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Positive and Negative Regulation of Poly(A) Nuclease

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PAN, a yeast poly(A) nuclease, plays an important nuclear role in the posttranscriptional maturation of mRNA poly(A) tails. The activity of this enzyme is dependent on its Pan2p and Pan3p subunits, as well as the presence of poly(A)-binding protein (Pab1p). We have identified and characterized the associated network of factors controlling the maturation of mRNA poly(A) tails in yeast and defined its relevant protein-protein interactions. Pan3p, a positive regulator of PAN activity, interacts with Pab1p, thus providing substrate specificity for this nuclease. Pab1p also regulates poly(A) tail trimming by interacting with Pbp1p, a factor that appears to negatively regulate PAN. Pan3p and Pbp1p both interact with themselves and with the C terminus of Pab1p. However, the domains required for Pan3p and Pbp1p binding on Pab1p are distinct. Single amino acid changes that disrupt Pan3p interaction with Pab1p have been identified and define a binding pocket in helices 2 and 3 of Pab1p's carboxy terminus. The importance of these amino acids for Pab1p-Pan3p interaction, and poly(A) tail regulation, is underscored by experiments demonstrating that strains harboring substitutions in these residues accumulate mRNAs with long poly(A) tails in vivo.

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 (11, 16, 40, 42). In *Saccharomyces cerevisiae*, newly synthesized poly(A) tails of different transcripts are relatively homogeneous, with their final lengths determined by the combined actions of poly(A) polymerase holoenzyme (Pap1p and Fip1p), poly(A)-binding protein (Pab1p), poly(A) nuclease (PAN), and the Pab1p-associated factor, Pbp1p (10, 24, 43).

Poly(A) tracts are generally bound by Pab1p, a highly conserved protein with four RNA recognition motifs (RRMs) connected to a carboxy-terminal helical domain via a proline- and methionine-rich segment (25, 31). Association of Pab1p with poly(A) requires a minimal binding site of 12 adenosines, and multiple molecules can bind via RRMs 1 and 2 to the same poly(A) tract, spaced approximately 25 nucleotides apart (1, 2, 31, 33). In yeast, the relatively abundant 70-kDa poly(A)-binding protein is encoded by the *PAB1* gene. Mutations in *PAB1* cause a significant increase in the average steady-state poly(A) tail length of total cellular mRNA (32), and these effects have been attributed to two apparently nuclear functions of Pab1p: the regulation of a switch between processive and distributive activities in poly(A) polymerase (43) and the stimulation of PAN activity (7, 9, 21).

Yeast poly(A) tails are initially synthesized to default lengths of 70 to 90 A's and then trimmed to mRNA-specific lengths by PAN. Analyses of three different mRNAs indicate that such trimmed tails have lengths ranging from 55 to 71 A's (8). PAN, a Pab1p-dependent 3′ to 5′ poly(A) exoribonuclease, requires magnesium, releases AMP as a product, and is regulated by *cis*-acting mRNA sequences (21). Purified PAN contains two proteins which are essential for nuclease activity: Pan2p is a 127-kDa protein with homology to the RNase T family of 3′→5′ exoribonucleases, while Pan3p is a 76-kDa protein which apparently acts as a positive activator of PAN activity (8). Deletion of *PAN2* and/or *PAN3* eliminates poly(A) nuclease activity but does not hinder cell growth. Physical interaction between Pan2p and Pan3p has been inferred from coimmunoprecipitation and two-hybrid analyses of the full-length proteins (9).

Pbp1p (Pab1p-binding protein 1) specifically interacts with a 74-amino-acid segment encompassing the proline- and methionine-rich domain of the Pab1p C terminus. *PBP1* is not essential for viability, but its disruption can suppress the lethality associated with a *PAB1* deletion (23). In the absence of Pbp1p, 3′ termini of pre-mRNAs are properly cleaved but receive poly(A) tails that are, on average, 15 to 30 nucleotides shorter than normal (23, 25). In vitro polyadenylation reactions using extracts from wild-type and *pbp1*Δ strains demonstrated that the mutant extracts initially produced full-length tails equivalent to their wild-type counterparts but subsequently trimmed the poly(A) at rates that were faster than normal. This accelerated poly(A) trimming did not occur in *pbp1*Δ/*pan2*Δ or *pbp1*Δ/*pan3*Δ extracts, indicating that Pbp1p is a negative regulator of PAN activity (25).

Once in the cytoplasm, yeast poly(A) tails are further shortened to 10 to 15 A's by the Ccr4p and/or Caf1p exoribonucleases (38), after which decay of the rest of the mRNA is triggered; the mRNA is cleaved at its 5′ end by the Dcp1p/Dcp2 decapping enzyme, and the uncapped and deadenylated mRNA is subsequently degraded exonucleolytically (5, 10, 15, 18, 24, 27). Poly(A) tail length is thus a negative regulator of

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decapping and, as might be inferred from this conclusion, some mRNAs whose poly(A) tails are lengthened as a consequence of *pan* mutations are also stabilized (8). The presence of a poly(A) tail also influences mRNA translation activity. Pab1p associated with the poly(A) tail interacts with the initiation factor eIF4G, and the network of ensuing interactions promotes early events in ribosome binding to the mRNA (24). These and other activities indicate that the Pab1p/poly(A) mRNP has several important functions in both the nuclear and cytoplasmic posttranscriptional control of gene expression (24).

In this study, we sought to elucidate the mechanism of poly(A) tail maturation by defining the interactions of the factors involved in this process. We utilized the two-hybrid system to map the domains required for interactions between Pan3p and Pan2p, Pab1p and Pan3p, and Pbp1p and Pan2p and for multimerization interactions of Pan3p and Pbp1p. Mutagenesis of Pab1p was also performed to further map the interaction domain of Pan3p onto Pab1p. Collectively, these analyses have led to the identification of the key components of a regulatory network controlling the maturation of mRNA poly(A) tails.

MATERIALS AND METHODS

General methods. Preparation of standard yeast media and methods of cell culture were as described previously (30). Transformation of yeast utilized the rapid method (36). DNA manipulations were performed according to standard techniques (34). PCR amplifications were performed with *Taq* DNA polymerase (41) and confirmed, where appropriate, with DNA sequencing according to the method of Sanger et al. (35) or by PCR sequencing by the Nucleic Acid Facility at the University of Massachusetts Medical School. Plasmid DNAs were propagated in *Escherichia coli* strain DH5α.

Yeast strains. Yeast strain L40 (yDM61) was used for all two-hybrid analyses (23). Yeast strain yAS1255 (*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 pab1*Δ::*HIS3*), a *pab1*Δ strain bearing a plasmid expressing a C-terminal truncation of *PAB1* (lacking the proline- and methionine-rich domains), was provided by Alan Sachs. Strain yAS320 (yDM197) (23), which bears a chromosomal deletion of *PAB1* covered by a wild-type *PAB1-URA3*-CEN plasmid, was used to introduce mutant *PAB1* alleles by the plasmid shuffle technique (14). Strain yME43, the *pan*3Δ strain, was created by PCR-based gene deletion as described previously (4). PCR using oligonucleotide pair PAN3-D5-L–PAN3-D3-L amplified the *LEU2* gene from plasmid pJJ252 (19). The PCR product was electrophoresed on an agarose gel and extracted, and the full-length product was recovered using a QIAquick gel extraction kit (QIAGEN Inc.). The recovered product was transformed into yeast strain yAS306 (yDM117) (23), and transformants were selected on appropriate media. Genomic DNA was isolated from cultures started from individual colonies, and the presence of the disruption was confirmed by PCR using the gene-specific primers, PAN3-5–PAN3-3.

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

Plasmid constructions. The numbers following the gene names indicate amino acid residues encompassed by the respective constructs. To create *lexA*(DB) constructs, fragments of *PAB1* were amplified by PCR from plasmid YPA3 (23) with oligonucleotide pairs PAB1.4a-PAB1.6 (*PAB1* 406 to 577), PAB1.15-PAB1.6 (*PAB1* 422 to 577), PAB1.5b-PAB1.6 (*PAB1* 488 to 577), PAB1.5a-PAB1.6 (*PAB1* 494 to 577), PAB1.4a-PAB1.10 (*PAB1* 406 to 492), PAB1.4a-PAB1.13 (*PAB1* 406 to 479), PAB1.7a-PAB1.10 (*PAB1* 422 to 492), PAB1.7a-PAB1.13 (*PAB1* 422 to 479), PAB1.4a-PAB1.14 (*PAB1* 406 to 457), PAB1.11-PAB1.10 (*PAB1* 429 to 492), PAB1.11-PAB1.13 (*PAB1* 429 to 479), PAB1.11-PAB1.14 (*PAB1* 429 to 457), PAB1.12-PAB1.10 (*PAB1* 435 to 492), PAB1.12-PAB1.13 (*PAB1* 435 to 479), and PAB1.12-PAB1.14 (*PAB1* 435 to 457). All other *PAB1* constructs were as described previously (23). The products of the reactions were digested with EcoRI and SalI and subcloned into pBTM116.

PAN2 lexA(DB) constructs were created by PCR amplification of *PAN2* fragments from plasmid pAS468 (9) by using the following oligonucleotide pairs: PAN2.1F-PAN2.1115R (*PAN2* 1 to 1115), PAN2.1F-PAN2.748R (*PAN2* 1 to 748), PAN2.333F-PAN2.1115R (*PAN2* 333 to 1115), PAN2.498F-PAN2.1115R (*PAN2* 498 to 1115), PAN2.619F-PAN2.1115R (*PAN2* 619 to 1115), PAN2.671F-PAN2.1115R (*PAN2* 671 to 1115), and PAN2.814F-PAN2.1115R (*PAN2* 814 to 1115). The products of the reactions were digested with EcoRI and PstI and subcloned into pBTM116.

To create *lexA*(DB) and *GAL4*(AD)-*PAN3* full-length (FL) constructs (*PAN3* 1 to 679), *PAN3* was amplified by PCR from genomic DNA with oligonucleotide pairs PAN3-ATG and PAN3-UAA. The products of each reaction were digested with BamHI and XhoI and subcloned into both pBTM116 and pGAD424. To create *lexA*(DB) and *GAL4*(AD)-*PAN3* truncations, fragments of *PAN3* were amplified by PCR from genomic DNA with oligonucleotide pairs PAN3.001-PAN3.548 (*PAN3* 1 to 548), PAN3.001-PAN3.437 (*PAN3* 1 to 437), PAN3.001-PAN3.249 (*PAN3* 1 to 249), PAN3.125-PAN3.679 (*PAN3* 125 to 679), PAN3.250-PAN3.679 (*PAN3* 250 to 679), PAN3.337-PAN3.679 (*PAN3* 337 to 679), PAN3.420-PAN3.679 (*PAN3* 420 to 679), PAN3.250-PAN3.548 (*PAN3* 250 to 548), PAN3.549-PAN3.679 (*PAN3* 549 to 679), PAN3.560-PAN3.679 (*PAN3* 560 to 679), PAN3.337-PAN3.548 (*PAN3* 337 to 548), PAN3.593-PAN3.679 (*PAN3* 593 to 679), PAN3.549-PAN3.662TAA (*PAN3* 549 to 662), PAN3.250-PAN3.437 (*PAN3* 250 to 437), and PAN3.337-PAN3.548 (*PAN3* 337 to 548). The products of the reactions were digested with EcoRI and SalI and subcloned into both pBTM116 and pGAD424.

Full-length and amino-terminal *GAL4*(AD) *PBP1* constructs were amplified by PCR from genomic DNA with oligonucleotide pairs PBP1-ATG–PBP1.722R (*PBP1* 1 to 722) and PBP1-ATG–PBP1.443 (*PBP1* 1 to 443). Carboxy-terminal *GAL4*(AD) *PBP1* constructs were amplified by PCR from plasmid *GAL4*(AD) *PBP1* (357 to 722) (24) with oligonucleotide pairs PBP1.357F-PBP1.695R (*PBP1* 357 to 695), PBP1.357F-PBP1.578R (*PBP1* 357 to 578), PBP1.421F-PBP1.722R (*PBP1* 421 to 722), PBP1.421F-PBP1.695R (*PBP1* 421 to 695), PBP1.421F-PBP1.578R (*PBP1* 421 to 578), PBP1.421F-PBP1.506R (*PBP1* 421 to 506), PBP1.454F-PBP1.722R (*PBP1* 454 to 695), PBP1.454F-PBP1.695R (*PBP1* 454 to 695), PBP1.454F-PBP1.578R (*PBP1* 454 to 578), and PBP1.454F-PBP1.506R (*PBP1* 454 to 506). The products of the reactions were digested with EcoRI and SalI and subcloned into pGAD424. All other *GAL4*(AD) *PBP1* constructs were originally isolated from a library in the two-hybrid screen with *lexA*(DB) *PAB1* P-H (24). The *lexA*(DB) *PBP1* constructs were made by subcloning EcoRI/SalI restriction fragments bearing the various *PBP1* alleles from the *GAL4*(AD) vector (pGAD424) to the *lexA*(DB) vector (pBTM116).

Two-hybrid analysis. Yeast strain L40 was transformed with the appropriate *lexA*(DB) and *GAL4*(AD) constructs described above. In each experiment, appropriate empty vector controls were also included. Transformants were initially selected on plates with synthetic complete medium lacking Leu and Trp (SC −Leu, −Trp plates) and subsequently screened for *lacZ* activity using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) filter lift assays. To determine the extent of *HIS3* reporter activity, colonies from each transformation were grown overnight in SC −Leu, −Trp broth and serially diluted on SC −Leu, −Trp and SC −Leu, −Trp, −His plates containing 0, 5, 10, 20, 40, 60, 80, or 100 mM 3-aminotriazole (3-AT).

Construction and screening of the *lexA*(DB)-*PAB1* (406 to 577) mutant library. PCRs were performed under suboptimal conditions (28) using oligonucleotides PAB1.4 and PAB1.6 to amplify the carboxy terminus of *PAB1* as described previously (23). Reactions were purified using QIAquick PCR purification kits (QIAGEN Inc.) and subcloned into the pBTM116 vector using SalI and BamHI restriction enzymes. *E. coli* transformants were pooled and amplified and plasmids were isolated using QIAfilter maxi-prep kits (QIAGEN Inc.). The resulting library was cotransformed with *GAL4*(AD)-*PAN3*-FL and plated on minimal medium plates containing X-Gal. Colonies that displayed reduced *lacZ* activity (white or light blue) were retained for further analysis. Total nucleic acid was isolated from each strain (17) and electroporated into *E. coli* strain JF1754 (14). The DNA-binding domain plasmids were selected by their inability to complement the *E. coli leuB* mutation when cells were replica plated to E −Leu plates with ampicillin (14, 39). Isolated plasmids were further characterized by restriction mapping with EcoRI and PstI to confirm the presence of the *PAB1* insert. DNA-binding domain plasmids representing each allele identified were then retransformed with empty vector, *GAL4*(AD)-*PBP1* (377 to 722), or *GAL4*(AD)-*PAN3*-FL plasmids, and the loss of interaction was reconfirmed by assaying both β-galactosidase activity and 3-AT resistance.

Site-directed mutagenesis of *lexA*(DB)-*PAB1* (406 to 577). Site-directed mutagenesis was carried out on the wild-type *lexA*(DB)-*PAB1* (406 to 577) plasmid using Stratagene Quik Change kits as directed by the manufacturer. The oligonucleotide pairs in these reactions were PAB1.444.G-D.For–PAB1.444.G-D.Rev (*PAB1* G444D), PAB1.451.V-A.For–PAB1.451.V-A.Rev (*PAB1* V451A), PAB1.506.R-G.For–PAB1.506.R-G.Rev (*PAB1* R506G), PAB1.514.Y-C.For–PAB1.514.Y-C.Rev (*PAB1* Y514C), PAB1.519.A-V.For–PAB1.519.A-V.Rev (*PAB1* A519V), PAB1.528.G-D.For–PAB1.528.G-D.Rev (*PAB1* G528D), and

TABLE 1. Oligonucleotides used in this study

Name	Sequence (5′–3′)
PAB1.4a	CCGGAATTCATGCAATTGGCTCAACAAATCCAAGCC
PAB1.4x	CAATTGGCTCAACAAATCCAAGCC
PAB1.5a	CCGGAATTCATGGCCAACGATAACAACCAATTTTATC
PAB1.5b	CCGGAATTCGGTGGTGGTGGTTTCCCAAGAAATGCCAACGATAAC
PAB1.6	TTACGCGTCGACTTAAGCTTGCTCAGTTTGTTGTTC
PAB1.6x	TTAAGCTTGCTCAGTTTGTTGTTC
PAB1.7a	CCGGAATTCATGCGTGACTCTCAATTGGAAGAGACTAAGGC
PAB1.10	ACGCGTCGACCTATCTTGGGAAACCACCTTGTGGGGGGACGCCG
PAB1.11	CCGGAATTCGCCGCTGCCGGTATGCCAGGTCAATTCATGCC
PAB1.12	CCGGAATTCGGTCAATTCATGCCTCCAATGTTCTATGGTG
PAB1.13	ACGCGTCGACCTAACCATTTCTAAATTGAGGTCGCAAGCCGTTCTTTGGC
PAB1.14	ACGCGTCGACCTAGTTTGGACCGTTGAATGGAACACCTCTTGGTGGC
PAB1.15	CCGGAATTCGCTACTGCTGCCGCTGCCGCCGCCGCTGCC
PAB1.444.G-D.For	GCCTCCAATGTTCTATGATGTTATGCCACCAAGAGGTGTTCC
PAB1.444.G-D.Rev	GGAACACCTCTTGGTGGCATAACATCATAGAACATTGGAGGC
PAB1.451.V-A.For	GCCACCAAGAGGTGCTCCATTCAACGGTCCAAACCC
PAB1.451.V-A.Rev	GGGTTTGGACCGTTGAATGGAGCACCTCTTGGTGGC
PAB1.506.R-G.For	CAACCAATTTTATCAACAAAAGCAAGGACAAGCTTTGGGTGAAC
PAB1.506.R-G.Rev	GTTCACCCAAAGCTTGTCCTTGCTTTTGTTGATAAAATTGGTTG
PAB1.514.Y-C.For	GCTTTGGGTGAACAATTATGCAAGAAGGTTTCTGCTAAGAC
PAB1.514.Y-C.Rev	GTCTTAGCAGAAACCTTCTTGCATAATTGTTCACCCAAAGC
PAB1.519.A-V.For	CAAGAAGGTTTCTGTTAAGACTTCAAATGAAGAAGCAGCTGG
PAB1.519.A-V.Rev	CCAGCTGCTTCTTCATTTGAAGTCTTAACAGAAACCTTCTTG
PAB1.528.G-D.For	GCTAAGACTTCAAATGAAGAAGCAGCTGATAAAATTACTGGTATGATTTTGG
PAB1.528.G-D.Rev	CCAAAATCATACCAGTAATTTTATCAGCTGCTTCTTCATTTGAAGTCTTAGC
PAB1.533.M-T.For	AGCTGGTAAAATTACTGGTACGATTTTGGATTTGCCACCTC
PAB1.533.M-T.Rev	GAGGTGGCAAATCCAAAATCGTACCAGTAATTTTACCAGCT
PAB1.519.Rvt.F	CAAGAAGGTTTCTGCTAAGACTTCAAATGAAGAAGCAGCTGG
PAB1.519.Rvt.R	CCAGCTGCTTCTTCATTTGAAGTCTTAGCAGAAACCTTCTTG
PAN2.1F	CGGAATTCATGAATAATTGGCAACATTTCTTCAAC
PAN2.333F	CGGAATTCATGTTTACCAATACCCCTGAAATGCTAGC
PAN2.498F	CGGAATTCCCCTTTAATAACACTGAGTATTCAGGA
PAN2.619F	CGGAATTCCCTCAAAAATTTAACAGATTCCTGCTCTCG
PAN2.671F	CGGAATTCCCCTCCTTATCAATATCAGGAATCAACAAAACC
PAN2.784R	AACTGCAGTTATATGTGGCTTGTGCCCTTCAATTCGG
PAN2.814F	CGGAATTCCCAAAAGAGAATTGCTTCAAGTGGCTC
PAN2.1115R	AACTGCAGTTATCCCTTTGAAGTTTCTGGAACTTTG
PAN3.1	CCGGAATTCATGGACAAAATCAAT
PAN3.125	CCGGAATTCTTAGTGAATAACAATAACAATAATAGTAATATAAGC
PAN3.250	CCGGAATTCGAAAGGTCTGCAGATCAGCTTTTCATT
PAN3.337	CCGGAATTCAACAAAATATCCAAAATATATCAGATATGG
PAN3.560	CCGGAATTCACCTATACAGAATACATGGAGTCTGTC
PAN3.593	CCGGAATTCGAATCAAGAATAGACATAAATTGGTCC
PAN3.437TAA	ACGCGTCGACTTAGTCCCCAGTAATCAAAACTTTTCTCCAG
PAN3.548TAA	ACGCGTCGACTTAATAAAAGTGACTAGTAAGATCATGAATGC
PAN3.662TAA	ACGCGTCGACTTAGGATATAATAATACAGTTTAACTCATGGGG
PAN3.679TAA	ACGCGTCGACTTATTGGGTGATGGATCGAAAGGTGG
PAN3-5	TACATATCAGACCTTTACAGGGTAACC
PAN3-3	GATGTGGCGAACAAGGGATGAATCGC
PAN3-D5-L	CGATGATAGGTAAGCCTAAAAGACTAGTCAGACGTATCTTACGCCCGCACAGAATCAAATTCGATGACTGG
PAN3-ATG	CGGGATCCTCATGGACAAAATCAATCCTGATTGGGCG
PAN3-UAA	CCCGCTCGAGTTATTGGGTGATGGATCGAAAGGTGG
PAN3-D3-L	GTATAATTAATATATATGTGTGTATCTACATTATTTGGTGTTGTTTAACTTTTTGTGTGGTGCCCTCCTCC
PBP1-ATG	CCCGCTCGAGGAATTCATGAAGGGAAACTTTAGGAAAAGAGATAGC
PBP1.357F	CCGGAATTCGCAGCAGCAGGTGCGCCAGAGGGAAAACCCCC
PBP1.421F	CCGGAATTCTTGCCTCCAAAACCGATCAGCAAAACCCC
PBP1.454F	CCGGAATTCAACATATCTCAAGGCCAATCATCTACCGGCC
PBP1.506R	ACGCGTCGACCTAGGATTTGATGAACATATTAAAACTTTTTCC
PBP1.578R	ACGCGTCGACCTAACCAGGCACAGCATTGCCCATGGAGTTC
PBP1.695R	ACGCGTCGACCTATGCCGACCCGTTCGCTGGCATGCCAGATGG
PBP1.722R	ACGCGTCGACCTATTTATGGCCACTGGATCATCTATTATTG

PAB1.533.M-T.For–PAB1.533.M-T.Rev (*PAB1* M533T). The *PAB1* G510R allele was created by reverting the A519V allele in the original 510/519 double mutant clone to wild type by using the primer pair PAB1.519.Rvt.F–PAB1.519.Rvt.R. The resulting *lexA*(DB)-*PAB1* (406 to 577) constructs were transformed into yeast cells and assayed for interaction with empty vector, *GAL4*(AD)-*PBP1* (357 to 722), or *GAL4*(AD)-*PAN3*-FL by using two-hybrid analysis. *PAB1*-FL single mutant alleles were created using the same primer pairs as above. To generate the *PAB1* G510R mutant in full-length *PAB1*, the mutation was amplified from the *lexA*(DB)-*PAB1* (406 to 577) G510R allele by PCR using *Pfu* Turbo polymerase (Stratagene) and primers PAB1.4x and PAB1.6x.

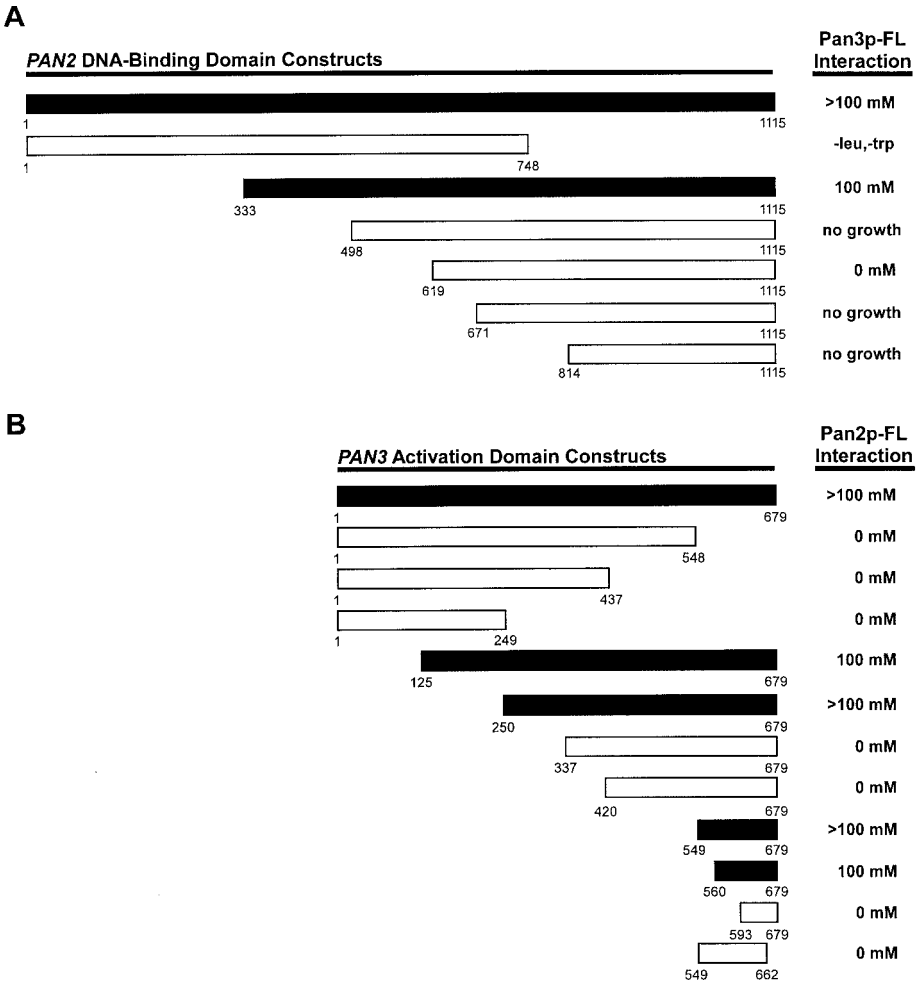

FIG. 1. Two-hybrid mapping of Pan2p-Pan3p interactions. Protein fragments used in two-hybrid analyses are denoted as bars with numbers to indicate the span of specific amino acid residues included in each. Proteins that interacted activated transcription of the *HIS3* gene, producing resistance to the competitive inhibitor 3-AT. Results are expressed as the highest millimolar concentration of 3-AT (on SC −His, −Leu, −Trp plates) that still allowed substantial cellular growth; no growth indicates cells that could grow in the presence of histidine but were unable to grow on medium lacking histidine. Bars for those proteins that produced 50 to 100% resistance to 3-AT are filled in black. Appropriate empty vector controls were performed for each analysis and are indicated only when they significantly contributed to 3-AT resistance levels. (A) Pan3p-FL was tested against Pan2p truncations lacking either the C terminus or N terminus in order to determine the binding domain of Pan3p on Pan2p. (B) Fragments of Pan3p were used to identify the binding domain of Pan2p on Pan3p.

The resulting product was then used as the forward and reverse template in a site-directed mutagenesis reaction.

Analysis of mRNA poly(A) tail lengths. Total yeast RNA was isolated by vortexing cells in the presence of glass beads and phenol (12). Poly(A)$^+$ RNA was isolated by binding to oligo(dT)-cellulose, as described previously (17), except that the RNA was bound, washed twice with binding buffer and twice with wash buffer, and then eluted in batch. 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 (32, 37). Autoradiographs of poly(A) tail lengths were scanned with a Molecular Dynamics personal densitometer SI and displayed graphically.

RESULTS

Mapping of Pan2p-Pan3p interaction domains. An earlier study demonstrated that Pan2p and Pan3p, the subunits of PAN, were interacting proteins (9). We utilized the two-hybrid genetic system (3) to delineate the respective interaction domains on these two proteins. To map the Pan3p-binding site on Pan2p, fragments of *PAN2* were tested for interaction with full-length *PAN3* (Fig. 1A). For these constructs, we were unable to create functional fusions of *PAN2* with the *GAL4* activation domain, and all *PAN2-PAN3* interaction studies were thus performed with *lexA*(DB)-*PAN2* fusions and *GAL4*(AD)-*PAN3* alleles. These experiments showed that full-length Pan3p interacted strongly with full-length Pan2p, producing resistance up to or greater than 100 mM 3-AT (Fig. 1A). A comparable, strong interaction was also observed between *PAN2* (333 to 1115) and full-length *PAN3*. Further truncations of the Pan2p N terminus led to a loss of interaction with Pan3p-FL, as did a C-terminal truncation (Fig. 1A). Tests of *PAN3* fragments against full-length *PAN2* demonstrated that only a small portion of the C-terminal region of Pan3p is necessary for interaction with Pan2p (Fig. 1B). The smallest C-terminal *PAN3* segment capable of producing 3-AT resistance equal to that of full-length *PAN3* was a fragment encom-

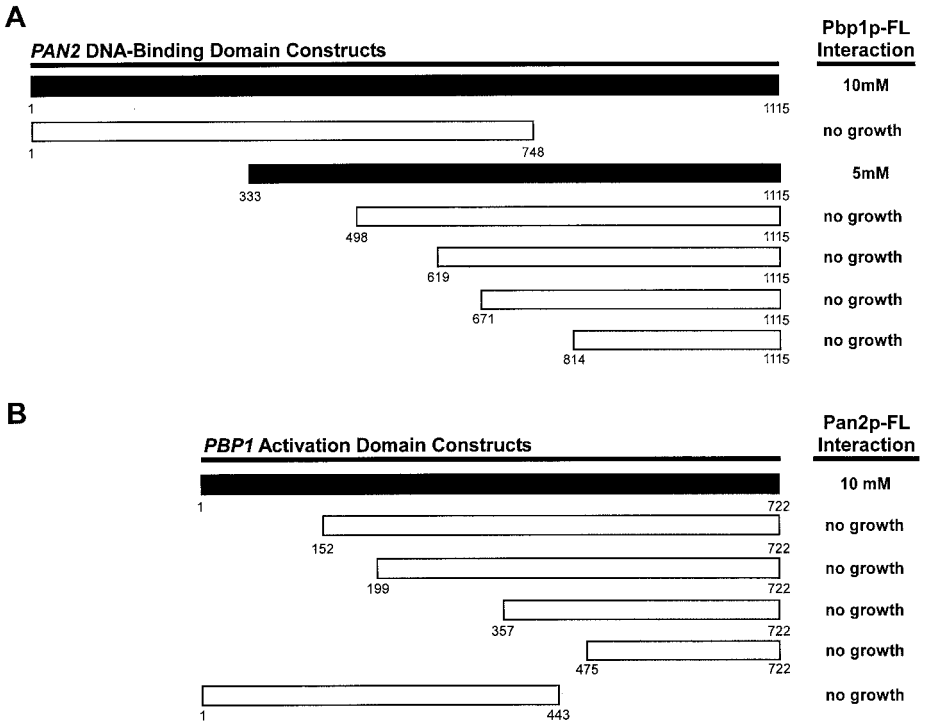

FIG. 2. Two-hybrid mapping of Pan2p-Pbp1p interactions. Two-hybrid analyses are as described in the legend to Fig. 1. (A) Pan2p truncations were tested against Pbp1p-FL to determine the binding domain of Pbp1p on Pan2p. (B) Pbp1p fragments were used to determine the binding domain of Pan2p-FL on Pbp1p.

passing amino acids 560 to 679. Interestingly, a *PAN3* N-terminal truncation that retained amino acids 250 to 679 also produced high-level 3-AT resistance, while the *PAN3* (337 to 679) and *PAN3* (420 to 679) segments were unable to activate transcription and supported growth only on plates lacking histidine (Fig. 1B). The inactivity of the last two fragments is probably attributable to protein misfolding, sequestration of the interaction domain, and/or protein instability, but we have not tested these hypotheses.

Identification of Pbp1p-Pan2p interaction domains. Pbp1p functions as a negative regulator of PAN (see the introduction), and directed two-hybrid analyses were conducted to determine whether this regulation might be manifested as a consequence of Pbp1p interaction with Pan2p or Pan3p. While we observed no *PAN3-PBP1* interactions (data not shown), full-length *PAN2* did interact with full-length *PBP1* in a two-hybrid assay, producing resistance up to 10 mM 3-AT (Fig. 2). Full-length Pan2p did not, however, show an interaction with the Pbp1p (357 to 722) fragment previously shown to interact with Pab1p (23). Pan2p (333 to 1115), a Pan2p N-terminal truncation fragment, also interacted with full-length Pbp1p, although at a reduced level (Fig. 2A). All other Pan2p fragments showed no interaction with either Pbp1p allele (Fig. 2A and data not shown). Pbp1p fragments lacking the N or C terminus of the protein were tested against full-length Pan2p but produced no significant interactions (Fig. 2B). Therefore, only full-length Pbp1p appears to interact with Pan2p in the two-hybrid system. While the interactions found were relatively weak, all were significantly above background levels. The weakness of this interaction, and the similarity of its sequence constraints to those observed for Pan3p interaction with Pan2p (Fig. 1A), suggest that it may be bridged.

Pab1p interacts with Pan3p. Cell-free polyadenylation reactions utilizing Pab1p lacking its C-terminal 137 amino acids produce poly(A) tails that exceed normal lengths (8). Figure 3 shows that the loss of poly(A) length control engendered by Pab1p C-terminal truncation is also manifested in vivo. Since the effects of Pab1p truncation on poly(A) length are similar to those caused by *pan* mutations (8), and since PAN activity requires Pab1p (7, 21), we considered the possibility that interactions between the Pab1p C terminus and PAN served a regulatory function. Pab1p-Pan2p interactions could not be tested in the two-hybrid system because both proteins were only functional as *lexA* DNA-binding domain fusions (data not shown; 23). Consequently, only Pab1p-Pan3p interactions were tested. Directed two-hybrid analyses demonstrated that *PAB1* (406 to 577), a *PAB1* C-terminal segment including both the spacer and helical domains, interacted with full-length *PAN3* and conferred resistance up to 60 mM 3-AT (Fig. 4A). Truncations of this fragment, isolating either the proline- and methionine-rich region or the helical domain, eliminated Pab1p-Pan3p interaction (Fig. 4A). Similarly, any truncation of *PAN3* tested against the entire C-terminal region of *PAB1* produced cells capable of growing on histidine-deficient medium but not on medium containing any 3-AT (Fig. 4B). Collectively, these experiments indicate that the entire Pan3p protein is required for interaction with Pab1p.

To further delineate the Pan3p-binding domain on Pab1p, two *PAB1* C-terminal constructs containing point mutations originally shown to eliminate interaction with Pbp1p were

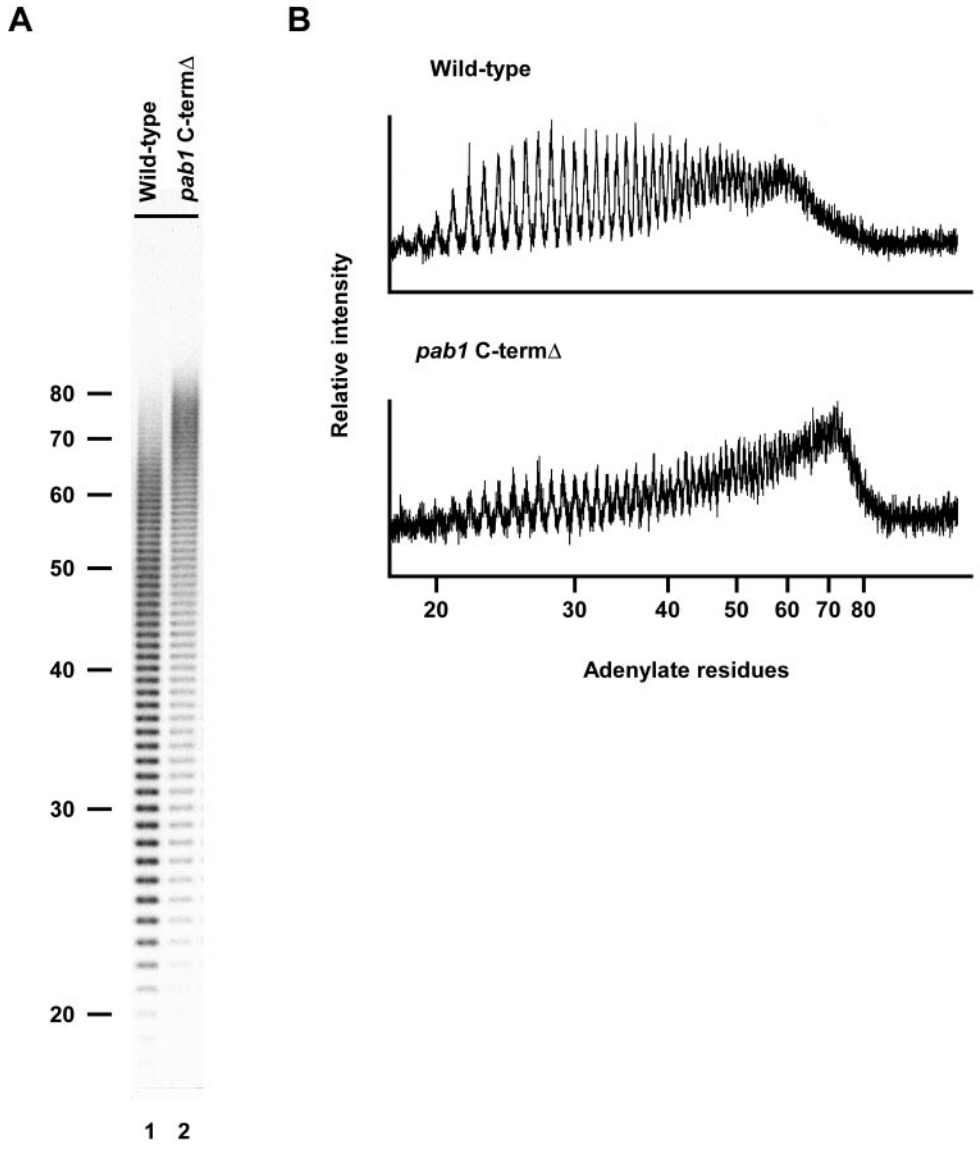

FIG. 3. Truncation of the Pab1p C terminus affects poly(A) length control in vivo. mRNA poly(A) tail lengths were determined in wild-type or *pab1* strains bearing a carboxy-terminal truncation (C-termΔ). The resulting autoradiograph (A) was analyzed by densitometric tracing (B). Numbers of adenylate residues were determined by comparison with a DNA sequence ladder.

tested for interaction with full-length Pan3p (23). The first construct contained a single mutation at codon 444, changing a G to a D. This mutation, located within the Pab1p proline- and methionine-rich segment, did not affect interaction with Pan3p since strains harboring this construct still produced resistance up to 60 mM 3-AT (Fig. 4A). The second construct contained two point mutations, one at codon 451, changing a V to an A, and a second at codon 514, changing a Y to a C. Two-hybrid reporter strains containing this construct were unable to grow on media lacking histidine, indicating that one or both of the two altered amino acid residues are critical for binding of Pab1p to Pan3p (Fig. 4A). To determine their individual effects, each mutation was independently created in *PAB1* (406 to 577). While the *pab1* 451 (V→A) substitution had no effect, the *pab1* 514 (Y→C) mutant exhibited reduced interaction with Pan3p (Table 2 and data not shown).

Identification of Pan3p interaction mutations within the *PAB1* (406 to 577) segment. To better understand the interaction between Pab1p and Pan3p, additional point mutants were generated in *PAB1* (406 to 577). The mutants were screened for a loss of interaction with full-length Pan3p, while maintaining interaction with Pbp1p. A total of four alleles were isolated in this screen: *pab1* 528 (G→D), *pab1* 481 (V→A)/506 (R→G), *pab1* 497 (N→D)/533 (M→T), and *pab1* 510 (G→R)/519 (A→V). These alleles were further manipulated to isolate the pertinent individual point mutants by using site-directed mutagenesis of the wild-type *lexA*(DB)-*PAB1* (406 to 577) construct to make each mutation. The selection of alleles to be isolated was dictated by the apparent requirement of the four Pab1p carboxy-terminal helices for interaction with Pan3p. This hypothesis stems from several observations, including those demonstrating that (i) the initially isolated mutant that

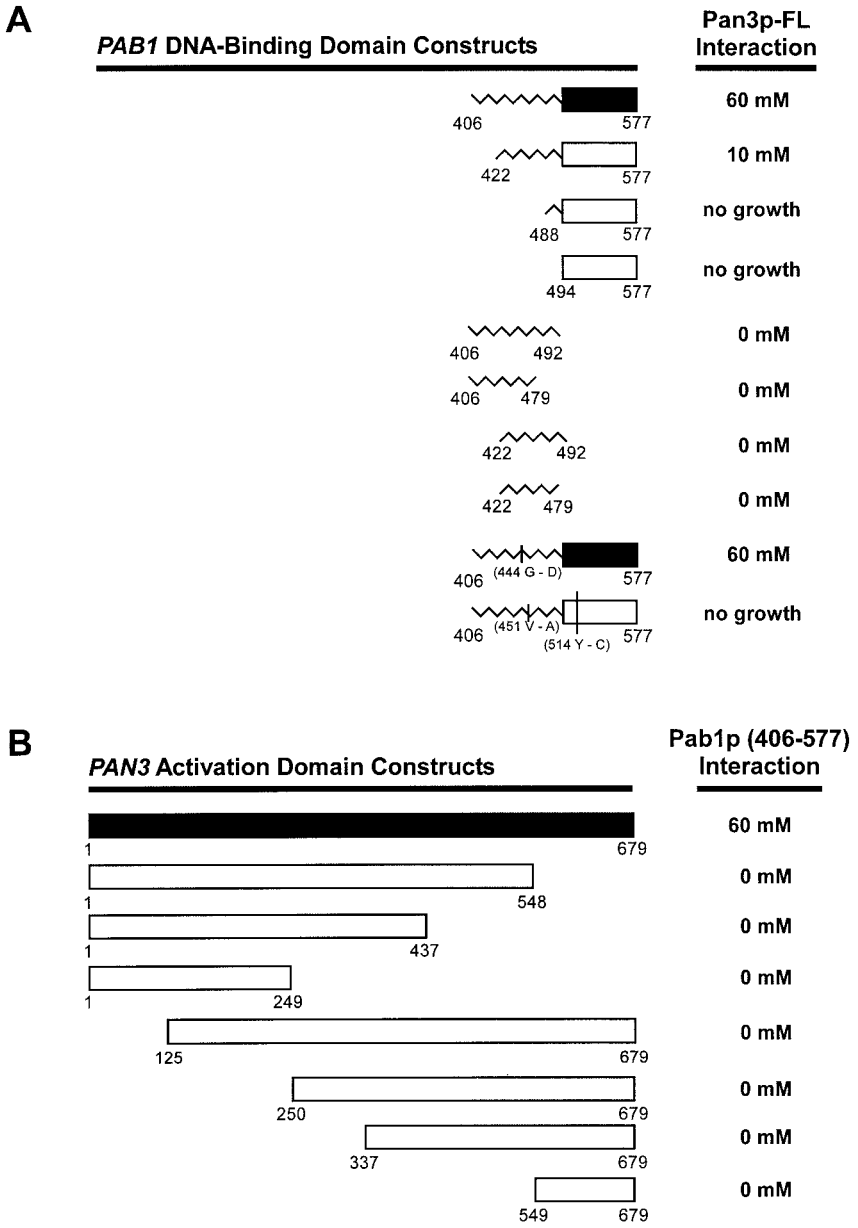

FIG. 4. Two-hybrid mapping of Pab1p-Pan3p interactions. Two-hybrid analyses are as described in the legend to Fig. 1. (A) Pab1p C-terminal fragments were tested with Pan3p-FL to determine the binding site of Pan3p on Pab1p. (B) Pan3p fragments were used to determine the location of Pab1p binding on Pan3p.

decreases interaction with Pan3p [*pab1* 514 (Y→C)] bears an amino acid change in helix 2, (ii) all of the new *pab1* alleles which fail to interact with Pan3p bear mutations in helix 2 or 3, and (iii) the sole single point mutant isolated, *pab1* 528 (G→D), is located within helix 3.

The set of individual mutants was then cotransformed and tested for interaction with empty vector, *PBP1* (357 to 722), or *PAN3*-FL. The *pab1* 506 (R→G), *pab1* 510 (G→R), *pab1* 528 (G→D), and *pab1* 533 (M→T) alleles all maintained wild-type interaction with *PBP1* (357 to 722) and eliminated interaction with *PAN3*-FL (Table 2). These results support the hypothesis that the binding site for Pan3p is located within the C-terminal helices of Pab1p. Interestingly, the *pab1* 519 (A→V) mutant, which is located between helices 2 and 3, interacted with Pan3p at wild-type levels (data not shown).

This collection of mutants provides strong support for a model in which Pan3p and Pbp1p have separate binding domains on Pab1p. The mutations affecting Pan3p binding were located in the four helices, while the one mutation affecting Pbp1p binding (24) was located in the N-terminal portion of the proline- and methionine-rich region (Fig. 5A). Examination of the point mutations in the context of the nuclear magnetic resonance (NMR) structure of Pab1p's carboxy-terminal helices showed that three of the five mutations (506, 510, and 514) were clustered on the same side of helix 2 (Fig. 5B). The identification of mutations at residues 528 and 533 of helix 3

TABLE 2. Single amino acid changes in Pab1p eliminate Pan3p interactions

PAB1 allele	3-AT resistance (mM) upon interaction with Pan3p-FL
PAB1 WT[a]	60
pab1 506 (R→G)	0
pab1 510 (G→R)	0
pab1 514 (Y→C)	20
pab1 528 (G→D)	0
pab1 533 (M→T)	0

[a] WT, wild type. Empty vector and *PBP1* (357 to 722) produced 0 and 100 mM 3-AT resistance, respectively, for all alleles.

further defines a pocket into which Pan3p can bind Pab1p (Fig. 5B).

Pab1p alleles that fail to interact with Pan3p have long poly(A) tails. As noted above, *pab1* mutations cause a significant increase in the average steady-state poly(A) tail length of total cellular mRNA (32), a defect thought to be at least partially attributable to the inability of the mutant Pab1p proteins to stimulate PAN activity (7, 9, 21). To determine if the loss of Pan3p binding to Pab1p resulted in similar defects in poly(A) tail maturation, Pan3p-noninteracting *pab1* alleles were introduced into full-length *PAB1* constructs and poly(A) tail lengths associated with total cellular mRNA were analyzed. When compared with wild-type strains, *pan3*Δ strains accumulate mRNAs with very long poly(A) tails (9) (Fig. 6A to C, compare lanes 1 and 2). Similarly, each of the *pab1* alleles deficient in Pan3p binding also increased poly(A) tail lengths to almost the same extent observed in the *pan3*Δ strain. This observation provides strong evidence that Pab1p plays a significant role in poly(A) tail maturation by, at a minimum, localizing PAN to the 3′ end of the mRNA.

Identification of Pab1p-Pbp1p interaction domains. Our previous mapping studies indicated that Pbp1p-Pab1p interaction required the Pab1p C-terminal segment, including the proline- and methionine-rich span and the helices (23). Recent NMR data indicates that several factors, including the ataxins and translation termination factor eRF3, specifically interact with the five C-terminal helices of metazoan poly(A)-binding protein (20). Since yeast *PBP1* shows some sequence homology with the human gene *SCA2*, encoding the factor for human spinocerabellar ataxia type II, it has been speculated that Pbp1p would interact in a similar fashion. To evaluate this hypothesis, several new *PAB1* fragments were created and tested against *PBP1* (357 to 722). Surprisingly, most trunca-

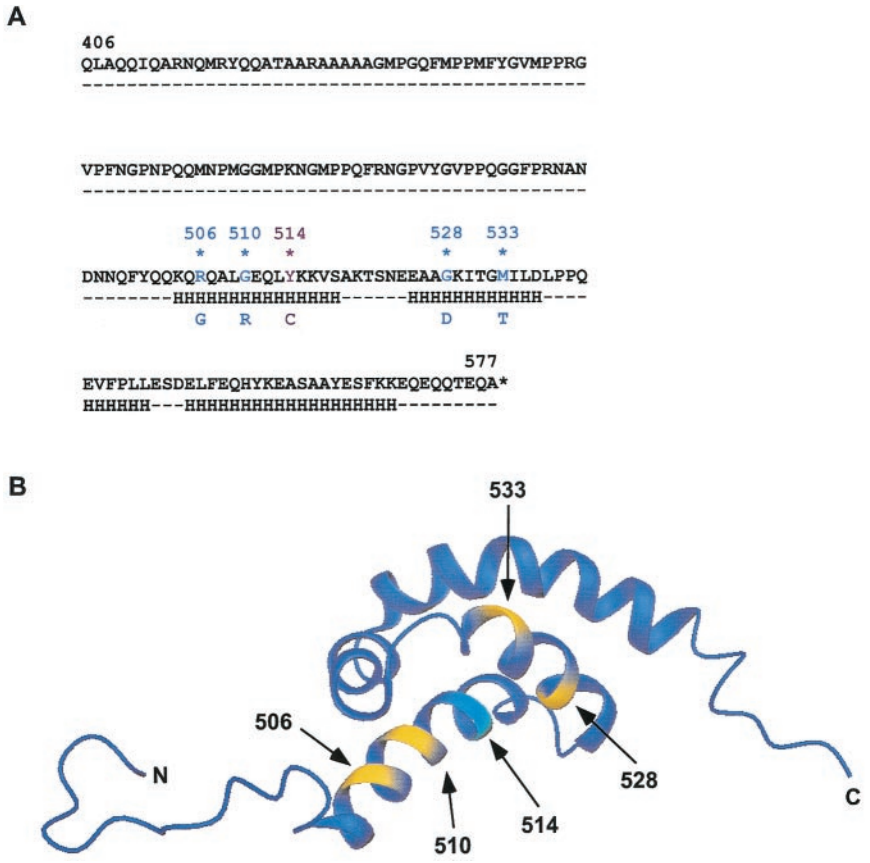

FIG. 5. Pab1p mutations affecting interaction with Pan3p. (A) Location in the primary structure of Pab1p of point mutants that alter Pan3p binding to Pab1p. Mutations eliminating Pan3p binding are colored blue, while the mutant with a partial phenotype is colored violet. (B) Location of *pab1* point mutations within the NMR structure of the C terminus of Pab1p. The NMR structure of the Pab1p C-terminal helices is from Protein Data Bank file 1IFW and the model was generated by DINO (29). Mutations eliminating Pan3p binding are colored yellow, while the mutant with a partial phenotype is colored light blue.

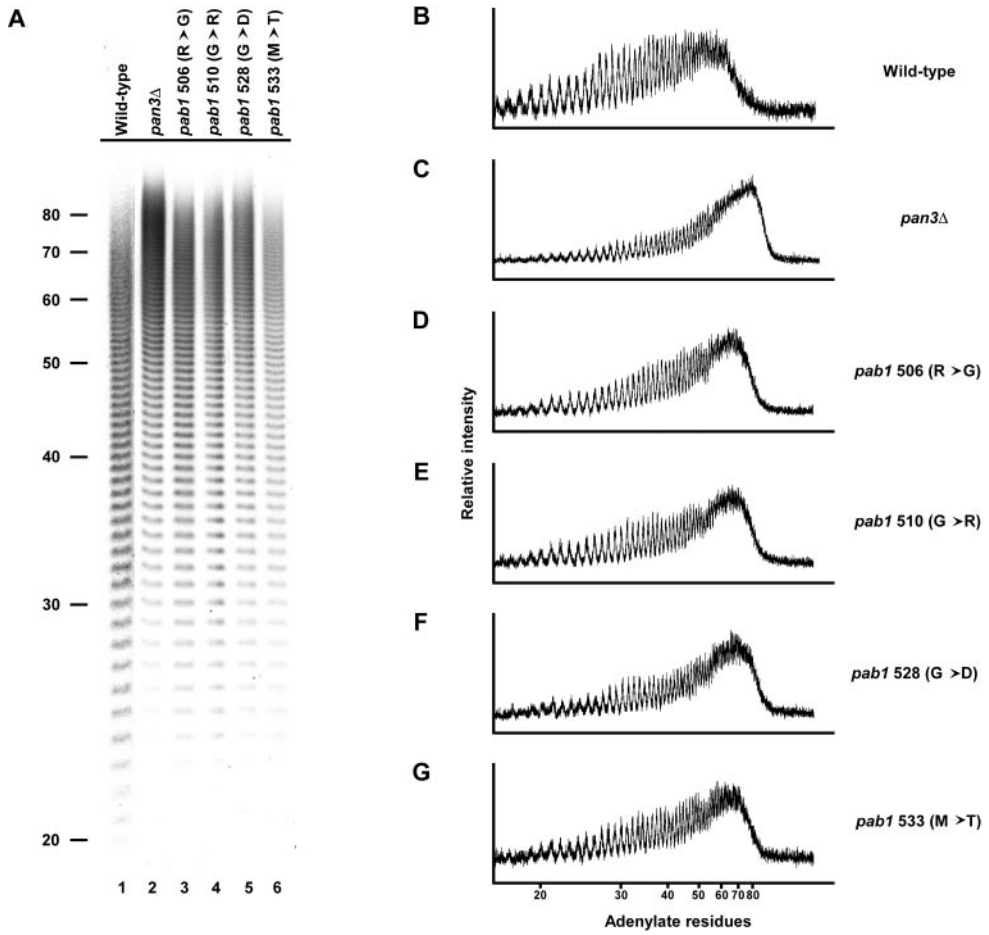

FIG. 6. *pab1* mutations affecting Pan3p interaction promote loss of poly(A) length control. RNA was isolated from wild-type (B), *pan3*Δ (C), *pab1* 506 (R→G) (D), *pab1* 510 (G→R) (E), *pab1* 528 (G→D) (F), and *pab1* 533 (M→T) (G) strains, and mRNA poly(A) tail lengths were analyzed by gel electrophoresis and densitometric tracing of the resulting autoradiographs (A). Numbers of adenylate residues were determined by comparison with an RNA ladder.

tions of the *PAB1* C terminus did not affect interaction strength with Pbp1p. However, truncations removing the proline- and methionine-rich region significantly reduced or eliminated 3-AT resistance (Fig. 7A). We conclude that a majority of the Pab1p proline- and methionine-rich region, between amino acids 406 and 479, is required for interaction with Pbp1p, thus casting doubt on the observed homology of *PBP1* and *SCA2*.

Conversely, we sought to determine if we could further refine our mapping of the Pab1p-binding site on Pbp1p. Pbp1p was originally identified in a two-hybrid screen that used *lexA*(DB)-*PAB1* (406 to 577) as bait (23). That study demonstrated that N-terminal truncations up to Pbp1p amino acid 357 had no effect on interaction. Here, we sought to determine if additional N-terminal truncation could be achieved and whether truncations from the C terminus were also possible. These experiments showed that (i) a *PBP1* fragment including amino acids 421 to 722 was still able to produce 3-AT resistance up to 80 mM, (ii) further truncation of the N terminus markedly reduced and, in some cases, eliminated interaction, and (iii) any deletion of the C terminus eliminated interaction (Fig. 7B). These data indicate that the Pab1p-binding activity of Pbp1p requires residues 357 to 722 to produce a full-strength interaction.

Pan3p interacts with itself. In an effort to better understand the regulatory mechanisms controlling PAN activity, we supplemented our analyses of Pan3p-Pab1p and Pan3p-Pan2p interactions with analyses of Pan3p self-interaction. In part, this aspect of our analysis was prompted by an initial observation that *PAN3*-FL fused to both *lexA*(DB) and *GAL4*(AD) promoted growth on plates containing up to 80 mM 3-AT, indicating a very strong interaction (Fig. 8A). Experiments with *PAN3* fragments demonstrated that those expressing the C-terminal two-thirds of the protein promoted self interaction. *PAN3* (250 to 679) expressed from both vectors promoted cell growth up to 60 mM 3-AT. The same construct tested with *PAN3*-FL (both orientations) yielded resistance up to 80 mM 3-AT (Fig. 8A). *PAN3* (549 to 679) expressed with itself showed growth on 40 to 60 mM 3-AT. However, cotransformation of this fragment with empty vector controls indicated that these levels were only slightly above background. Self-interaction tests of constructs lacking the C terminus, such as *PAN3* (250 to 437) and *PAN3* (250 to 548), failed to promote activation of transcription to levels above that of the empty

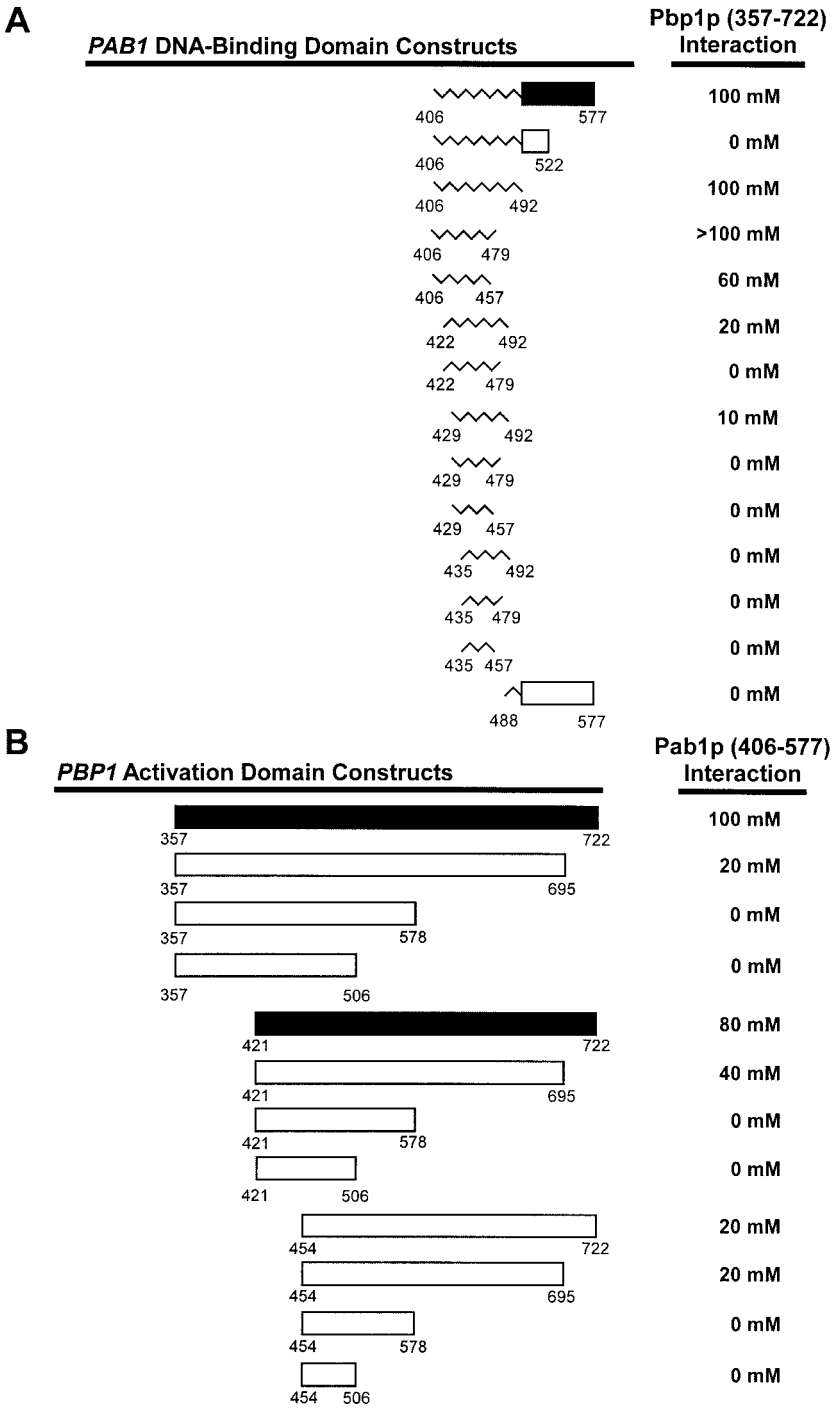

FIG. 7. Two-hybrid mapping of Pab1p-Pbp1p interactions. Two-hybrid analyses are as described in the legend to Fig. 1. (A) Pab1p fragments were tested against Pbp1p (357 to 722) to further map the Pbp1p-binding site on Pab1p. (B) C-terminal Pbp1p fragments were used to map the location of Pab1p binding on Pbp1p.

vector controls, further confirming the necessity of the carboxy terminus in multimerization (Fig. 8A). Together, these results suggest that the minimal domain for Pan3p multimerization resides between amino acids 250 and 679.

Pbp1p interacts with itself. Previous studies have shown that Pbp1p can also multimerize (23). To map the multimerization domain of this protein, we performed an analysis similar to that used for Pan3p. *PBP1* (199 to 722) was able to produce an interaction with full-length protein to levels yielding greater than 80 mM 3-AT resistance (Fig. 8B). This same fragment was also able to interact with itself, yielding resistance up to 60 mM 3-AT. A larger fragment, *PBP1* (152 to 722), was also found to interact with full-length *PBP1*, but that allele did not interact with itself (Fig. 8B). Interestingly, the full-length protein did

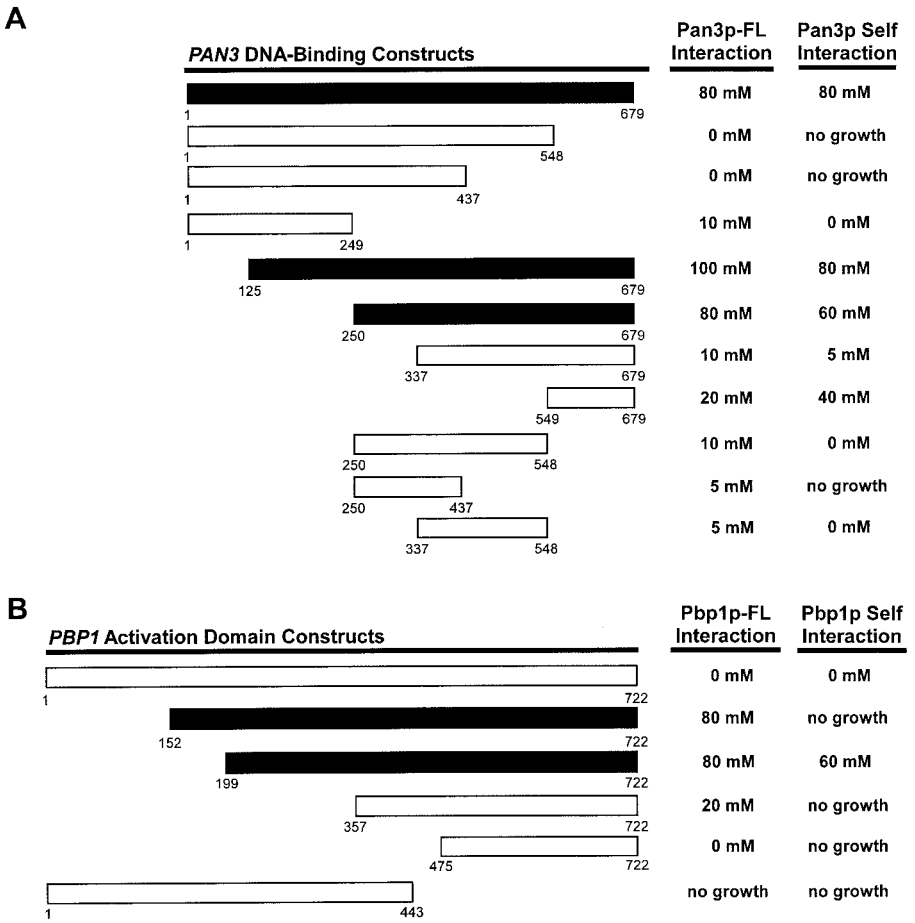

FIG. 8. Mapping Pan3p and Pbp1p self-interactions. Two-hybrid analyses are as described in the legend to Fig. 1. (A) Pan3p fragments were tested against full-length Pan3p and themselves to map the domain(s) necessary for self-interaction. (B) Pbp1p fragments were tested against full-length Pbp1p and themselves to map the domain(s) necessary for self-interaction.

not demonstrate an ability to interact with itself, suggesting that its N terminus may fold in such a way as to regulate and prevent multimerization.

DISCUSSION

Eukaryotic mRNAs are subject to considerable posttranscriptional modification, including capping, splicing, and polyadenylation. The process of polyadenylation adds a 3′-poly(A) tail and provides mRNAs with a binding site for a major class of regulatory factors, the poly(A)-binding proteins. These highly conserved polypeptides bind poly(A) by using one or more RRMs and then act as *cis*-acting effectors of specific steps in the polyadenylation, export, translation, and turnover of the transcripts to which they are bound. They provide a scaffold for the binding of factors that mediate these steps and also act as apparent antagonists to the binding of factors that enable the terminal steps of mRNA degradation. Collectively, these sequential nuclear and cytoplasmic contributions control mRNA function (24).

Previous experiments have suggested two possible roles for the major yeast poly(A)-binding protein, Pab1p, in the nuclear maturation of mRNA. Pab1p appears to have an indirect effect on the transition of poly(A) polymerase (Pap1p) from processive to distributive activity and a direct effect on the poly(A) tail-trimming activity of PAN (9, 43). The latter role of Pab1p, and the protein-protein interactions which underlie that role, have been addressed in this study. By using two-hybrid analysis, we have demonstrated that Pab1p interacts with Pan3p and Pbp1p, two proteins that respectively appear to act as positive and negative regulators of PAN activity (9, 23). Not surprisingly, our experiments have also shown that these two proteins also interact with the Pan2p subunit of PAN, i.e., the subunit whose homologies to previously characterized exonucleases (8, 26) suggest that it harbors the catalytic activity relevant to PAN function. Figure 9 summarizes this set of interactions and raises interesting possibilities for the precise role of Pab1p in poly(A) trimming. At a minimum, Pab1p provides a site through which PAN and its negative regulator, Pbp1p, gain access to the newly formed poly(A) tract. Additionally, as has been proposed for Pab1p interactions with translation initiation factors (6, 22), Pab1p may alter the abilities of Pan3p and/or Pbp1p to bind to additional factors, e.g., binding to Pab1p may enhance the ability of Pan3p to bind to, and/or activate, Pan2p. Additional analyses have shown that Pab1p-Pan3p and Pab1p-Pbp1p two-hybrid interactions still occur in *pan2*Δ or *pan3*Δ strains and are thus not dependent on the integrity of PAN (data not shown). It should be noted, how-

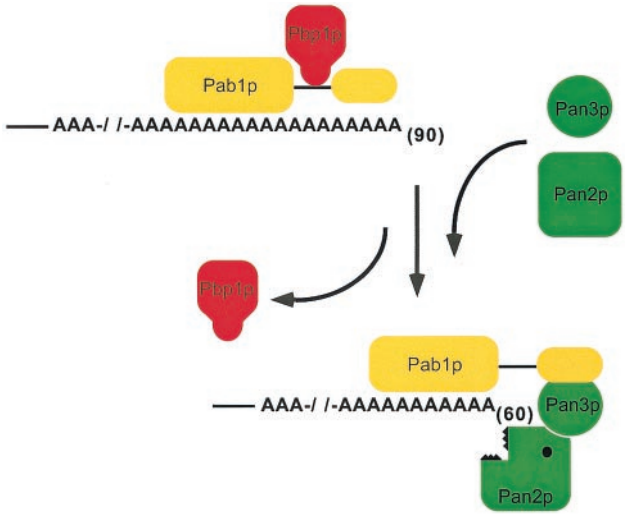

FIG. 9. Maturation of mRNA poly(A) tails is regulated by Pbp1p and Pan3p: a model. Pbp1p is shown associating with the proline- and methionine-rich segment of Pab1p, preventing trimming of the poly(A) tail until addition of approximately 90 adenylate residues is complete. Subsequently, Pan3p interacts with the Pab1p C-terminal helices, while Pbp1p is displaced. Binding of PAN may occur by initial recruitment of only Pan3p or by simultaneous recruitment of the Pan2p/Pan3p holoenzyme. Pan2p exonuclease activity is activated upon recruitment to the mRNA, and an average of 30 adenylate residues are removed from the poly(A) tail in an mRNA-specific manner. A bridged interaction between Pbp1p and Pan2p (see Discussion) could occur if dissociation of Pbp1p from Pab1p does not occur prior to PAN binding.

ever, that some of the interactions portrayed in Fig. 9 as direct have only been inferred from one experimental approach and may thus be bridged by other factors in the poly(A) maturation complex (including yet to be identified factors not shown in the figure). In this regard, two observations suggest that the Pbp1p-Pan2p interaction may be bridged: (i) the extent of 3-AT resistance observed for the relevant constructs is weak and (ii) the Pan3p- and Pbp1p-binding sites on Pan2p are identical (see Fig. 1A and 2A).

Pab1p's role as a possible stimulator (via Pan3p) or inhibitor (via Pbp1p) of PAN activity could be simplified if its interactions with the respective factors were mutually exclusive. However, our data demonstrate that, although Pan3p and Pbp1p both bind to the C-terminal domain of Pab1p, their respective binding sites differ. Pbp1p's binding site is principally localized to the proline- and methionine-rich domain, whereas Pan3p's binding site is concentrated within the C-terminal helices (Fig. 4, 5, and 7 and Table 2). The separation of these sites is underscored by experiments showing that mutations eliminating interaction with one of the two factors do not necessarily eliminate interaction with the other (Table 2 and reference 24). It is important to note, however, that the existence of apparently independent binding sites for Pan3p and Pbp1p does not guarantee that both proteins can bind Pab1p simultaneously. Regardless of the dynamics of Pan3p and Pbp1p binding to Pab1p, the localization of the Pan3p-binding site to the Pab1p C terminus provides an explanation for the extended poly(A) tails observed in *pab1* C-terminal truncation mutants (Fig. 3 and 6) (8).

The two-hybrid method utilized in this study was able to define a set of protein-protein interactions governing PAN activity. Some interactions were shown to require full-length or near-full-length fusion proteins (e.g., Pan2p-Pbp1p), whereas others could be narrowed to very limited domains (e.g., Pab1p-Pan3p). The resolving power of the technique is in part a reflection of the fact that not all proteins can fold and function as fusions with either *lexA*(DB) or *GAL4*(AD), i.e., some fusions yield proteins that are misfolded and/or unstable. Nevertheless, the value of this approach is underscored by the identification of point mutations that corroborate the importance of polypeptide interactions otherwise defined by only two-hybrid methodology and by the observation that specific protein fragments which failed to interact with one protein often were capable of interacting with another. In addition to defining heteromeric Pab1p-Pan3p, Pab1p-Pbp1p, Pbp1p-Pan2p, and Pan3p-Pan2p interactions, our analyses have also shown that the two regulatory factors, Pan3p and Pbp1p, can interact homomerically (Fig. 8). Multimer formation by regulatory proteins has considerable precedent, as does the opportunity for switching active and inactive forms as a consequence of additional, heteromeric interactions (13). As noted above, whether Pab1p serves such a regulatory role for Pan3p and Pbp1p remains to be determined.

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