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PAN3 Encodes a Subunit of the Pab1p-Dependent Poly(A) Nuclease in *Saccharomyces cerevisiae*

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The Pab1p-dependent poly(A) nuclease (PAN) from *Saccharomyces cerevisiae* copurifies with polypeptides of approximately 127 and 76 kDa. Previously, it was demonstrated that the 127-kDa Pan2 protein is required for PAN activity (R. Boeck, S. Tarun, M. Reiger, J. Deardorff, S. Müller-Auer, and A. B. Sachs, J. Biol. Chem. 271:432–438, 1996). Here we demonstrate that the 76-kDa protein, encoded by the nonessential *PAN3* gene, is also required for enzymatic activity. Deletion of *PAN3* resulted in the loss of PAN activity in yeast extracts, and immunodepletion of Pan3p from purified PAN fractions abolished enzymatic activity. We show by coimmunoprecipitation and directed two-hybrid studies that the Pan2 and Pan3 proteins physically interact. In addition, we demonstrate that a deletion of *PAN2*, *PAN3*, or both resulted in similar increases in mRNA poly(A) tail lengths in vivo. These data strongly suggest that both Pan2p and Pan3p are required subunits of the PAN enzyme and that PAN functions in vivo to shorten mRNA poly(A) tails.

When the poly(A) tail is added posttranscriptionally to the 3′ end of a pre-mRNA, it is synthesized to a homogeneous length that is organism specific, ranging from approximately 70 to 90 nucleotides in yeast cells to 200 to 250 nucleotides in mammalian cells (29). Following transport of the mRNA to the cytoplasm, the poly(A) tail is progressively shortened, resulting in tail lengths that are heterogeneous and that tend to decrease with increasing age of the mRNA. Increases in poly(A) tail lengths, as a result of cytoplasmic adenylation of mRNA, have also been reported during early development (38, 52), while the existence of cytoplasmic adenylation in somatic cells has not yet been firmly established. Thus, the length of the poly(A) tail is not static but changes throughout the life span of an mRNA.

The dynamic nature of the poly(A) tail appears to be important in modulating poly(A) tail function. One function of the poly(A) tail is to influence mRNA translational efficiency (reviewed in reference 29). For example, during early development in many species, regulated adenylation usually correlates with translational activation whereas deadenylation correlates with translational repression (reviewed in references 38 and 52; also see references 42 to 44 and 50). In addition, synthetic polyadenylated mRNAs are translated more efficiently than their unadenylated counterparts when added to in vitro translation extracts (28, 36), injected into *Xenopus* oocytes (19), or electroporated into plant, animal or yeast cells (20, 21). Characterization of the yeast poly(A)-binding protein (Pab1p), a highly conserved protein that binds to the mRNA poly(A) tail, demonstrates that Pab1p is required for translation initiation (40). In vitro studies have demonstrated that the Pab1p-poly(A) complex can stimulate the recruitment of the 40S ribosomal subunit to the mRNA in yeast extracts (49).

Poly(A) tails also influence mRNA stability (reviewed in references 4 and 39). The removal of the poly(A) tail can be the first step in the degradation of mRNA (see, for example, references 13, 33, 34, 45, and 53). Studies examining a synchronous pool of newly synthesized mRNAs in *Saccharomyces cerevisiae* have elucidated a deadenylation-dependent pathway for mRNA turnover, whereby mRNA decay is initiated by poly(A) tail removal followed by decapping and finally by rapid degradation of the mRNA (13, 33, 34). Why is poly(A) tail removal a prerequisite for subsequent steps in mRNA decay? It appears that the poly(A) tail inhibits mRNA decapping through the activity of Pab1p (10). Thus, it is believed that when poly(A) tails are shortened to an oligo(A) length incapable of binding Pab1p, the mRNA is decapped and degradation ensues.

Relatively little is known about the proteins involved in poly(A) tail shortening. Thus far, only two deadenylases, one from *S. cerevisiae* (see below) and one from mammalian cells (2, 3), have been purified and analyzed biochemically. Characterization of the deadenylation reaction in vivo suggests that tail removal is highly regulated. Poly(A) tail shortening rates are mRNA specific and can vary at least 10- to 20-fold in *S. cerevisiae* (13, 26) and 50- to 100-fold in mammalian cells (32, 45). Differential rates of deadenylation are often regulated by *cis*-acting sequences within the mRNA (9, 35, 46). Poly(A) shortening rates have at least two kinetically distinct phases: an initial slow phase followed by a more rapid phase (14, 45). Studies with *S. cerevisiae* suggest that Pab1p may function in both phases (10).

To further investigate the deadenylation reaction, a poly(A)-specific RNase (PAN) has been purified from *S. cerevisiae* (7, 41). The yeast PAN does not degrade poly(A) unless it is bound by the poly(A)-binding protein. Biochemical characterization of the PAN enzyme demonstrates that it is a 3′-to-5′ exonuclease which releases AMP as a product and requires magnesium for activity (30). PAN appears to have high affinity for both RNA and Pab1p, since it remains bound to poly(U)-Sepharose and Pab1p-Sepharose resins in high (>1 M) ionic strengths (7, 41). The characterization of PAN activity in vitro demonstrates that it can be regulated by *cis*-acting sequences within an RNA. For example, while PAN normally removes poly(A) in a distributive manner, certain mRNA sequences can cause its activity to become processive (30).

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TABLE 1. Yeast strains used in this study

Strain	Genotype
YAS306	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*
YAS307	*MAT*α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*
YAS308	*MAT***a**/*MAT*α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*
YAS1760	*MAT***a** *URA3*:*GAL*(*UAS*)-*lacZ LYS2*:*GAL*(*UAS*)-*HIS3 gal4 gal80 ade2-101 his3 leu2-3,112 trp1-901 ura3-52 cyh1* (Y190 [24])
YAS1924	YAS1760 with p*DNA-PAN2* (pAS468), p*PAN3-ACT* (pAS469)
YAS1925	YAS1760 with p*DNA-SNF1*, p*SNF4-ACT*
YAS1926	YAS1760 with p*DNA-PAN2* (pAS468), p*ACT*
YAS1927	YAS1760 with p*DNA-SNF1*, p*PAN3-ACT* (pAS469)
YAS1928	YAS1760 with p*DNA-PAN2* (pAS468), p*SNF4-ACT*
YAS1934	*MAT***a**/*MAT*α *pan3*::*HIS3*/*PAN3 ade2 his3 leu2 trp1 ura3 can1*
YAS1935	*MAT***a** *pan3*::*HIS3 ade2 his3 leu2 trp1 ura3 can1*
YAS1936	*MAT***a** *ade2 his3 leu2 trp1 ura3 can1*
YAS1937	*MAT*α *ade2 his3 leu2 trp1 ura3 can1*
YAS1938	*MAT*α *pan3*::*HIS3 ade2 his3 leu2 trp1 ura3 can1*
YAS1939	*MAT***a** *pan3*::*HIS3 ade2 his3 leu2 trp1 ura3 can1* p*PAN3TRP1CEN* (pAS471)
YAS1940	*MAT***a** *pan3*::*HIS3 ade2 his3 leu2 trp1 ura3 can1* p*PAN3URA3CEN* (pAS472)
YAS1941	*MAT***a**/*MAT*α *pan2*::*LEU2*/*PAN2 pan3*::*HIS3*/*PAN3 ade2 his3 leu2 trp1 ura3 can1*
YAS1942	*MAT***a** *pan2*::*LEU2 ade2 his3 leu2 trp1 ura3 can1*
YAS1943	*MAT***a** *pan3*::*HIS3 ade2 his3 leu2 trp1 ura3 can1*
YAS1944	*MAT*α *pan2*::*LEU2 pan3*::*HIS3 ade2 his3 leu2 trp1 ura3 can1*
YAS1945	*MAT*α *ade2 his3 leu2 trp1 ura3 can1*

Proteins of approximately 135 and 76 kDa copurify with PAN activity in two related purification strategies (7, 41). Microsequencing of the 135-kDa protein region from the first of these purification strategies led to the cloning of the *PAN1* gene (41). Subsequent work, however, showed that *PAN1* is not necessary for PAN enzymatic activity (for further discussion, see reference 7). More recently, a modified large-scale purification of PAN revealed that a 127-kDa protein (previously referred to as 135 kDa [7]) copurifying with PAN activity is encoded by the *PAN2* gene (7). Deletion of *PAN2* demonstrated that it is required for PAN activity but not for cell viability. In this paper, we show that the 76-kDa protein copurifying with PAN is encoded by the *PAN3* gene and is also required for enzymatic activity but not cell viability. Furthermore, we demonstrate a physical interaction between Pan2p and Pan3p. Our results suggest that the PAN enzyme is composed of at least two subunits, Pan2p and Pan3p, and that loss of function of either or both of these subunits results in abrogation of PAN activity.

MATERIALS AND METHODS

Yeast methods. Yeast strains used in this study are listed in Table 1. Cultures were grown at 30°C in either rich medium (YPD), minimal medium (YMD), or synthetic complete medium (SCD), supplemented with nutrients required for auxotrophic deficiencies. Standard methods were used for medium preparation, genetic crosses, sporulation, and tetrad analysis (23). Yeast cells were transformed with DNA by the lithium acetate method (22).

Cloning and disruption of *PAN3*. To identify Pan3p, the 76-kDa region from poly(U)-Sepharose eluates (7) was microsequenced as described previously (41).

To clone the *PAN3* gene, a 2.7-kb fragment (corresponding to nucleotides −335 to 2342 [see Fig. 2]) was amplified by PCR with 10 ng of yeast genomic DNA (27), 0.5 μM (each) primers OAS201 and OAS202 (Table 2), and 2.5 U of *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) in a 100-μl reaction mixture. Thermocycling conditions were as follows: 5-min denaturation at 94°C; 5 cycles of 1-min denaturation at 94°C, 1-min annealing at 45°C, and 3-min extension at 72°C; and 25 cycles under the same conditions except at a 55°C annealing temperature. The PCR product was digested with *Kpn*I and *Xba*I and ligated into the following *Kpn*I-*Xba*I-digested vectors: pBluescript KS+ (Stratagene), pSE358 (*pTRP1CEN4*, pUN15 derivative [17]), and pRS416 (*pURA3CEN6* [47]) to create pAS470, pAS471, and pAS472, respectively.

To construct a *pan3*::*HIS3* deletion, DNA fragments 5′ (primers OAS201 and OAS203 [Table 2]; nucleotides −335 to −4 [see Fig. 2]) and 3′ (primers OAS202 and OAS204 [Table 2]; nucleotides 2028 to 2342 [see Fig. 2]) to the *PAN3* open reading frame (ORF) were amplified by PCR under the reaction conditions described above. The 5′ PCR product was digested with *Kpn*I-*Eco*RI, and the 3′ product was digested with *Eco*RI-*Xba*I; these were then inserted into *Kpn*I-*Xba*I-digested pBluescript KS+ (Stratagene) to create pAS473. A 2.8-kb *HIS3*-Kan^r *Eco*RI-*Xho*I fragment from plasmid pJA9 (a gift from S. Elledge) was then ligated into *Eco*RI-*Xho*I-digested pAS473, yielding pAS474. *S. cerevisiae* strains with the entire *PAN3* open reading frame deleted were created by a single-step disruption (37) whereby the linear 3.0-kb *Bst*EII-*Xmn*I *pan3*::*HIS3* fragment from pAS474 was transformed into YAS308 and a His$^+$ transformant (YAS1934) was selected. Sporulation and dissection of tetrads from YAS1934 yielded haploid strains containing the *pan3*::*HIS3* deletion. Replacement of the *PAN3* gene with *HIS3* was confirmed by restriction enzyme digestion and Southern blot analysis.

PAN activity assay. PAN activity was assayed as described previously (41), except that about 50,000 cpm of homogeneously labeled [α-^{32}P]poly(A)$_{200}$ was used per reaction. The [α-^{32}P]poly(A)$_{200}$ substrate was prepared with yeast poly(A) polymerase under the following reaction conditions: 0.5 μM oligo(A)$_{12}$ (Pharmacia, Alameda, Calif.), 167 μM ATP, and 50 μCi of [α-^{32}P]ATP (3,000 Ci/mmol) in 20 μl of polymerase buffer with 500 U of yeast poly(A) polymerase (U.S. Biochemicals, Cleveland, Ohio). The reaction mixture was incubated at 30°C for 60 min, heat inactivated at 55°C for 10 min, and brought up to 100 μl with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES [pH 7.5]). Unincorporated nucleotide was removed by spin column (S-400) chromatography (Pharmacia).

Recombinant expression of Pan3p and antibody production. The *PAN3* ORF (nucleotides +1 to 2131 [see Fig. 2]) was fused in frame to a derivative of pET-11d (Novagen, Madison, Wis.) that encodes an N-terminal His$_7$ tag (a gift from S. Buratowski). The 2.1-kb *Nco*I-*Xmn*I fragment excised from pAS471 was ligated into pET-11d:His$_7$, yielding pAS475, which directs the expression of His$_7$-tagged Pan3p (the first 15 amino acids are MHHHHHHHHM**AMDKIN** [authentic Pan3p residues are in boldface type]) under the control of the T7 promoter.

A 600-ml culture of *Escherichia coli* BL21(DE3) harboring plasmid pAS475 was grown at 30°C in Luria-Bertani medium supplemented with 50 μg of ampicillin per ml and 500 μg of methicillin per ml to an optical density at 600 nm of 0.4, induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h, and then harvested. Bacterial pellets were weighed, resuspended in 3 ml of sonication buffer (50 mM Tris [pH 8.0], 500 mM NaCl, 0.05% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) per g of cells, and stored as 1-ml aliquots at −70°C.

Since the majority of induced Pan3p is insoluble, Pan3p was purified from *E. coli* inclusion bodies. Per thawed aliquot, phenylmethylsulfonyl fluoride and lysozyme were added to final concentrations of 1 mM and 0.25 mg/ml, respectively, and the suspension was incubated on ice for 20 min. Cells were broken by being sonicated three times for 10 to 15 s, and the lysate was clarified by

TABLE 2. Oligonucleotides

Name	Sequence
OAS186	5′ CCGGAATTCCATGGATAATTGGCAACATTTCTTC (*Eco*RI linker)
OAS187	5′ CCGGAATTCATATTATCCCTTTGAAGTTTC (*Eco*RI linker)
OAS201	5′ GGGGTACCAAATCGAAAGTCGTCCGT (*Kpn*I linker)
OAS202	5′ GCTCTAGAACCTCGCCGTGCACA (*Xba*I linker)
OAS203	5′ GGAATTCTAAGATACGTCTGACTAGTCTTTTAGG (*Eco*RI linker)
OAS204	5′ GGAATTCCTCGAGTAAAACAACACCAAATAATGTAGA (*Eco*RI-*Xho*I linker)

microcentrifugation (14,000 rpm in an Eppendorf 5415 C centrifuge for 15 min at 4°C). The pellets were washed twice by sonication in 1 ml of sonication buffer and subjected to microcentrifugation. Recombinant Pan3p was extracted from the inclusion bodies by rotating pellets end-over-end for 2 h at 4°C in 1 ml of inclusion body (I) buffer (6 M urea in sonication buffer).

Recombinant His-tagged Pan3p was purified by nickel-Sepharose chromatography. A typical purification used 1 ml of resin for a 1-cm-diameter column, and all steps were carried out at room temperature. Solubilized Pan3p in I buffer was loaded onto a ProBond metal-binding resin (Invitrogen, San Diego, Calif.) at a flow rate of 10 column volumes/h. Subsequently, the column was washed with 10 column volumes of I buffer–5 mM imidazole followed by 5 column volumes of I buffer–25 mM imidazole. Pan3p was eluted in 5 column volumes of I buffer–200 mM imidazole and collected in 1-ml aliquots. Peak fractions of purified His-tagged Pan3p were concentrated by ultrafiltration (Centricon-10; Amicon, Beverly, Mass.) to approximately 1 mg/ml.

For Pan3p antibody production, purified recombinant Pan3p was resolved on a 1.5-mm-thick preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel, stained with 0.1% Coomassie R-250 (in double-distilled H_2O), and excised from the gel. Two rabbits were immunized six times over a 6-month period with the Pan3p-embedded gel suspension. The first injection consisted of 500 μg of Pan3p per rabbit; all other injections consisted of 250 μg of Pan3p per rabbit. For Pan2p antibody production, recombinant Pan2p was gel purified from *E. coli* inclusion bodies. Two rabbits were immunized four times over a 2-month period with 250 μg of a Pan2p-embedded gel suspension.

Protein and immunological techniques. Yeast S100 extracts were prepared from 25 ml of cells grown to mid-log phase (optical density at 600 nm, 0.5 to 1.0), harvested by centrifugation, washed once in IP buffer (see below), and resuspended in 200 μl of IP buffer plus 0.2 g of acid-washed glass beads. The cells were lysed by two cycles of 30-s vortexing and 1-min cooling on ice. The lysates were cleared by microcentrifugation (14,000 rpm for 5 min at 4°C) and then by centrifugation once at 30,000 × *g* for 30 min in a TLA 100.3 rotor (Beckman Instruments) and once at 100,000 × *g* for 60 min in a TL-100 ultracentrifuge (Beckman). All protein concentrations were determined by the Bradford dye-binding assay (8) (Bio-Rad, Hercules, Calif.).

Proteins were resolved on 0.75-mm-thick SDS-polyacrylamide gels (7.8% polyacrylamide) unless stated otherwise (31). To detect proteins directly, the gels were stained with either Coomassie blue R-250 (51) or silver (6). Western blot (immunoblot) analysis was performed by procedures described elsewhere (31). Briefly, the gel was electroblotted onto nitrocellulose (Amersham, Arlington Heights, Ill.) in Tris-glycine transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol, 0.05% SDS). The blot was blocked with 5% milk in Tris-buffered saline (TBS)–0.1% Tween 20 (TBS-T) (31) and incubated for 1 h with the primary antibody diluted in 5% milk in TBS-T. Pan2p and Pan3p antibodies were diluted 1:1,000 and 1:5,000, respectively. After the blots were washed with TBS-T, they were incubated for a minimum of 30 min with anti-rabbit horseradish peroxidase-conjugated antibodies (Amersham) diluted 1:5,000 in TBS-T, washed again as described above, and then developed by the enhanced chemiluminescence detection system (Amersham).

For immunoprecipitations studies, either anti-Pan3p or preimmune antibodies were chemically coupled to protein A-Sepharose (Pharmacia) with dimethylpimelimidate (Sigma, St. Louis, Mo.), as described elsewhere (24). In general, 20 μl of serum was incubated with 100 μl of protein A-Sepharose in a final volume of 500 μl for 1 h. Beads were washed twice with 10 volumes of 0.2 M sodium borate (pH 9.0) and resuspended in 10 volumes of 0.2 M sodium borate (pH 9.0), and dimethylpimelimidate solid was added to 20 mM. The coupling reaction was carried out for 30 min (rotating the mixture end-over-end) at room temperature and then stopped by washing beads once in 0.2 M ethanolamine (pH 8.0) and incubating them in 0.2 M ethanolamine (pH 8.0) for 2 h (rotating the mixture end-over-end). Following coupling, antibody-protein A-Sepharose complexes were washed once with phosphate-buffered saline (PBS) (31), twice with 0.1 M glycine (pH 3.0), and twice with PBS, and then stored at 4°C.

Immunoprecipitation studies were carried out at 4°C with IP buffer (50 mM Tris [pH 7.4], 2 mM magnesium acetate, 100 mM potassium acetate, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 7 mM 2-mercaptoethanol, 1 μM pepstatin, 1 μM leupeptin). In general, 20 to 40 μl of antibody-protein A-Sepharose complexes, washed with IP buffer, was mixed with 10 μl of poly(U) eluate (7) in a final volume of 100 μl and rotated end-over-end for 2 h. Supernatants were removed and assayed for PAN activity or analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) with silver staining or Western blot analysis. Pellets were washed three times with IP buffer plus 1 M potassium acetate and three times with PBS and eluted in 2× SDS sample loading buffer. To measure PAN activity or to detect Pan3p by Western blot analysis, 2 μl of IP supernatant was used [0.2 μl of poly(U) equivalent]. To detect PAN by silver staining or Pan2p by Western blot analysis, 50 μl of IP supernatants [5 μl of poly(U) equivalent] was precipitated in 10% trichloroacetic acid, collected by microcentrifugation (14,000 rpm for 20 min at 4°C), washed once with −20°C acetone, and resuspended in 2× SDS sample loading buffer.

Directed two-hybrid assay. Plasmid pAS468 containing the *PAN2* ORF fused to the *GAL4* DNA-binding domain was constructed by inserting the *PAN2* ORF into plasmid pAS455 (pAS2 [25]). The *PAN2* ORF was amplified by PCR with 0.5 μM primers OAS186 and OAS187, 50 ng of YAS306 DNA (27), and 5 U of *Pfu* polymerase in 100 μl as specified by the manufacturer (25 cycles of 1-min denaturation at 94°C, 2-min annealing at 50°C, and 6-min extension at 75°C). The resulting 3.4-kb DNA fragment was treated with T4 DNA polymerase and T4 polynucleotide kinase, digested with *Nco*I, and inserted into *Nco*I-*Sma*I-digested pAS455, yielding pAS468. Plasmid pAS469 containing the *PAN3* ORF fused to the activation domain of *GAL4* was constructed by inserting the *PAN3* ORF into plasmid pAS456 (pACT2 [16]). The *PAN3* ORF was isolated from plasmid pAS470 as a 2.3-kb *Apa*I-*Xba*I fragment, treated with T4 DNA polymerase in the presence of deoxynucleoside triphosphates, and inserted into *Sma*I-digested pAS456. Plasmids containing the *SNF1* ORF fused to the *GAL4* DNA-binding domain and *SNF4* ORF fused to activation domain II of *GAL4* were both kind gifts of S. Elledge and have been described elsewhere (18). All plasmids were transformed into yeast strain YAS1760 (Y190 [25]). Each transformant was tested for growth on SCD medium minus His, Trp, and Leu but in the presence or absence of 75 mM 3-aminotriazole. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) colony filter assays were performed as described by Chevray and Nathans (12).

RNA methods. RNA was prepared by a modified hot-phenol extraction procedure. The equivalent of 20 ml of cells with an optical density at 600 nm of 1 was harvested, washed in HS buffer (20 mM Tris-HCl [pH 8.0], 10 mM EDTA, 300 mM NaCl), and stored at −70°C. Thawed yeast cell pellets were resuspended in 700 μl of HS buffer plus 80 μl of 10% SDS, lysed by the addition of 600 μl of hot phenol (65°C), vortexed for 30 s, and then incubated at 65°C for 2 min. After microcentrifugation (14,000 rpm for 2 min at 4°C), the supernatant was extracted again with hot phenol, once with phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform-isoamyl alcohol (24:1) and precipitated with ethanol. The RNA pellet was washed with 70% ethanol, and the resulting 0.2 to 0.5 mg of RNA was resuspended in 100 μl of double-distilled H_2O.

For poly(A) tail gels, the labeling-reaction mixture consisted of 0.4 μg of yeast RNA, 5 μCi of [α-^{32}P]cytidine 3′,5-bis(phosphate) (pCp), 16.5 U of RNasin (Promega, Madison, Wis.), and 10 U of T4 RNA ligase (New England BioLabs, Beverly, Mass.) in 4 μl of buffer supplied by the manufacturer. After being labeled for 4 h at room temperature or overnight at 4°C, the reaction mixture was diluted to 25 μl in HS buffer and 10 to 15 μl was used for subsequent RNase A digestion. RNase A digestion was carried out in 20 mM Tris-HCl (pH 8.0)–1 mM $MgCl_2$–0.5 M NaCl–1 mg of tRNA per ml–0.1 mg of RNase A per ml in a 100-μl reaction for 30 min at 37°C, followed by extraction with phenol-chloroform-isoamyl alcohol, ethanol precipitation, and washing with 70% ethanol. Precipitated RNA was resuspended in formamide loading buffer (95% formamide, 20 mM EDTA, 0.5% bromophenol blue, 0.5% xylene cyanol), loaded onto a 12% polyacrylamide–7 M urea sequencing gel in 0.5× TBE (31), and electrophoresed at 50 W in 0.5× TBE for 2.5 to 3 h. The gel was exposed directly by autoradiography.

Nucleotide sequence accession number. The *PAN3* sequence has been deposited in GenBank under accession number Z28025.

RESULTS

Identification and characterization of the *PAN3* gene. A large-scale purification of the PAN enzyme from yeast S100 extracts has recently been described (7). For the work reported here, the purified PAN fraction refers to the poly(U)-Sepharose eluate from this purification. A silver-stained SDS-PAGE gel of this purified PAN fraction reveals several polypeptides copurifying with the enzymatic activity (Fig. 1). The 127-kDa protein is encoded by *PAN2*, a gene required for PAN activity (7). The 110-kDa protein has not yet been identified. It has been observed, however, that the levels of p110 diminish upon further purification of the enzyme, suggesting that it may not be required for PAN activity (7).

To identify the gene(s) encoding the 76-kDa protein doublet, this region of the gel from the purified PAN fraction was microsequenced. The sequences of two tryptic fragments (see the underlined sequences in Fig. 2) corresponded to a protein encoded by the putative ORF *YKL025c*, found on chromosome XI of *S. cerevisiae* (15). We have designated this gene *PAN3*, for poly(A) nuclease 3.

The *PAN3* ORF predicts a novel protein of 679 amino acids, with a molecular mass of 76,454 Da (Fig. 2). The size of the Pan3 protein is consistent with the electrophoretic mobility of the purified doublet. Pan3p does not exhibit strong homology to any other protein with a known function by comparison with sequences in the GenBank database when the BLASTP algorithm is used (1). In addition, Pan3p does not have any readily recognizable protein motifs when the PROSITE pattern search program is used. However, the C terminus of the Pan3

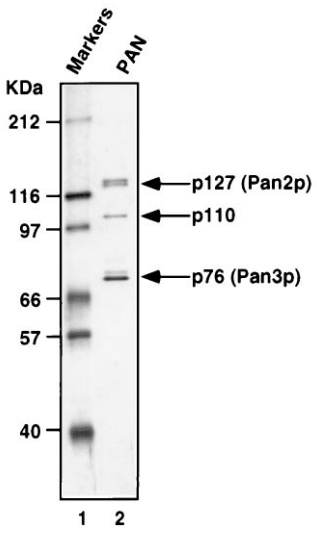

FIG. 1. The highly purified PAN fraction contains proteins of 127 kDa (Pan2p [7]), 110 kDa, and 76 kDa (Pan3p). A silver-stained SDS-PAGE gel is shown. Positions of the molecular mass markers are indicated to the left.

protein is highly homologous to a putative protein, ZK632.7, identified by the *Caenorhabditis elegans* genome-sequencing project (48). Over the last 130 amino acids, the two proteins are approximately 45% identical and 70% similar. This sequence homology may represent a conserved motif within the two proteins or potentially the evolutionary conservation of Pan3p and hence the PAN enzyme.

To determine if *PAN3* is a single-copy gene in haploid *S. cerevisiae*, a Southern blot of yeast genomic DNA digested with either *Eco*RI or *Bgl*II was probed with a 2.7-kb DNA fragment containing the entire *PAN3* gene (Fig. 2, −335 to 2342). This experiment failed to detect any DNA other than *PAN3*, suggesting that *PAN3* is not a duplicated gene (data not shown). A Northern (RNA) blot with the *PAN3* probe revealed a transcript of approximately 2.2 kb, a result which is in agreement with the size of the predicted ORF (data not shown).

To assess whether *PAN3* is required for yeast viability, the entire *PAN3* ORF was replaced with the *HIS3* gene and introduced into a diploid yeast strain (YAS308) by homologous recombination (Fig. 3A). When the *pan3*::*HIS3*/*PAN3* heterozygous diploid yeast cells were sporulated and the tetrads dissected, all four spores were viable (Fig. 3B). The disruption of the *PAN3* gene was confirmed by Southern analysis (Fig. 3C). Thus, it was concluded that *PAN3* is not required for yeast viability. Yeast cells with *PAN3* deleted grew equivalently to isogenic wild-type yeast cells at all temperatures tested (17 to 37°C) (data not shown) and were not sensitive to a variety of different growth conditions, including 3% formamide, alternative carbon sources (3% glycerol–3% ethanol and 2% galactose), a high salt concentration (0.9 M NaCl), heavy metals (1 mM $CuSO_4$), and translational inhibitors (250 ng of cycloheximide per ml and 100 μg of hygromycin B per ml) (data not shown).

To confirm that the *PAN3* mRNA was translated, rabbit polyclonal antibodies were raised against recombinant, histidine-tagged Pan3p that was expressed in *E. coli* and purified by metal affinity chromatography. Western blot analysis with this serum demonstrated that wild-type yeast cells express a protein doublet at approximately 76 kDa. This protein doublet corresponds to Pan3p, since it was absent in strains with *PAN3* deleted (Fig. 4A, lanes 1 and 2). The 63-kDa cross-reacting protein detected in Western blots of yeast extracts (Fig. 4A) is not a breakdown product of Pan3p, since it was also present in extracts prepared from yeast cells with *PAN3* deleted. The antibodies directed against Pan3p were also used to confirm that Pan3p is present in the purified PAN fraction. As demonstrated in Fig. 5A (lane 1), the antibodies directed against Pan3p recognized the 76-kDa protein doublet. The Pan3p doublet in yeast extracts and purified PAN fractions migrates at the same position by SDS-PAGE (data not shown), and the relative ratio between the upper and lower Pan3p bands appears to be similar. It is possible that this doublet represents two forms of Pan3p resulting from a posttranslational modification.

***PAN3* encodes a component of the PAN enzyme that is required for catalytic activity.** Several experimental approaches were taken to investigate the role of Pan3p in PAN function. First, the effect of a *PAN3* deletion on PAN enzymatic activity in yeast S100 extracts was examined. Extracts were prepared from wild-type yeast cells (YAS1937), yeast cells with *PAN3* deleted (YAS1938), or yeast cells with *PAN3* deleted in the genome but expressing the *PAN3* gene on a plasmid (YAS1939 and YAS1940). Western analysis with anti-Pan3p serum confirmed the presence or absence of Pan3p in these extracts (Fig. 4A). PAN activity was assayed by adding radiolabeled poly(A) with or without recombinant Pab1p to the S100 extracts. Since PAN releases AMP as a product (30), the Pab1p-stimulated degradation of poly(A) can be measured by quantifying the amount of released (trichloroacetic acid-soluble) [α-^{32}P]AMP. Assays were performed in the linear range for PAN enzymatic activity, and each result presented represents the average nuclease activity measured in three or more extracts. As shown in Fig. 4B, poly(A) degradation was stimulated in wild-type extracts by the addition of Pab1p. However, extracts of yeast cells harboring a *PAN3* deficiency did not show this stimulation. Restoration of greater than 85% of the Pab1p-stimulated PAN activity in Pan3P-deficient *S. cerevisiae* was achieved by expressing the cloned *PAN3* gene on a centromere-based plasmid. These results are consistent with the hypothesis that Pan3p is required for PAN activity.

To further address the role of Pan3p in PAN function, immunoprecipitation studies were performed. Anti-Pan3p and preimmune antibodies were chemically cross-linked to protein A-Sepharose beads and incubated with the purified PAN fraction. As shown in Fig. 5A, the Pan3p antibodies efficiently depleted Pan3p from this fraction (Figure 5A, lanes 2 and 3) whereas preimmune antibodies did not (lane 4 and 5). To determine the effect of depleting Pan3p on PAN enzyme activity, the supernatants of the immunodepleted fractions were tested for Pab1p-stimulated PAN activity (Fig. 5B). As expected, addition of Pab1p greatly stimulated the degradation of poly(A) in the purified PAN fraction. The supernatant that was immunodepleted of Pan3p lacked PAN activity. In contrast, the supernatant from the preimmune immunodepletion retained approximately 85% of PAN activity. Immunodepletion of Pan3p from more crude yeast extracts, such as S100 extracts, also abolished PAN activity (data not shown). Taken together, these experiments demonstrate that the *PAN3* gene product is a component of the PAN enzyme.

The Pan3 and Pan2 proteins physically interact. Since both Pan2 and Pan3 are required for PAN activity and both copurify with the PAN enzyme, it was of interest to determine whether these two proteins are physically associated. In the first experiment to address this question, coimmunoprecipitation studies were performed. Antibodies in the α-Pan3p or the preimmune serum were cross-linked to protein A-Sepharose beads and then incubated with the purified PAN fraction. Proteins bound to the antibodies and those remaining in the supernatant were

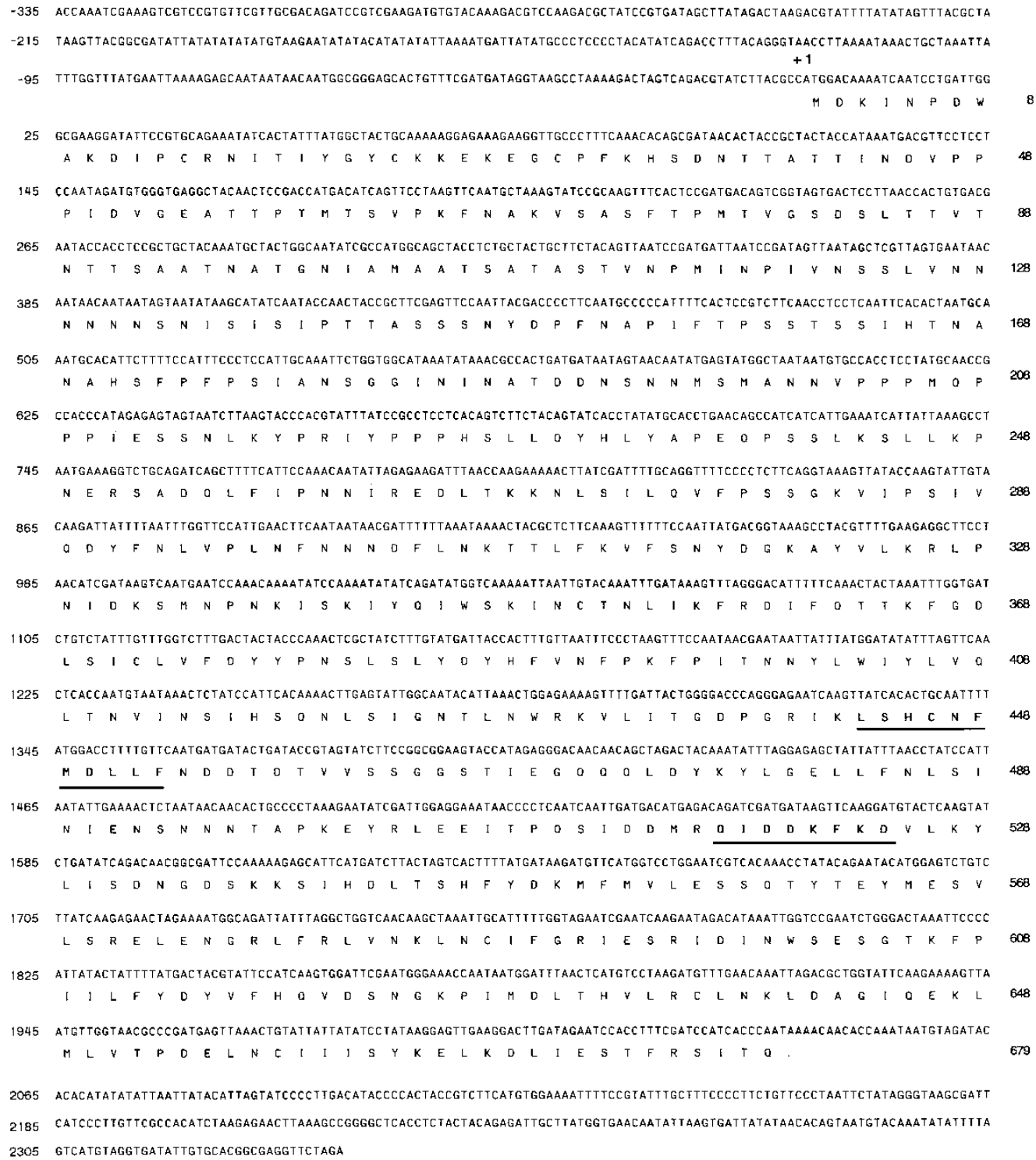

FIG. 2. Nucleotide and predicted amino acid sequences of the *PAN3* gene. The nucleotide sequence is numbered on the left. +1 is the first nucleotide of the *PAN3* ORF. The deduced amino acid sequence is numbered on the right. The two peptides microsequenced from the 76-kDa region of the purified PAN fraction are underlined.

resolved by SDS-PAGE and visualized by silver staining (Fig. 6A). Note that immunoglobulin G from the protein A beads interferes with Pan3p detection by silver staining (Fig. 6A, lane 3), but Western blot analysis confirmed its presence (Fig. 6B). α-Pan3p but not preimmune antibodies coprecipitate the 127-kDa Pan2 protein (Fig. 6A, lanes 3 and 4). An α-Pan2p Western blot with immune (Fig. 6C) but not preimmune (data not shown) sera of these samples demonstrated that the 127-kDa protein corresponds to Pan2p. The 127-kDa region of the purified PAN fraction contains a protein doublet (Fig. 1).

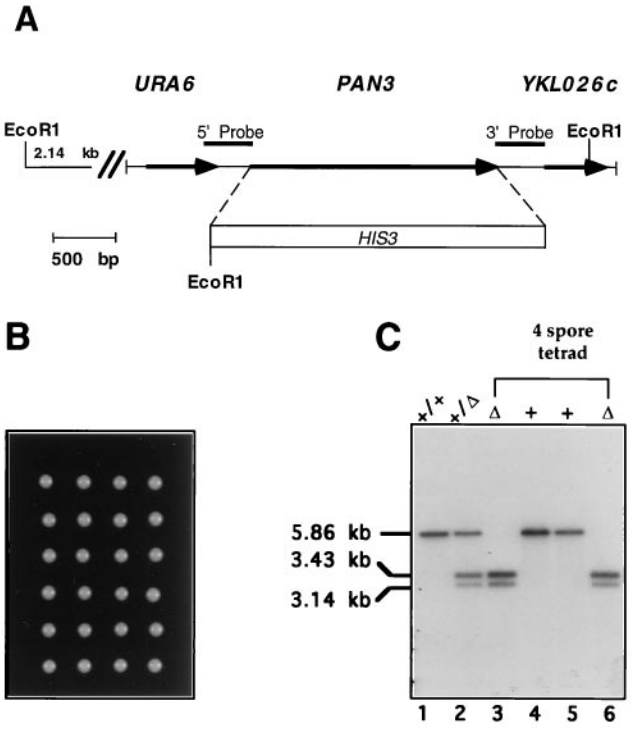

FIG. 3. *PAN3* is not essential for yeast viability. (A) Schematic of the *PAN3* genomic locus and gene replacement with the *HIS3* gene. The diagram indicates the relative positions of the *Eco*RI restriction sites and the positions of the *PAN3* probes. (B) Tetrad analysis of *pan3*::*HIS3*/*PAN3* diploid yeast strain YAS1934. (C) Southern blot analysis confirms the disruption of the *PAN3* gene. A Southern blot of yeast genomic DNA digested with *Eco*RI was probed with radiolabeled probes 5′ and 3′ to the *PAN3* gene (see above). Lanes: 1, wild-type diploid yeast strain (YAS308); 2, diploid yeast strain heterozygous for the *pan3*::*HIS3* disruption (YAS1934); 3 to 6, four-spore tetrad derived from YAS1934 (YAS1935 to YAS1938). The 5.86-kb band represents the wild-type *PAN3* locus. The 3.43- and 3.14-kb bands represent the replacement of *PAN3* with the *HIS3* gene.

When the 127-kDa doublet is better resolved by SDS-PAGE, the Pan2p antibodies recognize both of these polypeptides (data not shown). The interaction between Pan2p and Pan3p is presumably of high affinity, because immune complexes washed with 1 M potassium acetate and/or 1 M guanidine HCl still contained both proteins. The p110 protein is not coimmunoprecipitated with α-Pan3p antibodies (Fig. 6A, lanes 5 and 6). Likewise, Western blot analysis demonstrated that Pan1p is also not coimmunoprecipitated with α-Pan3p antibodies (data not shown). From the coimmunoprecipitation experiments, we conclude that Pan2p and Pan3p interact.

As an alternative strategy to determine if Pan2p and Pan3p physically interact, a directed two-hybrid approach was used. This approach allows the detection of protein interactions via the reconstitution of a functional transcriptional activator in *S. cerevisiae* (18). The entire *PAN2* ORF was fused to the DNA-binding domain of *GAL4* (DNA-PAN2) whereas the entire *PAN3* ORF was fused to the *GAL4* activation domain (PAN3-ACT) by using vectors described by Harper et al. (25). Plasmids were transformed into a yeast strain (YAS1760) which carries two chromosomally located reporter genes regulated by the *GAL1-10* promoter: the *E. coli lacZ* gene and the yeast *HIS3* gene. Transcriptional activation, and therefore physical interaction of the fusion proteins, can be measured by histidine prototrophy in the presence of 3-AT, as well as by an X-Gal colony filter assay.

When either DNA-PAN2 or PAN3-ACT was transformed into YAS1760, no His^+ prototrophy in the presence of 75 mM 3-AT or blue colonies could be observed (data not shown). However, simultaneous transformation of both plasmids into YAS1760 yielded a strain (YAS1924) that could grow in the absence of histidine but with 75 mM 3-AT (Fig. 7), and which strongly induced the *lacZ* gene as judged by its dark blue color in the presence of the indicator X-Gal (Fig. 7). Cotransformation of DNA-PAN2 with only the activation domain (YAS1926) failed to activate the reporter gene expression. To further confirm that the observed activation of the *GAL1-10*

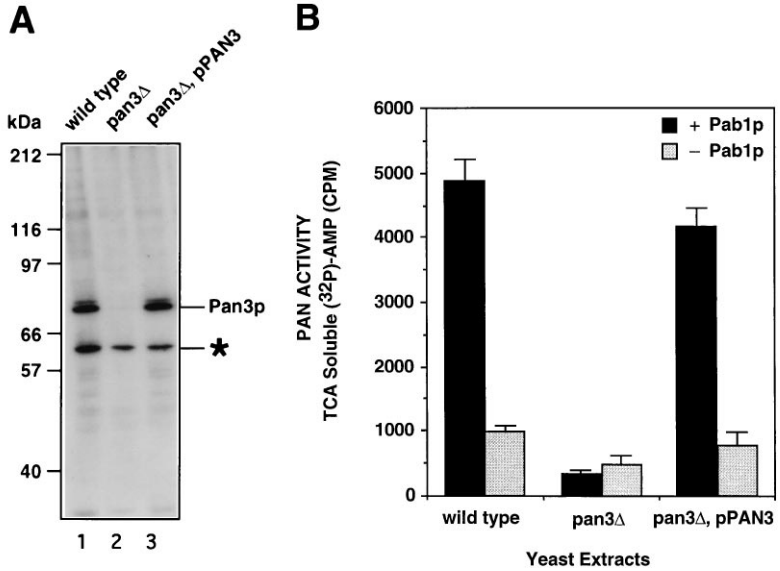

FIG. 4. *PAN3* is required for PAN activity in yeast extracts. (A) Western blot analysis of Pan3p in yeast S100 extracts (10 μg of protein). Lanes: 1, wild-type yeast strain (YAS1937); 2, *PAN3*-deficient yeast strain (YAS1938); 3, strain YAS1938 harboring *PAN3* on a centromere-based plasmid (YAS1939). The positions of Pan3p and a cross-reacting polypeptide (asterisk) are indicated. (B) Pab1p-stimulated PAN activity in the yeast S100 extracts described above. Radiolabeled poly(A), plus or minus recombinant Pab1p, was incubated with yeast extracts (3.5 μg of total protein) for 40 min at 30°C (see Materials and Methods). Nuclease activity was measured by quantifying the release of trichloroacetic acid-soluble [^{32}P]AMP.

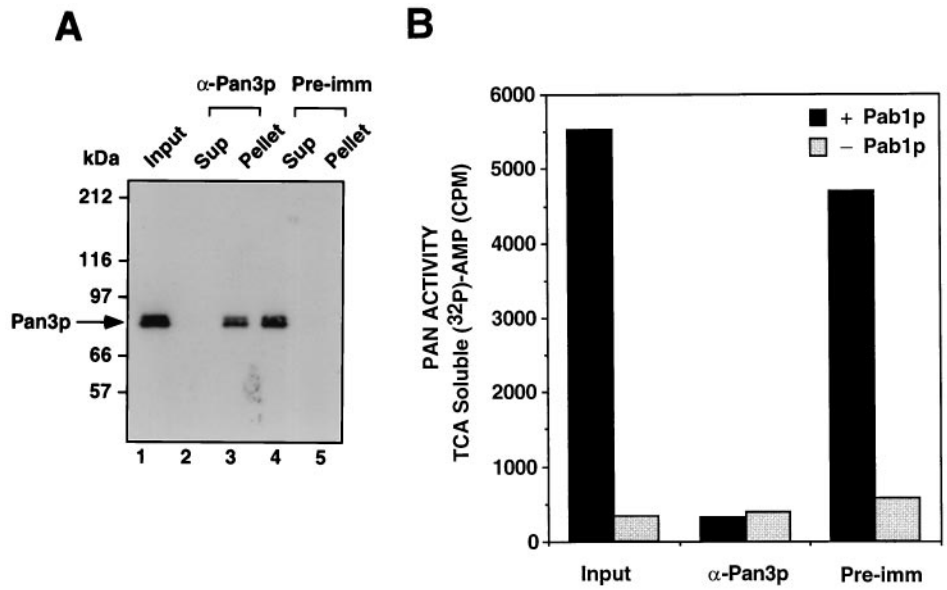

FIG. 5. Immunodepletion of Pan3p abolishes PAN activity. (A) Western blot analysis with α-Pan3p antiserum confirms that Pan3p is immunodepleted from the purified PAN fraction. Lanes: 1, input purified PAN fraction; 2 and 4, supernatant fractions (Sup) following immunodepletion with Pan3p or preimmune antibodies, respectively; 3 and 5, immunoprecipitate fractions (pellet) with either Pan3p or preimmune antibodies, respectively. The positions of the molecular mass markers are indicated to the left. (B) Pab1p-stimulated PAN activity for the same input and supernatant fractions described in panel A. Radiolabeled poly(A), plus or minus recombinant Pab1p, was incubated with supernatant fractions for 20 min at 30°C (see Materials and Methods). Nuclease activity was measured by quantifying the release of trichloroacetic acid-soluble [^{32}P]AMP.

promoter was due to a specific interaction between Pan2p and Pan3p, both fusion proteins were coexpressed in the presence of nonrelated proteins fused to the DNA-binding domain (DNA-SNF1) or activation domain (SNF4-ACT) of *GAL4*. Cotransformation of yeast cells with DNA-PAN2 and SNF4-ACT (YAS1928) or with DNA-SNF1 and PAN3-ACT (YAS1927) did not result in any observable activation of the *GAL1-10* promoter. On the other hand, cotransformation of DNA-SNF1 and SNF4-ACT (YAS1925), which are known to physically interact (11), did result in growth on plates without His but with 75 mM 3-AT, as well as light blue coloring in the presence of X-Gal. Together with the above immunoprecipitation studies, these data suggest that Pan2p and Pan3p are physically associated.

mRNA poly(A) tail lengths are increased in *PAN2*- and *PAN3*-deficient *S. cerevisiae* strains. Given that Pan2p and Pan3p

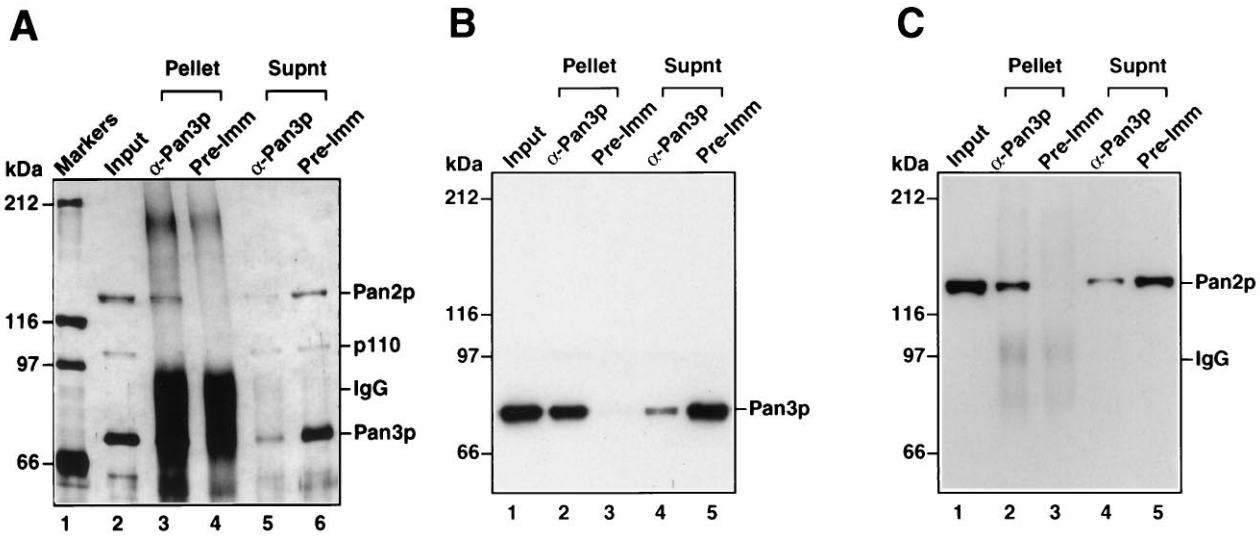

FIG. 6. Coimmunoprecipitation of Pan2p and Pan3p. Immobilized anti-Pan3p or preimmune antibodies were incubated with the purified PAN fraction. (A) Supernatant and coprecipitating proteins were resolved by SDS-PAGE and visualized by silver staining. Lanes: 1, protein markers with corresponding molecular masses indicated to the left; 2, input purified PAN fraction (Input); 3 and 4, immunoprecipitates (Pellet) with either anti-Pan3p or preimmune antibodies, respectively; 5 and 6, supernatants (Supnt) following immunodepletion with either anti-Pan3p or preimmune antibodies, respectively. The locations of Pan2p, Pan3p, and p110 are indicated. (B) Western blot of immunoprecipitated fractions described for panel A with Pan3p antibodies. (C) Western blot of immunoprecipitated fractions described for panel A with Pan2p antibodies.

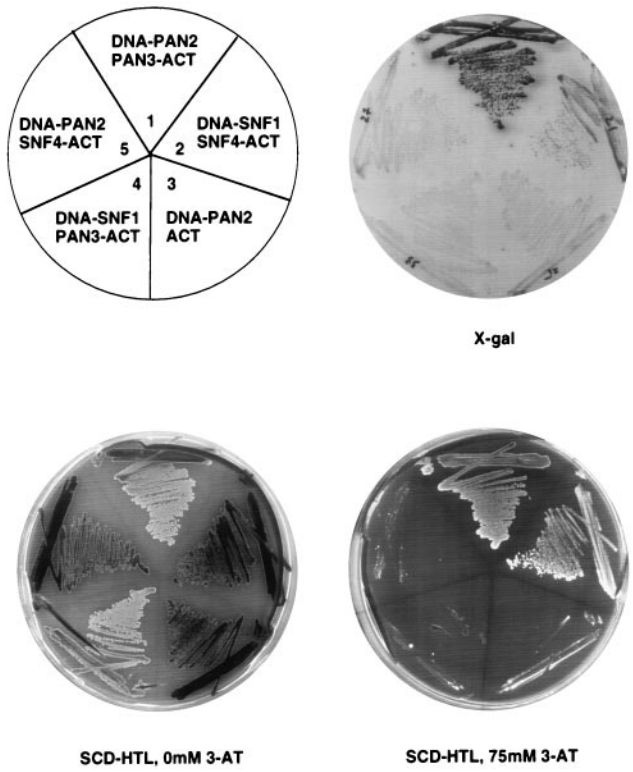

FIG. 7. Pan2p and Pan3p interact by a directed two-hybrid assay. Yeast strain YAS1760 was transformed with plasmids containing the Gal4p DNA-binding domain (DNA-X) in combination with plasmids expressing the Gal4p activation domain (Y-ACT) fused to different proteins (1 to 5 = YAS1924 to YAS1928). Each strain was grown on SCD −His, −Trp, −Leu (SCD-HTL) plates in the presence or absence of 75 mM 3-AT. The yeast strains growing on SCD-HTL without 3-AT were transferred to a nitrocellulose membrane and tested for β-galactosidase activity in the presence of X-Gal.

are required for PAN activity and appear to physically interact, it was of interest to analyze the potential phenotypes associated with a *pan2 pan3* double deletion (*pan2*Δ *pan3*Δ). Haploid *pan2*::*LEU2* yeast cells were mated to haploid *pan3*::*HIS3* yeast cells, and the resulting diploid strain (YAS1941) was sporulated. Tetrad analysis demonstrated that a *pan2*Δ *pan3*Δ mutant was viable and grew equivalently to either a single-deletion mutant or a wild-type yeast strain between 17 and 37°C (data not shown). In addition, *pan2*Δ *pan3*Δ strains were not sensitive to the growth conditions tested for *pan3*Δ strains (see above).

To address whether deletions in both *PAN2* and *PAN3* exacerbated the PAN-specific deadenylation defect, in vivo poly(A) tail lengths of total steady-state mRNA were examined. Bulk mRNA poly(A) tail lengths can be analyzed by 3′ end labeling total RNA with [^{32}P]pCp, digesting it with RNase A, and resolving the remaining intact poly(A) tails on polyacrylamide gels. As previously reported, a mutation in *PAN2* (7) led to an increase in average poly(A) tail lengths (Fig. 8, lanes 2 and 5). Similarly, a deletion in *PAN3* also resulted in an increase in the average poly(A) tail lengths (lanes 2 and 4). The changes in poly(A) tail lengths on mRNA in a *pan2*Δ *pan3*Δ strain were comparable to those found in either the *pan2*Δ or *pan3*Δ single mutants (lane 3 versus lanes 4 and 5). Therefore, it appears that the PAN-specific deadenylation defect is not exacerbated in a *pan2*Δ *pan3*Δ mutant.

The distribution of poly(A) tails (20 nucleotides in length or greater) was quantified by phosphoimager analysis, and it was found that approximately 18% of the poly(A) tails are 70 nucleotides in length or greater in PAN-deficient strains. This is in contrast to wild-type strains, in which only 7% of the poly(A) tails are in this length range. The greater percentage of longer poly(A) tails found in a PAN-deficient strains demonstrates that the PAN deadenylase functions in some aspect of poly(A) tail metabolism in vivo. However, whether PAN functions during a poly(A) maturation event or to regulate mRNA stability has not yet been established (see Discussion). In addition, although the average poly(A) tail length increases in PAN-deficient strains, poly(A) tails of all sizes are detected for bulk mRNA. This observation indicates that poly(A) tails can be degraded in the absence of PAN.

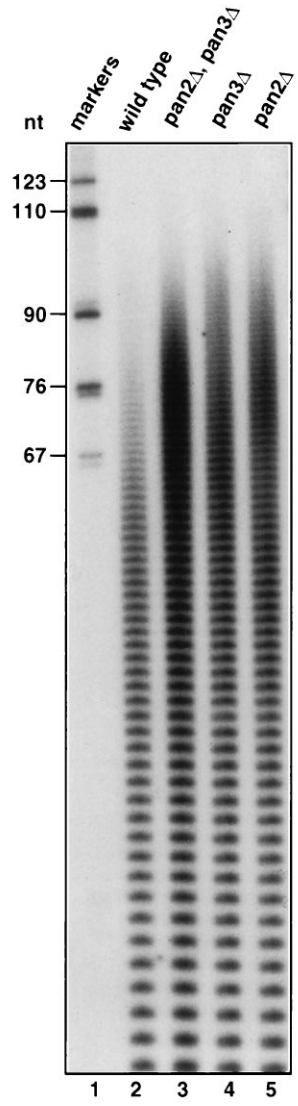

FIG. 8. mRNA poly(A) tail lengths are increased in *PAN2* and *PAN3* mutants. Total RNA was 3′ end labeled with [^{32}P]pCp and digested with RNase A. The remaining intact mRNA poly(A) tails were separated on a 12% polyacrylamide–7 M urea gel and visualized by autoradiography (see Materials and Methods). Lanes: 1, *Msp*I fragments of pBR322; 2, wild-type strain (YAS1945). 3, *pan2*Δ *pan3*Δ yeast strain (YAS1944); 4, *pan3*Δ yeast strain (YAS1943); 5, *pan2*Δ yeast strain (YAS1942).

DISCUSSION

The 127-kDa Pan2 protein was previously reported to be present in highly purified poly(A) nuclease fractions and to be required for enzymatic activity (7). Here we extend our characterization of the yeast PAN and show that the 76-kDa Pan3 protein present in highly purified PAN fractions is also required for enzymatic activity. Moreover, we find through coimmunoprecipitation and two-hybrid interaction studies that the Pan2 and Pan3 proteins physically interact. In addition, yeast cells harboring a *pan2*Δ *pan3*Δ double deficiency were found to have poly(A) tail lengths and growth phenotypes similar to those found in strains with either single deficiency. From these data, we conclude that the *S. cerevisiae* PAN is composed of at least two subunits, Pan2p and Pan3p, and that removing either or both of these subunits from the cell leads to an equivalent poly(A) tail metabolism defect. Our identification of the proteins that make up the yeast PAN should allow a more thorough understanding of the process by which deadenylation occurs and how it is regulated in vivo.

Our data demonstrate that Pan2p and Pan3p are subunits of the PAN enzyme, although they do not rule out the possibility that the enzyme has other subunits. Possible candidates are the Pan1 and p110 proteins. However, neither Pan1p nor p110 seems to be required for enzymatic activity (7), and neither of them coimmunoprecipitates with Pan3p. Further work involving larger-scale purifications of PAN or genetic investigations such as a two-hybrid screen will be useful in identifying other proteins associated with the PAN enzyme. Ultimately, the reconstitution of PAN activity with recombinantly expressed Pan2p and Pan3p will be necessary to determine if these proteins are alone sufficient for enzymatic activity.

Our demonstration of the physical interaction between Pan2p and Pan3p raises many questions about the functions of each protein. For instance, which PAN subunit is responsible for its high-affinity binding to RNA, for interacting with Pab1p, and for performing the nucleolytic reaction? Furthermore, our past demonstration that the normally distributive PAN enzyme can be converted to a highly processive enzyme by specific mRNA sequences (30) raises questions about which of the subunits is responsible for this regulation and how this regulation can occur. Similarly, the finding that the Pan3p subunit appears to exist naturally in at least two resolvable forms on SDS-PAGE indicates the potential for posttranslational regulatory mechanisms impinging on this enzyme.

The yeast PAN is the first deadenylase whose subunits have been cloned and characterized. It is surprising that *PAN2* and *PAN3* are not required for yeast viability and do not result in any detectable growth deficiencies. The most likely reason for the lack of a growth phenotype is that *S. cerevisiae* contains other deadenylases that are redundant with PAN function. This conclusion is supported by work presented here and previously (7), which revealed the presence of shortened poly(A) tails in yeast strains lacking Pab1p-activated PAN activity. Whether these other putative deadenylases are Pab1p stimulated has not yet been established, although no other Pab1p-stimulated nuclease activity is detected in PAN-deficient extracts under our assay conditions.

The possibility that other PANs are poly(A)-binding protein (PAB) independent is supported by several different studies. mRNA poly(A) tails in *S. cerevisiae* are shortened in the absence of Pab1p, albeit less efficiently (10, 40). The overexpression of PAB in *Xenopus* oocytes has been observed to inhibit rather than stimulate the maturation-specific deadenylation process (54). In addition, PAB has been reported to stabilize mRNAs from 3′→5′ exonucleolytic attack in a mammalian in vitro mRNA decay system (5). These observations suggest that other PANs may not require PAB for activity and that under certain conditions, PAB may even inhibit deadenylation.

On what mRNA substrate might PAN be functioning? The longer poly(A) tails detected in vivo for PAN mutants could result from inefficient cytoplasmic deadenylation, which has been shown to consist of at least two kinetically distinguishable phases (10, 45). One of these appears to involve shortening of the poly(A) tail on mRNA soon after it is exported from the nucleus, and the second involves the shortening of the poly(A) tail down to a length that can stimulate mRNA decapping and subsequent mRNA degradation. Pab1p functions in both phases (10), possibly because it is needed to activate PAN. However, because many different phenotypes are associated with a Pab1p deficiency (10, 40), it cannot be determined whether the Pab1p-specific tail phenotypes were due to the loss of PAN activation or to the secondary consequence of disrupting another Pab1p-dependent process. Alternatively, the longer mRNA poly(A) tails appearing in these strains could be due to some other poly(A) metabolism defect, such as aberrant length control for the polyadenylation reaction. Given our production of PAN-deficient yeast cells, it should now be possible to directly address the role of this enzyme in poly(A) tail metabolism. This and future work will lead to a better understanding of how the poly(A) tail shortening reaction is used to control the expression of mRNAs in eukaryotic cells.

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