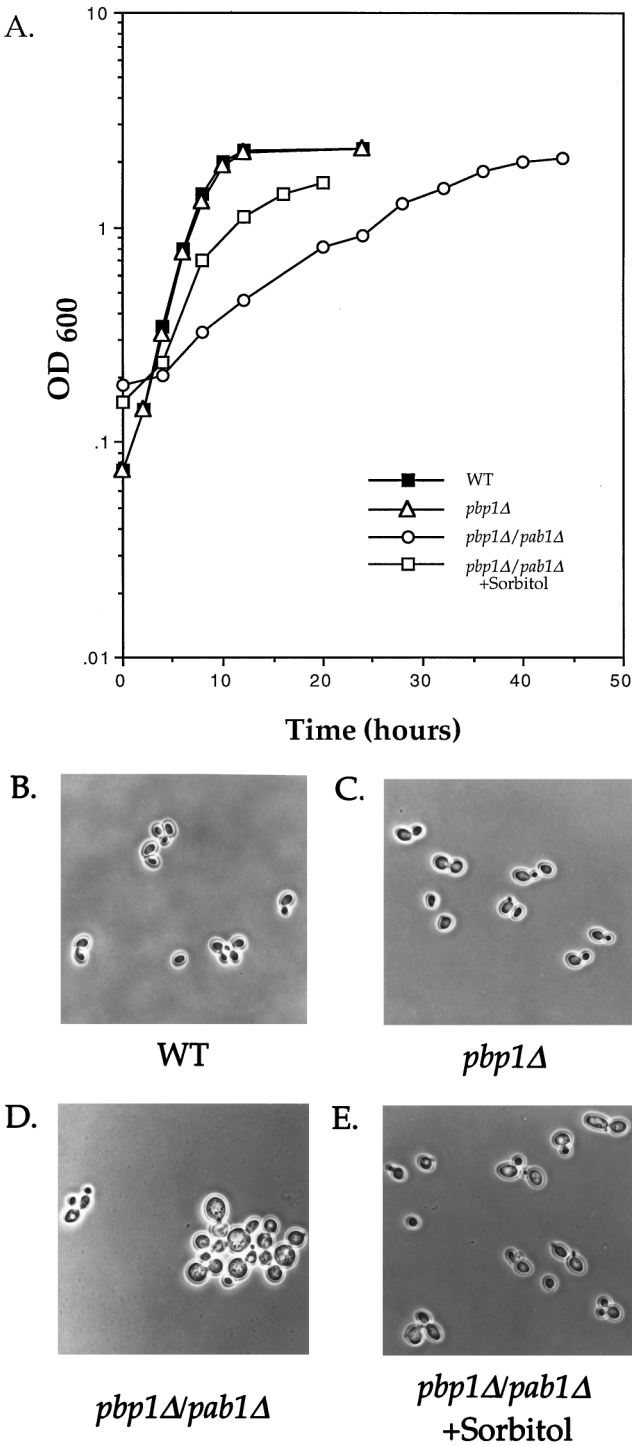

FIG. 3. Disruption of *PBP1* suppresses a deletion of *PAB1*. Cells were grown on rich medium without or with 10% sorbitol and monitored by measuring the optical density at 600 nm (OD_{600}) (A) and by phase-contrast microscopy (B to E). The strains tested were yDM117 (wild type [WT]), yDM146 (*pbp1*Δ), and yDM206 (*pbp1*Δ/*pab1*Δ).

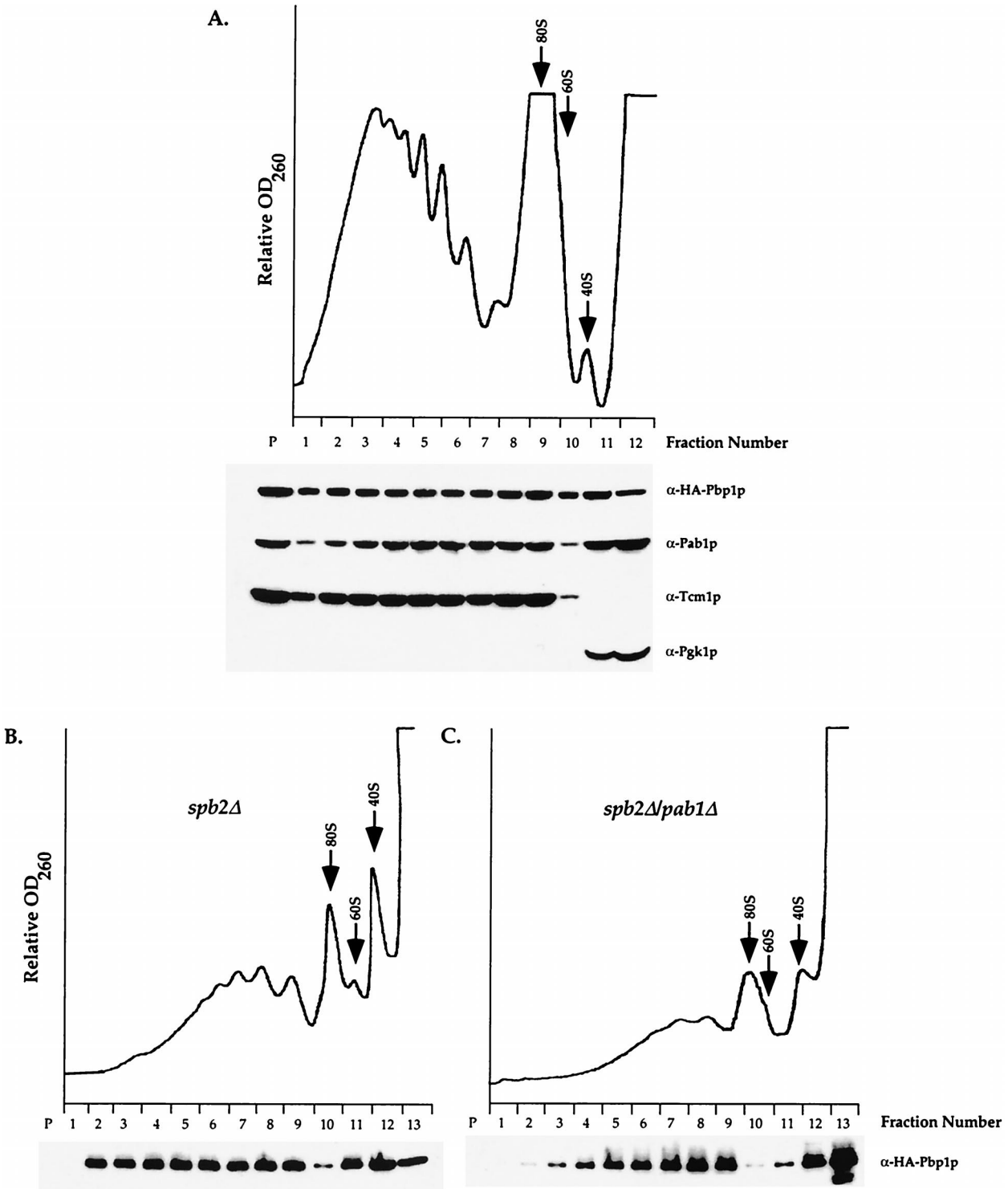

FIG. 4. Pbp1p fractionates with polysomes on sucrose gradients. Extracts from various strains bearing an HA-*PBP1* allele were fractionated on 15 to 50% sucrose gradients that were subsequently analyzed by Western blotting. (Top) Profile of optical density at 260 nm (OD_{260}), with sedimentation proceeding from right to left. The 80S, 60S, and 40S peaks are indicated by arrows. (Bottom) Western blot analysis of the gradient fractions. Panels were serially stripped and reprobed with the indicated antibodies. Fractions 1 to 9 and the pellet fraction (P) included the entire sample, whereas fractions 10 to 12 or 13 included only one-fifth of the sample. (A) yDM157 (wild type). (B) yDM227 (*spb2*Δ). (C) yDM214 (*spb2*Δ/*pab1*Δ).

suppress the lethality associated with *pab1*Δ mutations, since cells were viable when the loss of the *URA3-PAB1* plasmid was selected in the presence of 5-fluoro-orotic acid. The *pbp1*Δ/*pab1*Δ strain grew very slowly (doubling time, 7 to 9 h; Fig. 3A) compared with the wild-type strain or a *pbp1*Δ strain (doubling time, 1.5 to 2 h; Fig. 3A). Phase-contrast microscopy of the same strains showed that the *pbp1*Δ/*pab1*Δ cells were greatly enlarged, tended to adhere to each other, and contained dense

reflecting particles when compared with wild-type or *pbp1*Δ cells (compare Fig. 3D with Fig. 3B and C). The large size and shape of the *pbp1*Δ/*pab1*Δ cells were reminiscent of those cells that are osmotically sensitive, a phenotype often associated with defects in cell wall biosynthesis. When we grew the *pbp1*Δ/*pab1*Δ cells in the presence of 10% sorbitol, the growth defect was significantly suppressed (the doubling time was reduced from 7 to 9 h to ~3 h; Fig. 3A), and the visual phenotype of the cells returned to normal (compare Fig. 3E with Fig. 3B). Similarly, the addition of sorbitol to media partially suppressed the growth defect associated with an *spb2*Δ/*pab1*Δ strain (data not shown), suggesting that deletion of *PAB1* alters the expression of genes encoding cell wall components.

Translation initiation is inhibited in *pbp1*Δ/*pab1*Δ strains. Since the previously identified *spb1* to *spb7* suppressors of a *pab1* temperature-sensitive mutation altered translation (61, 62), we sought to determine if *PBP1* functioned in a similar manner. Initially, we monitored the rate of incorporation of ^{35}S-labeled amino acids into mutant and wild-type strains. These data indicated that *PBP1* must have a function distinct from that of *SPB2* since, as expected, protein synthesis was markedly diminished in an *spb2*Δ strain, but translation in a *pbp1*Δ strain was comparable to that in the wild-type strain (data not shown). Furthermore, a comparison of the rates of ^{35}S incorporation into the *pbp1*Δ/*pab1*Δ and *spb2*Δ/*pab1*Δ strains demonstrated that *pbp1*Δ-suppressed cells were more competent for translation (data not shown).

Further evidence that disruption of *PBP1* does not affect translation in a manner analogous to the effects of the *spb* mutations was obtained by analyzing the cellular distribution of polysomes and ribosome subunits. The *spb1* to *spb7* suppressors had altered ratios of 40S and 60S ribosome subunits (61) (Fig. 4B), but the relative abundances of polysomes and 80S, 60S, and 40S ribosomes were unaltered in a *pbp1*Δ strain (compare Fig. 5A with Fig. 4B). Interestingly, the polysome profiles of *pbp1*Δ/*pab1*Δ strains were indicative of a marked reduction in the efficiency of translation initiation; i.e., these strains showed few polysomes and an accumulation of 80S ribosomes (Fig. 5B), but they did not show the altered ratios of 40S and 60S ribosome subunits characteristic of *spb2*Δ/*pab1*Δ strains (Fig. 4C). Since an *spb2*Δ mutation alone had the latter phenotype (Fig. 4B) and since a *pbp1*Δ mutation had essentially no effect on polysome profiles, we infer that the profiles of the *pbp1*Δ/*pab1*Δ strains largely reflected the defect wrought by the deletion of *PAB1*. This profile is similar to but more severe than that observed previously for a *pab1* temperature-sensitive strain assayed after extended incubation at the restrictive temperature (61).

mRNAs in *pbp1*Δ/*pab1*Δ strains have long poly(A) tails. It was originally observed that most steady-state mRNAs in *spb*Δ/*pab1*Δ cells contained relatively long poly(A) tails (approximately 90 adenylate residues), although tails of shorter lengths were still readily apparent (61) (Fig. 6D). To determine if *pbp1*Δ/*pab1*Δ strains were similarly deficient in poly(A) metabolism, poly(A) tail lengths associated with total cellular mRNA were analyzed by 3′ end labeling followed by RNase digestion of the RNA and subsequent fractionation of the digestion products on denaturing polyacrylamide gels. As was seen with an *spb2*Δ mutant, a *pbp1*Δ strain showed no alteration in poly(A) tail length relative to the wild type (Fig. 6A to C). Surprisingly, the fraction of total mRNA molecules possessing long poly(A) tails (90 adenylate residues) was even larger in *pbp1*Δ/*pab1*Δ cells than in *spb2*Δ/*pab1*Δ cells, with almost no tails of shorter lengths (Fig. 6E). This observation is consistent with either a loss of regulation of poly(A) tail synthesis or a decrease in poly(A) removal in these mutant cells.

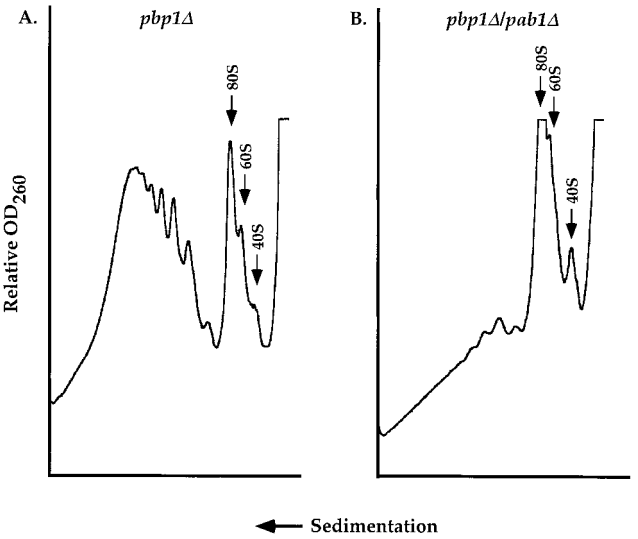

FIG. 5. Translation initiation is inhibited in *pbp1*Δ/*pab1*Δ strains. Extracts from strains yDM146 (*pbp1*Δ) (A) and yDM206 (*pbp1*Δ/*pab1*Δ) (B) were fractionated on 15 to 50% sucrose gradients, and the optical density at 260 nm (OD_{260}) was monitored. The direction of sedimentation and the positions of the 80S, 60S, and 40S peaks are indicated by arrows.

Pbp1p cofractionates with polyribosomes but does not require Pab1p for association. To consider the mechanism by which the deletion of *PBP1* suppresses a deletion of *PAB1*, we assessed the subcellular localization of Pbp1p. Pab1p associates with the poly(A) tails of mRNAs actively undergoing translation, i.e., polysomes (54). Therefore, it would be expected that factors which bind Pab1p might be present in polyribosomal fractions. A triple-HA epitope-tagged form of Pbp1p (determined to be functional in vivo by a complementation test demonstrating its inability to suppress a deletion of *PAB1*; data not shown) was constructed, and its subcellular localization was assayed by fractionating cytoplasmic extracts on sucrose gradients and subsequently analyzing the gradient fractions by Western blotting. As demonstrated in Fig. 4A, HA-Pbp1p and Pab1p sedimented in the gradient with similar distributions. Evidence for the specificity of this cosedimentation with polyribosomes included its coincidence with Tcm1p (ribosomal protein L3) and its separation from the soluble protein Pgk1p (Fig. 4A), as well as its disruption by EDTA, a Mg^{2+} chelator that dissociates ribosomes from mRNA (data not shown). Interestingly, significant portions of both Pbp1p and Pab1p were present in the lighter fractions of the gradient (lanes 10 to 12 in Fig. 4A) and may have represented a free pool of these factors and/or mRNPs that were not being translated.

Since Pbp1p was identified as a Pab1p-interacting protein, we determined if its association with polyribosomes depended on the presence of Pab1p. Fractionation of extracts from *spb2*Δ and *spb2*Δ/*pab1*Δ strains bearing the HA-*PBP1* allele showed that Pbp1p was still associated with polyribosomes in the absence of Pab1p (compare Fig. 4B with Fig. 4C). It remains to be determined whether this association was due to a direct association of Pbp1p with RNA (mRNA or rRNA) or with some other factor(s).

Pbp1p is also present in nuclei, and its accumulation is posttranscriptionally regulated. Like the ubiquitous Pab1p, Pbp1p localizes to both the cytoplasm (by its specific association with polysomes and crude cytoplasmic lysates; Fig. 4 and 7A) and the nucleus (by its association with purified nuclei; Fig. 7A). However, the relative ratios of Pbp1p to Pab1p are

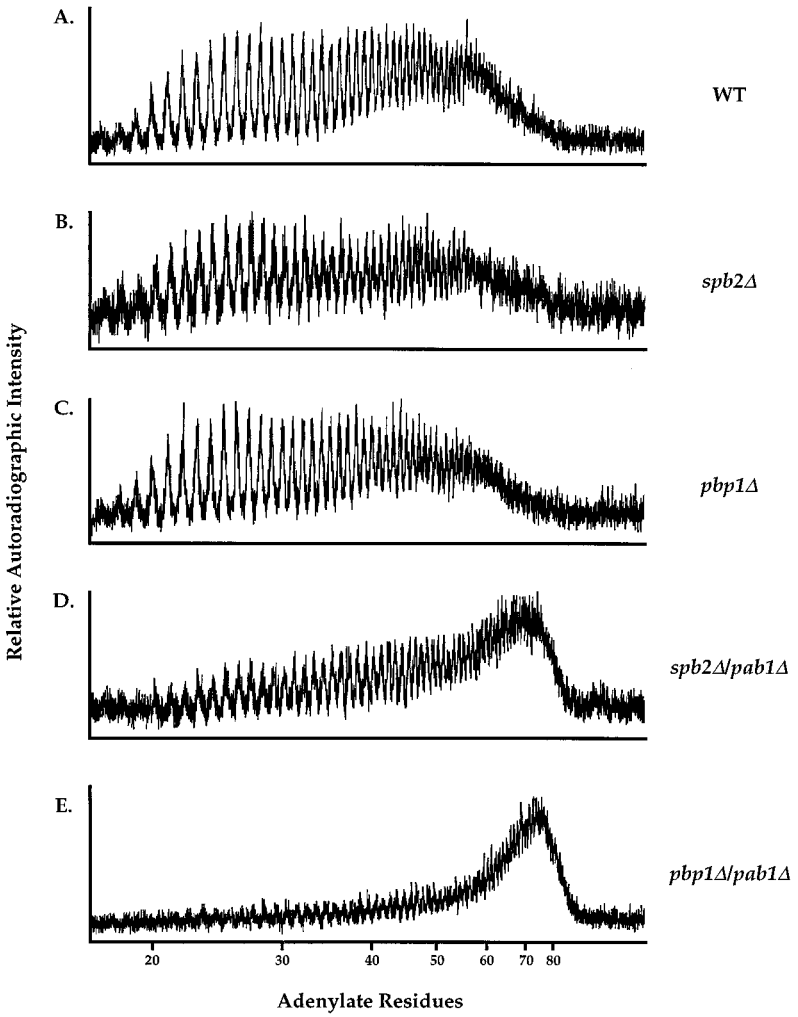

FIG. 6. mRNAs in *pbp1*Δ/*pab1*Δ strains have long poly(A) tails. RNA was isolated from strains yDM117 (wild type [WT]) (A), yDM119 (*spb2*Δ) (B), yDM146 (*pbp1*Δ) (C), yDM119 (*spb2*Δ/*pab1*Δ) (D), and yDM206 (*pbp1*Δ/*pab1*Δ) (E), and mRNA poly(A) tail lengths were analyzed by gel electrophoresis and densitometric tracing of the resulting autoradiographs. Numbers of adenylate residues were determined by comparison with a DNA sequence ladder.

not constant in the two subcellular locations. Pbp1p is considerably more abundant in the nucleus than in the cytoplasm, while Pab1p has the opposite distribution. The significance of these differences is supported by (i) the relative enrichment of the RNA polymerase II subunit, Rpo21p, and the loss of the cytoplasmic protein, Pgk1p, in the nuclear fraction (Fig. 7A) and (ii) the observation that a *PBP1-lacZ* fusion construct promotes the nuclear localization of β-galactosidase (58a). Expression of *PBP1* driven by the strong *ADH1* promoter overexpresses the mRNA only three- to fourfold, suggesting that Pbp1p, like Pab1p, is a very abundant protein (data not shown). The protein is expressed at maximal levels in the log phase and is almost completely absent in the stationary phase. This result is in contrast to the expression of Pab1p, whose abundance in stationary-phase cells decreases only modestly (Fig. 7C). The expression of *PBP1* mRNA, however, is not growth phase dependent (Fig. 7B), suggesting that, in the stationary phase, either the protein is more unstable or its mRNA is translationally repressed.

Pbp1p regulates polyadenylation. Since Pab1p was recently implicated in the control of polyadenylation (4, 37, 49) and a substantial fraction of Pbp1p is present in the nucleus (Fig. 7A), we sought to determine if Pbp1p was involved in cleavage and/or polyadenylation. To increase the amount of starting material for biochemical purification, reactions to assay 3′-end processing have typically been performed with extracts from stationary-phase yeast cultures. However, since Pbp1p was differentially expressed in the log phase and stationary phase (Fig. 7C), we assayed extracts made from cells at both stages of growth. In cleavage reactions performed with in vitro-transcribed *CYC1* mRNA precursors, no changes were observed with extracts from either growth stage or when extracts were made from strains bearing a *PBP1* deletion (Fig. 8, lanes 1 to 5). However, in polyadenylation assays that used precleaved *CYC1* transcripts as substrates, poly(A) tails were substantially shorter in extracts made from stationary-phase cells than in those made from log-phase cells (Fig. 8, compare lanes 7 and 9 with lanes 8 and 10). Extracts from *pbp1*Δ strains also showed a decrease in the extent of polyadenylation (Fig. 8, compare lanes 7 and 8 with lanes 9 and 10). This size change reflected a loss of approximately 20 adenylate residues in the "absence" of Pbp1p, caused by either disruption of the gene or decreased expression in the stationary phase. This change was not, however, attributable to changes in Pab1p levels, since they were unaffected by the growth phase (Fig. 7C) or by deletion of *PBP1* (data not shown). Poly(A) tail lengths in each condition

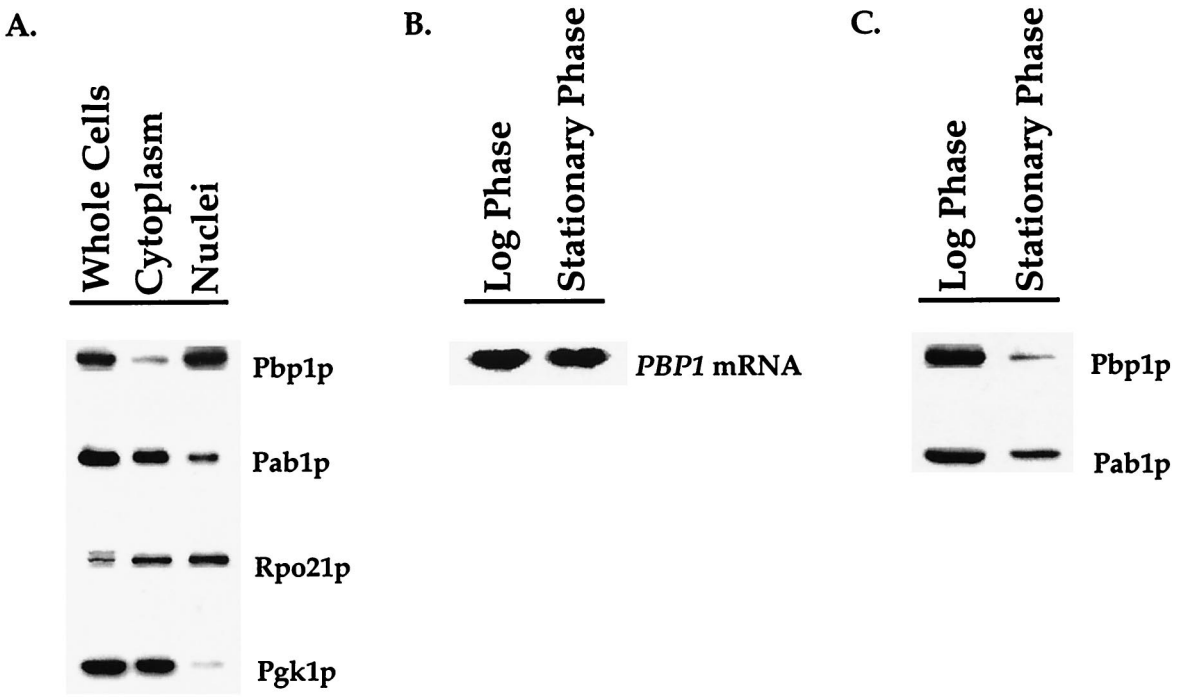

FIG. 7. Pbp1p copurifies with nuclei, and its expression is regulated posttranscriptionally. (A) Western blot analysis of Pbp1p and Pab1p as well as control proteins (Rpo21p and Pgk1p) in a whole-cell lysate, a cytoplasmic extract, and purified nuclei from strain yDM33. (B) Northern analysis of *PBP1* in strain yDM117 grown to log or stationary phase. (C) Western blot analysis of Pbp1p and Pab1p in strain yDM117 grown to log or stationary phase.

did not increase with extended incubation time (data not shown), indicating that polyadenylation was not simply proceeding at different rates but had terminated at different lengths. Deletion of *PBP1* combined with growth of the cells to stationary phase resulted in a further loss of polyadenylation, suggesting that other factors required for maximal polyadenylation are also downregulated during the stationary phase (Fig. 8, compare lanes 9 and 10). Consistent with this idea and with the notion that such factors can be titrated, mixing of equal amounts of extracts from log-phase wild-type and *pbp1*Δ strains led to the synthesis of poly(A) tails of intermediate lengths (Fig. 9, compare lanes 1 and 2 with lane 3).

DISCUSSION

The poly(A)-binding protein is a multifunctional posttranscriptional regulator. It is now well established that the poly(A) status of an mRNA can be an important determinant of both mRNA translational efficiency and the time of onset of mRNA decay (17, 34). It also appears likely that qualitative and quantitative aspects of polyadenylation influence other posttranscriptional events within the nucleus and the cytoplasm (4). The mediator of most of these processes is the ubiquitous and highly conserved poly(A)-binding protein. This conclusion follows primarily from experiments with yeast, where mutations in the *PAB1* gene or depletion of Pab1p have been shown to inhibit translation initiation (61), delay mRNA decay (16), and promote increases in overall mRNA poly(A) tail lengths (4). While the poly(A)-binding protein is the first of the 3′-untranslated region-binding proteins to be shown to have such a central and multifunctional role in the posttranscriptional regulation of gene expression, it is not unique. For example, metazoan poly(A)$^-$ histone mRNAs terminate in a highly conserved stem-loop (47). This structure is bound by a specific protein, the stem-loop-binding protein (27, 75), and the resulting RNA-protein complex has been shown to be essential for the 3′ processing of histone pre-mRNA, the transport of histone mRNA out of the nucleus, and the translation and regulated stability of histone mRNA in the cytoplasm (20, 22, 27, 52, 75). Clearly, identification of the factors with which such proteins interact will provide substantial insight into their function.

Pbp1p is a novel Pab1p-interacting protein. In an effort to better understand the functional role of Pab1p, we conducted a two-hybrid screen by using fragments of the yeast *PAB1* gene as bait. This analysis led to the identification of three previously uncharacterized genes, *PBP1* to *PBP3*, and two known genes, *PKC1* and *KRE6*, which encode factors that putatively interact with the C terminus of Pab1p. Since the *PBP1* gene was isolated most frequently and its product had the strongest apparent interaction with Pab1p, it was studied in detail.

Sequence analysis showed that Pbp1p is a serine-rich protein with a proline- and methionine-rich domain at its C terminus. While lacking a functional homolog in the available databases, Pbp1p does, nevertheless, have weak homology to a domain in the product of *SCA2*, a human gene implicated in spinocerebellar ataxia type 2 (55), and to cell proliferation antigen Ki-67 (23, 68). The interaction between Pbp1p and Pab1p appears to take place between the proline- and methionine-rich domains of the proteins. Other factors which interact through domains rich in proline include profilin, a cytoskeletal protein which binds poly-L-proline (48), and factors involved in mitogenic signalling, whose Src homology (SH3) domains bind a proline- and serine-rich sequence (40). The similarity of the Pab1p- and Pbp1p-interacting domains may allow for the formation of Pab1p-Pab1p or Pbp1p-Pbp1p homomultimers or Pab1p-Pbp1p heteromultimers (41). Such interactions may enable the cooperative binding of Pab1p to the poly(A) tail, stabilize Pab1p-poly(A) interactions after RNA binding, or allow for the regulation of Pab1p activity.

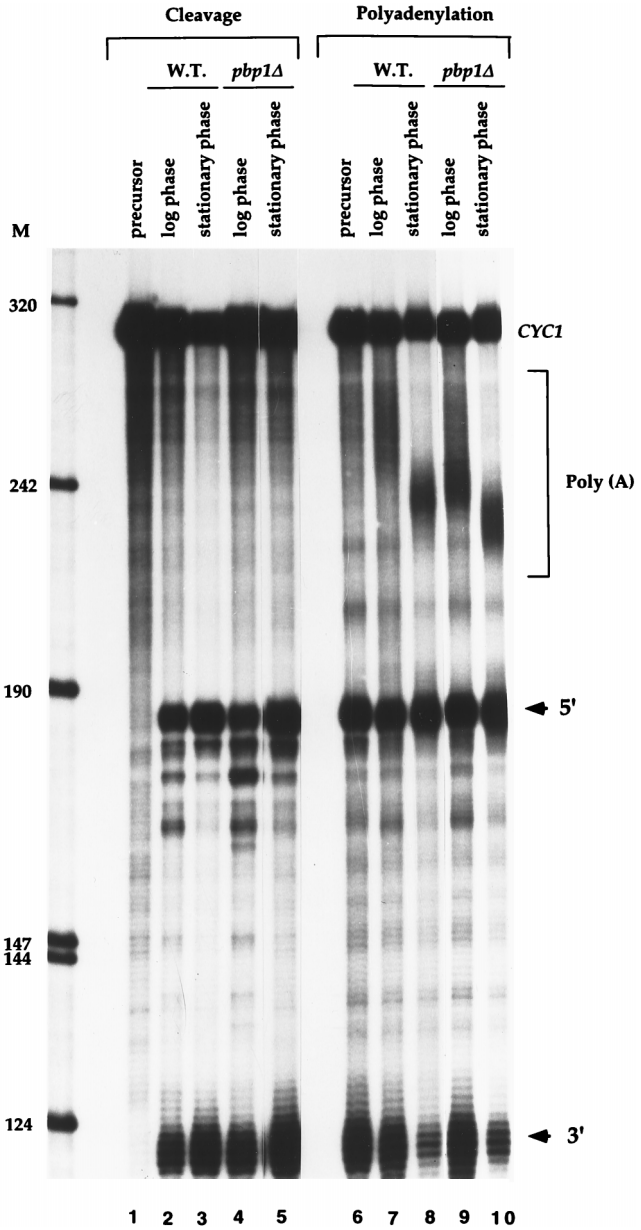

FIG. 8. Pbp1p regulates polyadenylation. RNA processing extracts were prepared from a wild-type (W.T.) (yDM117) or *pbp1*Δ (yDM146) strain grown to log or stationary phase. Cleavage reactions were performed with a full-length *CYC1* precursor (lanes 1 to 5). Polyadenylation assays were performed with a precleaved *CYC1* precursor (lanes 6 to 10). The bracket marks the position of the polyadenylated substrate. *CYC1* indicates the full-length precursor. The arrowheads indicate the 5′ and 3′ cleavage products. M, radiolabeled DNA markers of indicated sizes (in nucleotides).

Deletion of *PBP1* suppresses a *PAB1* deletion. Although two-hybrid interactions are often an excellent indicator of bona fide in vivo protein-protein interactions (9, 10), independent indications of such interactions tend to make the evidence more compelling. Hence, a putative association between Pab1p and Pbp1p is underscored by the observation that a *pbp1*Δ allele is a suppressor of a *PAB1* deletion. This observation is not restricted to the alleles analyzed here, since other studies, in which transposon insertion mutagenesis was used, have identified a *pbp1* allele (dubbed *spb9*) as a *pab1*Δ suppressor (59a). This genetic relationship, where the loss of two factors is required for viability, strongly suggests that both proteins participate in the same biochemical event and that one of the proteins regulates the other.

Mutations that alter the 60S subunit of the ribosome, as well as those that inhibit mRNA decay, have been identified as suppressors of *PAB1* mutations (12, 16, 29, 61, 62). The mechanism by which a *pbp1*Δ allele suppresses a *PAB1* deletion must be different from those of the previously isolated suppressors, since there are no obvious defects in translation or mRNA decay in strains harboring only the *pbp1*Δ mutation.

Pbp1p negatively regulates *PAB1* to control polyadenylation. Further evidence that Pbp1p and Pab1p are involved in the same metabolic event was obtained from analyses of poly(A) tail lengths on bulk mRNA and the ability of cell extracts to promote polyadenylation (Fig. 6, 8, and 9). At steady state, the cellular mRNA population has poly(A) tails that predominantly range from ~20 to 60 nt in length (Fig. 6). However, in *PAB1* mutants (i.e., *spb2*Δ/*pab1*Δ strains), the majority of mRNAs have long poly(A) tails, although some shorter tails are observed (Fig. 6) (61). This defect could be the result of either a loss of poly(A) tail shortening or an increase in polyadenylation. Disruption of *PBP1* has no effect on the poly(A) tail length of this pool of cytoplasmic mRNA; therefore, Pbp1p is not required for normal poly(A) tail shortening. However, the number of mRNAs with short tails is greatly reduced in a *pbp1*Δ/*pab1*Δ strain (Fig. 6). Possible explanations for this result are that Pbp1p is required for deadenylation in the absence of Pab1p or that Pbp1p plays a role in nuclear polyadenylation. The latter possibility is favored in light of the observation that polyadenylation is reduced in extracts from *pbp1*Δ strains (Fig. 8 and 9).

Since deletion of *PAB1* results in long poly(A) tails, we infer that *PAB1* negatively regulates the activity of the polyadenylation complex. Pab1p could thus be a negative regulator of

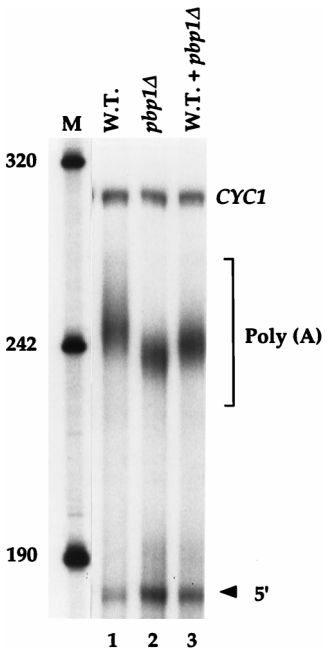

FIG. 9. Wild-type extracts partially complement the polyadenylation defects of *pbp1*Δ extracts. Equal amounts of processing extracts from a log-phase wild-type (W.T.) (yDM117) or *pbp1*Δ (yDM146) strain were mixed and tested for polyadenylation activity with a precleaved *CYC1* precursor as described in the legend to Fig. 8. The bracket marks the position of the polyadenylated substrate. *CYC1* indicates the full-length precursor. The arrowhead indicates the 5′ cleavage product. M, radiolabeled DNA markers of indicated sizes (in nucleotides).

poly(A) polymerase (Pap1p) or could be required for the activity of a nuclease (PAN and/or others) to create poly(A) tails of specific lengths prior to export of the mRNA to the cytoplasm. Since the loss of Pbp1p results in shorter poly(A) tails, Pbp1p could be a negative regulator of Pab1p or could control nuclease activity. Surprisingly, the inability of extracts from *pbp1*Δ strains to synthesize full-length poly(A) tails in vitro is not reflected by alterations in mRNA steady-state levels or poly(A) tail lengths in vivo. This result suggests that (i) the polyadenylation defect is limited to a subset of mRNAs, (ii) a rapid, initial poly(A) tail shortening event is bypassed in *pbp1*Δ strains, or (iii) other factors compensate for the absence of Pbp1p in vivo but are inactive in vitro.

Many of the factors required for 3′-end processing have been identified by biochemical purification. One such factor, CFI, was recently shown to be a complex of proteins that includes Pab1p (37). Using fractions from the purification of yeast processing factors (a generous gift from Marco Kessler and Claire Moore, Tufts University School of Medicine), we determined that Pbp1p partially copurifies with CFI but is absent from the most purified preparations of this complex (data not shown) (37). This observation is consistent with the notion that Pbp1p is necessary for maximal polyadenylation but not absolutely required for polyadenylation. Pbp1p probably has not been identified in biochemical fractions characterized by others because extracts frequently have been prepared from stationary-phase cells, in which Pbp1p levels are greatly reduced (Fig. 7C).

A role for Pbp1p in the regulation of polyadenylation within nuclei raises the question of the significance of the cytoplasmic fraction of this protein. The observation that Pbp1p and Pab1p cosediment with polysomes with similar distribution patterns in sucrose gradients (Fig. 4) is consistent with the association of these two proteins and with the known cytoplasmic functions of Pab1p (59). The ability of Pbp1p to associate with polysomes in the absence of Pab1p suggests that it either binds directly to mRNA or rRNA or interacts with other RNA-associated factors or both. Since *pbp1*Δ strains lack an mRNA decay or translation phenotype, however, the cytoplasmic function of Pbp1p remains elusive. The presence of large fractions of Pab1p and Pbp1p that do not cosediment with polysomes suggests that these proteins are present in excess. The "free" pool of Pab1p may ensure efficient translation of poly(A)$^+$ mRNAs, be involved in the regulation of translationally inactive mRNAs, or simply be a reflection of the recycling of this factor that must occur when poly(A) tails are shortened (34, 65).

Other Pab1p interacting proteins. The significance of the weaker Pab1p-interacting proteins Pbp2p, Pbp3p, Pkc1p, and Kre6p remains to be determined. Previously reported genetic interactions between *PKC1* and *KRE6* (57) suggested that their identification may be more than a coincidence and raised the possibility that the regulated phosphorylation of translation initiation factors (51) could be mediated by Pkc1p-Pab1p interactions. Consistent with this possibility are recent experiments which demonstrate that Pkc1p cosediments with polysomes in sucrose gradients (45a).

Earlier studies suggested that Pab1p also interacts with other proteins. Strains bearing C-terminally truncated *pab1* alleles accumulate mRNAs with long poly(A) tails (59a). This observation and others led to the purification of a Pab1p-dependent PAN (13, 15) and to the demonstration that the C-terminal domain of Pab1p is required for PAN activity in vitro (59a). Likewise, interactions between Pab1p and a factor required for pre-mRNA cleavage, Rna15p, are indicated by (i) the ability of a strain overexpressing *PAB1* to partially suppress an *rna15-2* temperature-sensitive allele (4), (ii) the specific interaction of the two proteins in a directed two-hybrid assay (4), and (iii) cochromatography and coimmunoprecipitation of both proteins (4, 37, 49). Our failure to identify any significant two-hybrid interactions between Pab1p and known PAN subunits or Rna15p raises the possibility that the screen was not capable of detecting very weak interactions or was limited by other aspects peculiar to two-hybrid analysis.

Limitations inherent in our two-hybrid analysis are also the most likely reason for the absence of any detectable interactions between Pab1p and eIF4G (72, 73). The latter protein has been reported to bridge mRNA 5′-3′ interactions, but such interactions are RNA dependent and are mediated by the second RRM of Pab1p (38). Since the only screen yielding interacting clones used, as bait, a construct lacking all of the *PAB1* RRMs, only RNA-independent interactions were observed and the detection of Pab1p-eIF4G interactions was precluded.

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