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Ronald Boeck

Salvador Zipagun Tarun Jr.
SUNY Geneseo

Michael Rieger

Julie A. Deardorff

Silke Muller-Auer

See next page for additional authors

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The Yeast Pan2 Protein Is Required for Poly(A)-binding Protein-stimulated Poly(A)-nuclease Activity*

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Ronald Boeck‡§¶, Salvador Tarun, Jr.‡§, Michael Rieger‖, Julie A. Deardorff‡, Silke Müller-Auer‖, and Alan B. Sachs‡**

From the ‡Department of Molecular and Cell Biology, 401 Barker Hall, University of California at Berkeley, Berkeley, California 94720 and ¶Genotype, Molekularbiologische und Biotechnologische Forschung GmbH, Angelhofweg 39, D-69269 Wilhelmsfeld, Federal Republic of Germany

The removal of the mRNA poly(A) tail in the yeast *Saccharomyces cerevisiae* is stimulated by the poly(A)-binding protein (Pab1p). A large scale purification of the Pab1p-stimulated poly(A) ribonuclease (PAN) identifies a 76-kDa and two 135-Da polypeptides as candidate enzyme subunits. Antibodies against the Pan1p protein, which is the minor 135-kDa protein in the preparation, can immunodeplete Pan1p but not PAN activity. The protein sequence of the major 135-kDa protein, Pan2p, reveals a novel protein that was also found in the previously reported PAN purification (Sachs, A. B., and Deardorff, J. A. (1992) *Cell* 70, 961–973). Deletion of the non-essential *PAN2* gene results in an increase of the average length of mRNA poly(A) tails *in vivo*, and a loss of Pab1p-stimulated PAN activity in crude extracts. These data confirm that Pan2p and not Pan1p is required for PAN activity, and they suggest that ribonucleases other than the Pab1p-stimulated PAN are capable of shortening poly(A) tails *in vivo*.

The poly(A) tail on messenger RNA (mRNA) is utilized in several different cytoplasmic reactions (reviewed in Ref. 2). For instance, the poly(A) tail is needed for efficient translation initiation in eucaryotic cells. Evidence for this involvement comes from *in vivo* studies in *Xenopus laveis* oocytes (for example, see Ref. 3) and from studies on the poly(A)-binding protein (Pab1p) in the yeast *Saccharomyces cerevisiae* (4). The poly(A) tails role in translation has recently been shown to be similar to the cap structures role, in that it stimulates the recruitment of the 40 S ribosomal subunit to the mRNA.[1]

The poly(A) tail is also used as a target for the mRNA degradation system (see Refs. 6 and 7, for reviews). The destruction of the poly(A) tail is an early step in the degradation pathway for many mRNAs. Subsequent to this deadenylation step in yeast, mRNAs can become substrates for the decapping enzyme (reviewed in Ref. 6), thereby making them accessible to the potent 5′ to 3′ exonuclease Xrn1p activity in the cell (8). The rate of an mRNAs deadenylation, as well as the efficiency of coupling between deadenylation and decapping, are determined by mRNA sequences within the body of the message.

In order to more thoroughly understand the process by which deadenylation occurs and how it is regulated by mRNA sequences, the purification of poly(A)-specific ribonucleases (PAN)[2] has been undertaken. In yeast, a PAN was identified based on its requirement for the poly(A) binding protein (Pab1p) for activity (1). This activity requires magnesium ions and releases 5′-AMP as a product. Yeast PAN substrate specificity is determined by the binding specificity of Pab1p, since non-poly(A) sequences bound by Pab1p are adequate substrates (9). Yeast PAN has been shown to be subject to mRNA specific regulation since its normally distributive mechanism is converted to a processive one when challenged with poly(A) attached to particular mRNAs sequences (9). In mammalian cells, a poly(A)-specific ribonuclease has been partially purified (10). This activity also requires magnesium and releases 5′-AMP as a product, but it does not appear to require an RNA binding protein for its function (11).

Previously we reported that the purification of yeast PAN required almost a 100,000-fold enrichment to positively identify proteins consistently co-purifying with the nuclease activity (1). In the most pure fraction, proteins of 135, 76, and 50 kDa sizes were visualized by silver staining. The p135 protein was chosen as the most likely candidate for containing PAN activity since it consistently co-purified with the activity. Protein microsequencing from four peptides derived from a slightly less pure p135 preparation was performed. These peptide sequences were used to clone the *PAN1* gene, which was determined to encode a protein in the 135-kDa preparation since three of the four peptide sequences were found in its predicted open reading frame. A genetic and biochemical analysis of the essential *PAN1* gene revealed that non-lethal mutations within it had mild effects on poly(A) tail metabolism, and that lethal mutations within it led to a rapid cessation of translation initiation (1). Although the toxicity of Pan1p in bacteria precluded its overexpression and absolute confirmation that it encoded a nuclease gene, the similarities of its associated loss of function phenotypes with those for loss of function mutations in the *PAB1* gene (4) were deemed sufficient evidence to conclude that it was indeed part of the enzyme.

Here we report that antibodies against a recombinant fragment of Pan1p could immunodeplete Pan1p from a new large scale preparation of PAN without depleting the PAN activity. The microsequencing of the other 135-kDa protein in the new preparation, Pan2p, and the subsequent mutagenesis of its

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§ These authors contributed equally to this work.

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** To whom correspondence should be addressed. Tel.: 510-643-7698; Fax: 510-643-5035; E-mail: alan@mendel.berkeley.edu.

[1] Tarun, S., and Sachs, A. B. (1995) *Genes & Dev.* **9,** 2997–3007.

[2] The abbreviations used are: PAN, poly(A)-specific ribonuclease; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); ORF, open reading frame; GdnHCl, guanidine hydrochloride; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

gene revealed it was not essential for cell viability but was essential for PAN activity. These data suggest that Pan2p and not Pan1p is an integral part of the Pab1p-stimulated PAN in yeast, and that ribonucleases other than this PAN are capable of destroying poly(A) tails *in vivo*.

MATERIALS AND METHODS

Large Scale Purification of PAN Activity from Yeast—1.2 kg of yeast cells (A_{595} = 1) harvested from a 200-liter culture of YPD (12) were washed 2 times in 1 liter of buffer A (50 mM Tris, pH 7.4, 2 mM MgAc, 14 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol), resuspended in 100 ml of the same buffer containing 100 mM KAc to a cell concentration of about 1 g/ml, and quick frozen by being dripped into a pool of liquid N_2. 250-g aliquots of frozen cell pellets were lysed in a metal blender (Fisher) filled with liquid N_2 by blending continuously for 8–10 min. Efficiency of lysis was checked by thawing a lysed aliquot and viewing under the light microscope. The resulting lysed cell powder was stored at −70 °C and thawed as needed.

All subsequent steps were performed at 4 °C. 500 g of frozen cell paste were slowly thawed, and then clarified for 30 min at 27,000 × *g* in a GSA rotor. This S27 was then clarified at 100,000 × *g* for 60 min in a 45TI rotor. This S100 was placed in a 500-ml beaker, and solid $AmSO_4$ was added to 60% final concentration with slow stirring over 30 min. The pH was adjusted to 7.4 with 0.8 M KOH. The precipitated protein (about 55 g) was collected at 7.5 × g for 15 min in a GSA rotor, resuspended in 100 ml of buffer A, and dialyzed for 10 h against 2 liters of buffer A containing 0.1 M KAc with 2 changes. This was then loaded at 180 ml/h onto a 500-ml DEAE-fast flow (Pharmacia Biotech Inc.) column (5 cm × 22 cm) pre-equilibrated with buffer A containing 0.1 M KAc. This column was washed in 1 liter of the same buffer, and PAN activity was eluted at the same flow rate in 600 ml of buffer A containing 0.2 M KAc. Approximately 200 ml of the 0.2 M KAc DEAE elution (containing the peak protein fractions) contained the PAN activity. This fraction was diluted in buffer A to a final KAc concentration of 100 mM, loaded at 120 ml/h onto a 30-ml Q-Sepharose (Pharmacia) column (2 cm × 10 cm) pre-equilibrated with buffer A containing 100 mM KAc. Following a 120-ml wash with the same buffer, PAN activity was eluted with a 75-ml ionic gradient spanning 100 to 500 mM KAc in buffer A at 1 ml/min. PAN activity in each third 1-ml fraction was assayed, and the peak activity fractions (typically between 130 and 180 mM KAc) were pooled. This Q-Sepharose fraction was either quick-frozen and stored at −70 °C for use in a 1-kg scale purification, or diluted to 70 mM KAc final with buffer B (20 mM HEPES, pH 7.4, 2 mM MgAc, 14 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.05% Nonidet P-40, 10% glycerol) for loading onto a phosphocellulose column. For a 1-kg scale purification, another 500 g of frozen lysed cell powder was processed as described, and both thawed Q-Sepharose PAN fractions were pooled and adjusted to 70 mM KAc with buffer B. This pool was loaded at 1 ml/min onto 70 ml of freshly prepared phosphocellulose (Whatmann) (2.5 cm × 15 cm) pre-equilibrated with buffer B containing 70 mM KAc. Following a 200-ml wash with the same buffer, PAN activity was eluted with a 200-ml ionic gradient spanning 70 to 500 mM KAc in buffer B at 1 ml/min. PAN activity in each third 1-ml fraction was assayed, and peak fractions (typically between 150 and 200 mM KAc) were pooled and directly loaded by gravity flow onto a 1.5-ml poly(U)-Sepharose (Pharmacia) column pre-equilibrated in buffer B containing 200 mM KAc and 1.5 mM EDTA. Following a 50-ml wash in buffer B containing 1.0 M KAc and 1.5 mM EDTA and a 5-ml wash in buffer B containing 0.2 M KAc and 1.5 mM EDTA, PAN activity was eluted by gravity flow with 8 ml of buffer B containing 1.0 M GdnHCl and 1.5 mM EDTA. All of the 1-ml fractions were dialyzed against 1 liter of buffer B containing 70 mM KAc and 1.5 mM EDTA for 6–10 h with 2 changes, and then assayed for activity. Peak activity fractions (typically 2–5 ml) were pooled and loaded by gravity flow onto a 0.65-ml recombinant Pab1p-Sepharose column pre-equilibrated in buffer B containing 0.2 M KAc. Following washes with 5 ml of the same buffer and 15 ml of buffer B containing 1.0 M KAc, PAN activity was eluted by gravity flow with 1.2 ml of buffer B containing 1.0 M GdnHCl. All of the 0.4-ml fractions were dialyzed for 6 h against 1 liter of buffer B containing 70 mM KAc with 2 changes, and then assayed for PAN activity. Fractions containing the peak of activity (typically between 0.4 and 1.2 ml) were pooled, aliquoted, and stored at −70 °C.

Preparation of the Pab1p-Sepharose Column—Pab1p-Sepharose was prepared by cross-linking recombinant Pab1p to CNBr-activated Sepharose-4B (Pharmacia). To prepare a 1-ml column, 0.6 g of CNBr resin was first dissolved in 1 mM HCl and subsequently washed over a glass sintered filter for 15 min with 1 mM HCl. 10 mg of recombinant Pab1p (see Ref. 13 for preparation) was cross-linked to the resin by rocking the protein with the resin in a 2:1 slurry of coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) to resin overnight at 4 °C. Uncoupled Pab1p was removed by washing with 5 ml of coupling buffer. Remaining active groups were blocked by incubating the resin in 0.1 M Tris-HCl, pH 8.0, for 2 h at 4 °C. The column was then washed with three cycles of 5 column volumes of buffer A and buffer B, consisting of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 4.0, and 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8, respectively. Verification that the majority of Pab1p cross-linked to the resin was obtained by analyzing the coupling buffer which had been incubated with the resin for Pab1p by SDS-PAGE and silver staining. The resin was shown to bind both rabbit polyclonal antibodies generated against Pab1p and poly(A) RNA.

Poly(A) Nuclease Enzymatic Assay—PAN activity was assayed as described previously (1). Briefly, up to 10 μl of protein sample was mixed with 1 μl of 200 ng/μl recombinant Pab1p and incubated for 15 min on ice. 5 μl of a cold RNA mixture consisting of 2 μg of tRNA and 5,000 cpm of homogeneously labeled ^{32}P-poly(A) in water was added, and the reaction was then started by the addition of 185 μl of room temperature dilution buffer (5 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 14 mM β-mercaptoethanol). After incubation at 30 °C for 30–45 min, the reaction was quenched by the addition of 200 μl of cold 20% trichloroacetic acid. Following a 10-min incubation on ice, the material was centrifuged at 14,000 × *g* for 10 min and 200 μl of the supernatant was added to an equal volume of 1 M unbuffered Tris base. Lastly, 6 ml of Aquasol (DuPont NEN) was added, and radioactivity was determined by scintillation counting.

Small scale S100 extracts used to investigate the existence of PAN activity in the mutant and wild type *PAN2* strains (Fig. 6*B*) were prepared from 12 ml of A_{600} = 0.8 yeast cultures grown in YPD medium or in YM medium supplemented with the required additives. Cells were pelleted and resuspended in 100 μl of ice-cold buffer A containing 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin in a 500-μl microcentrifuge tube. Following the addition of 150 μg of acid-washed glass beads, the cells were vortexed for 1.5 min and the extract was centrifuged for 5 min at 14,000 × *g* through a hole punched into the bottom of the tube into a 1.5-ml Microfuge tube. The extracts were then centrifuged in a TLA 100.3 rotor (Beckman Instruments) once at 40,000 × *g* for 30 min, and once at 100,000 × *g* for 60 min in a Beckman TL-100 ultracentrifuge. All steps were performed at 4 °C. The protein concentration was measured using the Bio-Rad protein assay kit.

Overproduction of a Pan1p Fragment—The intergenic region of *PAN1* encoding the polypeptide between amino acids 392 and 662 was amplified from a plasmid born *PAN1* gene (pAS313), and then subcloned into a IPTG inducible $(His)_6$-tagged vector for subsequent overproduction in bacteria and purification on a Nickel-agarose column. Specifically, synthetic oligonucleotide primers, OAS 94 and OAS 100 (1) were used to amplify the *PAN1* gene from nucleotides 1677 to 2490 (see (1) for nucleotide numbering) by polymerase chain reaction. Primers were used at 1 μM with 100 ng of plasmid pAS313, 2 mM $MgCl_2$, 200 μM dNTP, and 2.5 units of *Taq* polymerase (Promega) in a final volume of 100 μl of buffer (provided by Promega). Reaction coordinates were a 1.5-min denaturation at 94 °C, followed by 30 cycles of 1.5 min at 42 °C, 2 min at 72 °C, and 1.5 min at 94 °C, and finally 7 min at 72 °C. The amplified DNA fragment was digested with *Kpn*I and then cloned into *Kpn*I-digested pBRSETB vector (Invitrogen). Proper orientation of the subcloned fragment was confirmed by sequencing. This subcloning places the *PAN1* fragment C-terminal to a 41 amino acid His-tagged T7 phage gene 10 protein fragment. The resulting plasmid, pAS386, encodes a 311-amino acid PAN1 fusion protein.

BL21(DE3) bacteria cells were transformed with pAS386 to yield strain BAS1693. A single colony was innoculated into 1.5 liters of LB culture at 30 °C. At an A_{595} = 0.5, expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside to 0.5 mM final. Cells were grown for 3 more hours, harvested, and resuspended in 15 ml of 50 mM Tris, pH 8.0, 1 mM phenylmethylsulfonyl fluoride. Resuspended cells were quick frozen in liquid nitrogen, thawed, and treated at room temperature for 10 min with lysozyme (Sigma) at 100 μg/ml final. Following a brief sonication, the extract was clarified by centrifugation at 25,000 rpm for 40 min at 4 °C in a 50Ti rotor. For purification, 7.5 ml of the soluble extract was loaded onto a 1-ml nickel-agarose column (Invitrogen ProBond Resin) at 4 °C at 10 column volumes/h. Loading buffer was 20 mM Tris, pH 7.9, 100 mM NaCl, 2 mM imidazole. Following a wash with 10 column volumes of loading buffer, 3 column volumes of loading buffer plus 20 mM imidazole, and 3 column volumes of loading buffer plus 40 mM imidazole, the His-tagged PAN1 peptide was eluted with 5 column volumes of loading buffer plus 80 mM imidazole. This eluate was dialyzed for several hours against 200 volumes of 20 mM

Tris, pH 7.5, 50 mM KAc and loaded at 0.5 ml/min onto a Mono Q column (Pharmacia) pre-equilibrated in this buffer. Following a 10-ml wash with this buffer at the same flow rate, the protein was eluted with a 10-ml gradient of KAc (spanning 50 mM KAc to 500 mM KAc) in 20 mM Tris-HCl, pH 7.5. Pan1p fusion protein was detected in each 1-ml fraction by SDS-PAGE and silver staining of protein gels (as described in Ref. 14).

A total of 200 μg of the 38-kDa purified PAN1 fusion protein was purified from 3 liters of bacteria harvested at $A_{595} = 3$. The protein (0.5 mg/ml) was sent to Pocono Rabbit Farm and Laboratory (Canadensis, PA) for subsequent injections into rabbits. Rabbits were injected five times over a 4-month period.

Immunological Techniques—Pan1p was detected by Western analysis of crude extracts from 0.3 OD_{600} equivalents of yeast cells that was separated on a 7.8% SDS-polyacrylamide gel and then transferred onto Immobilon nitrocellulose filters (Amersham). The transfer to the filter was carried out for 45 min at 17.5 volts/cm in transfer buffer (10% methanol, 10 mM CAPS). Following blocking with 7% dry milk, 0.1% Tween 20 in TBS (139 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl, pH 7.5) for a minimum of 2 h, the primary antibody (diluted 1:1000 in TBS and 0.1% Tween (TBS-T buffer)) was incubated with the blot for a minimum of 1 h at room temperature. Blots were washed for at least 0.5 h, with three 10-min washes in TBS-T buffer. The blots were then incubated with a 1:5000 dilution of anti-rabbit horseradish peroxidase-conjugated antibodies (Amersham) in TBS-T buffer for a minimum of 1 h. Blots were washed three times for 10 min each in TBS-T buffer, and then developed using the ECL detection system (Amersham).

For Pan1p immunoprecipitations, either 6 μl of preimmune or immune sera were incubated by rocking with 20 μl of a 10% slurry of protein A-Sepharose (Sigma) in buffer A (see above) containing 0.1 M KAc in a 1.5-ml tube at 4 °C. After 2 h, the suspension was centrifuged for 15 s at 14,000 × *g* and the supernatant was discarded. The pellet was washed three times with 100 μl of cold buffer A containing 0.1 M KAc and then resuspended with 20 μl of the PAN fraction from the poly(U)-Sepharose column (9 units of PAN). Bovine serum albumin was added to 5 mg/ml final concentration to stabilize PAN activity. After end to end mixing for 90 min at 4 °C, the suspension was centrifuged for 30 s at 14,000 × *g*. The supernatant was collected and assayed for PAN activity. The pellet was washed three times with 100 μl of cold buffer A containing 0.1 M KAc and resuspended in 20 μl of Laemmli loading buffer. Immunoblotting was performed with 10 μl of the poly(U)-Sepharose fraction, 10 μl of the supernatant, and 10 μl of the pellet suspension.

Nucleic Acid Techniques—The *PAN2* gene was disrupted in the yeast strain YAS306 (MAT**a** ade2–1 his3–11, 15 leu2–3, 112 trp1–1 ura3–1 can1–100) according to Baudin *et al.* (15) by amplifying a 1.6-kb fragment containing the yeast LEU2 gene flanked by 45 nucleotides upstream and downstream of the *PAN2* initiation and stop codon, respectively. Polymerase chain reaction was performed on 10 ng of plasmid YIplac128 (16) with 20 μM primers OAS184 (5′-CTAGAACACAATGCTATACTGAGTTTCTGAATGGTGAATGTATTAATAGGCGTATCACGAGGCC-3′) and OAS185 (5′-GTGCGCTGGTGGCTCTTGAGATTACGTGAAAGGCACTGCACCATACACCGAAACGCGCGAGACGAAAGGG-3′) using 0.5 units of *Taq* DNA polymerase (Perkin-Elmar) in 20 μl of buffer supplied by the manufacturer (25 cycles of 1 min denaturation at 94 °C, 2 min annealing at 50 °C, 2 min extension at 72 °C). Following agarose gel purification of the 1.6-kb product, the DNA was transformed into YAS306 by LiAc transformation (17) giving rise to strain YAS1837 (MAT**a** PAN2::LEU2 ade2–1 his3–11,15 leu2–3, 112 trp1–1 ura3–1 can1–100).

Plasmid pAS464 containing the *PAN2* ORF fused to the GAL1 promoter on a URA3CEN4 plasmid was constructed by inserting the *PAN2* ORF downstream of the GAL1 promoter of plasmid pAS135. The *PAN2* ORF was amplified using 20 μM primers OAS186 (5′-CCGGAATTCCATGGATAATTGGCAACATTTCTTC-3′) and OAS187 (5′-CCGGAATTCATATTATCCCTTTGAAGTTTC-3′). Polymerase chain reaction was performed on 50 ng of YAS306 DNA in 100 μl of buffer supplied by the manufacturer (25 cycles of 1 min denaturation at 94 °C, 2 min annealing at 50 °C, 4 min extension at 75 °C) using 5 units of *Pfu* polymerase (Stratagene). The 3.4-kb fragment was digested with *Bgl*II/*Xba*I, purified on agarose gel, and inserted into pAS135 previously digested with *Bam*HI/*Xba*I. pAS464 was used to transform by LiAc the PAN2::LEU2 and wild type strains to yield strains YAS1838 (MAT**a** PAN2::LEU2 ade2–1 his3–11,15 leu2–3, 112 trp1–1 ura3–1 can1–100 pGAL1-PAN2URA3CEN4) and YAS1839 (MATα ade2–1 his3–11,15 leu2–3, 112 trp1–1 ura3–1 can1–100pGAL1-PAN2URACEN), respectively.

Yeast genomic DNA was prepared according to the method of Hoffman and Winston (5), digested with the indicated restriction enzymes (New England Biolabs), and transferred to a Zeta-Probe membrane (Bio-Rad) using standard techniques. The *PAN2* probe used for the Southern analysis consisted of a 0.45-kb DNA fragment, amplified using 20 μM OAS188 (5′-GAAGATCTATCCGGCTTCTTTCATCCACATT-3′) and OAS189 (5′-CTCAGTATAGCAATTGTGTTC-3′), corresponding to 450 nucleotides upstream of the *PAN2* initiation codon. Polymerase chain reaction was performed on 50 ng of YAS306 DNA using 1 unit of *Taq* DNA polymerase (25 cycles of 1 min denaturation at 94 °C, 1 min annealing at 48 °C, 2 min extension at 72 °C). The amplified fragment was then labeled to high specific activity with [α-^{32}P]dCTP using random hexamer priming. The membrane was incubated for 14 h at 65 °C in 1 mM EDTA, O.5 M Na_2PO_4, pH 7.2, 7% SDS containing 2 × 10^5 cpm/ml *PAN2* probe and then washed in 1 mM EDTA, 40 mM Na_2PO_4, pH 7.2, 5% SDS for 30 min at 25 °C, 30 min at 65 °C, and exposed to a Kodak X-Omat AR film. Poly(A) tails were visualized from preparations of total yeast RNA as described previously (1).

RESULTS

A Large Scale Purification of PAN Activity Identifies 135- and 76-kDa Candidate Polypeptides—In order to obtain large amounts of the Pab1p-stimulated PAN enzyme (referred to throughout as PAN) for biochemical studies, a modified large scale purification procedure for the PAN activity was developed (Fig. 1*A* and Table I). This procedure is capable of handling kilogram quantities of starting yeast cell paste, and produces PAN with a specific activity nearly equal to that achieved in the small scale purification previously reported (1). The significant differences in this procedure from the earlier one include an ammonium sulfate precipitation step, an extra anion exchange column, linear gradients of increasing ionic strength instead of bumps for elution, and the introduction of a recombinant Pab1p-Sepharose column as the final step in the procedure. Most importantly, the use of low levels of the detergent Nonidet P-40 to stabilize the enzyme activity allows for the apparent purification to homogeneity of an active enzyme.

Nearly all of the Pab1p-stimulated PAN activity bound to DEAE, Q-Sepharose, and phosphocellulose resins, and the vast majority of it eluted between 150 and 200 mM potassium acetate (Fig. 1*A*). The active phosphocellulose fractions were pooled and loaded onto a poly(U)-Sepharose column, which was then extensively washed with buffer containing 1 M potassium acetate before elution with buffer containing 1 M guanidine hydrochloride (GdnHCl). Following dialysis of each of the fractions to standardize the ionic strengths, almost all of the PAN activity was reproducibly found in the 1 M GdnHCl eluate. Resolution of the polypeptides within each of the fractions from the poly(U)-Sepharose column by SDS-PAGE, followed by silver staining to visualize the proteins, revealed three major polypeptides of 135, 110, and 76 kDa (Fig. 1*B*, *load*).

Subsequent chromatography of the active fraction from the poly(U)-Sepharose column on a recombinant Pab1p-Sepharose column revealed that both the 135- and 76-kDa proteins have a very high affinity for Pab1p (Fig. 1*B*). These proteins did not elute off of this column in high (1 M KAc) ionic strength but did elute off in the presence of 1 M GdnHCl. The 110-kDa protein, on the other hand, appeared to be partially washed off the column before the GdnHCl elution step since its yield relative to the other two polypeptides were diminished. Most importantly, an analysis of the PAN activity across each of the fractions of the Pab1p-Sepharose column showed that it is bound quantitatively and elutes only after exposure to GdnHCl (Fig. 1*B*). Because the 135- and 76-kDa proteins bind stoichiometrically to the Pab1p column, and because the PAN activity exhibits similar behavior, we conclude that each of these two peptides are required for the enzyme's activity. The p76 protein was also found in the previously reported purification (1), but was discounted due to its variable yields. Given the current results, we conclude this variability was due to p76 proteolysis

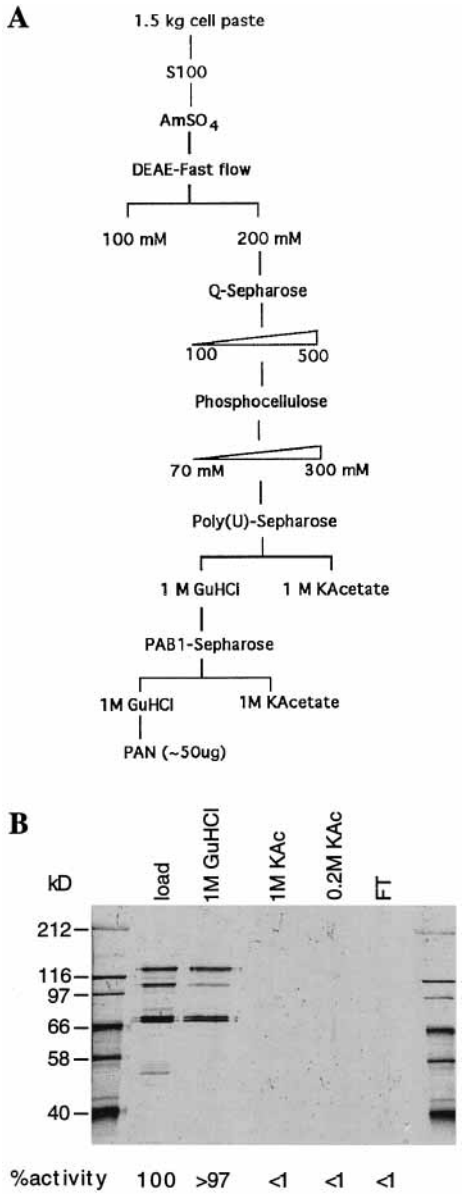

FIG. 1. **Large scale purification of PAN activity.** *A*, schematic diagram of the large scale purification procedure for PAN activity. *Triangles* represent linear gradient elutions spanning the indicated ionic strengths from the different resins. *B*, elution profile of PAN activity from Pab1p-Sepharose. After dialysis into a low salt buffer, the active PAN fraction from the poly(U)-Sepharose column (*load*) was chromatographed on a Pab1p-Sepharose column. The flow through (*FT*), 0.2 M KAc (0.2 M KAc), 1.0 M KAc (1 M KAc), and 1 M GdnHCl (1 M *GuHCl*) eluates were collected. Following separation of an equal percentage of each fraction by SDS-PAGE, proteins were visualized by silver staining. The relative amount of PAN activity in each fraction (percent of total recovered) is shown below.

during purification.

Similar enrichments for both the p76 and p135 polypeptides and the PAN activity were obtained using Superose-6 chromatography instead of Pab1p-Sepharose and by Pab1p-Sepharose chromatography of eluates from the Q-Sepharose column (data not shown). Attempts to separate p76 and p135 without loss of PAN activity on many other chromatographic resins have been unsuccessful, thereby adding support to the conclusion that the association of these two proteins is required for PAN activity.

As in the small scale purification, the PAN activity needs to be purified almost 100,000-fold from S100 extracts before candidate polypeptides can be identified (Table I). The yield from the large scale purification is quite good, although it is difficult to evaluate the amount of PAN activity prior to the DEAE step due to contaminating nonspecific nucleases, endogenous nucleic acids, and cellular Pab1p. This cellular Pab1p is separated from the PAN activity by the DEAE column. The specific activity of the preparation after the Pab1p-Sepharose column was difficult to calculate given the low amounts of protein present in these samples. However, we estimate that approximately 50 μg of pure PAN can be obtained from 1.5 kg of yeast cell paste. Given the nearly quantitative yield of enzyme activity off of the Pab1p-Sepharose column, this would give a specific activity for purified PAN that is approximately six times greater than that found on the poly(U)-Sepharose column. Overall, it is surprising that PAN is in such low abundance in the yeast cell, although we cannot rule out at this time the possibility that the majority of it is lost as insoluble material during the preparation of the S100 extract.

The Minor 135-kDa Polypeptide is Pan1p, Which Is Not Required for PAN Activity—The 135-kDa protein in this procedure was identical in molecular mass to the Pan1p microsequenced from preparations using the original purification procedure. Antibodies to Pan1p were used to investigate whether the 135-kDa protein purified in the large scale procedure was Pan1p.

The toxicity of the *PAN1* gene in many bacterial vectors prevented the overexpression of its encoded protein using modern recombinant techniques. However, a fragment of Pan1p that represents a repeated sequence motif in the protein (Fig. 2*A*) was maintained in a T7 expression vector, and large quantities of soluble, histidine-tagged protein were produced. This material was purified to near homogeneity over two columns and injected into rabbits for antibody production.

The specificity of the resulting antisera is shown in Fig. 2*B*. Crude extracts from yeast harboring either the full-length or a truncated version of Pan1p were prepared and resolved by SDS-PAGE. Subsequent to transfer onto nitrocellulose membranes, the Pan1p antigen was detected by Western analysis. As can be seen from these data, Pan1p was specifically recognized by the antisera used at a dilution of 1:1000. Its apparent molecular mass is nearly 175 kDa, a size significantly different than that of the previously purified 135-kDa protein. The cross-reacting material running at molecular weights smaller than the full-length Pan1p are presumably proteolytic fragments since they disappeared in extracts from a strain expressing the truncated Pan1p. We conclude from these data that the rabbit antiserum specifically recognizes the Pan1p antigen.

The co-purification of Pan1p in the new purification procedure was then evaluated by Western analysis using the Pan1p antisera. Surprisingly, most of the Pan1p did not bind to the phosphocellulose column (data not shown). In contrast, the phosphocellulose elution fraction contained almost 100% of the PAN activity found in the original Q-Sepharose load. Although most of the Pan1p fragments flowed through this column, a proteolytic 135-kDa fragment which did bind and elute with the PAN activity continued to co-purify with the activity over the poly(U)-Sepharose and the Pab1p-Sepharose columns. Like the PAN activity and the p135 and p76 proteins, the Pan1p fragment eluted only when the columns were washed with GdnHCl (Fig. 2*C*). However, the lack of co-purification of the bulk of Pan1p with the PAN activity on the phosphocellulose column strongly suggested that Pan1p was not required for catalytic activity.

Antibodies directed against Pan1p efficiently immunodepleted the residual Pan1p found in the poly(U)-Sepharose eluate from the large scale purification (Fig. 3*A*). However, the amount of the 135-kDa protein detected by silver staining in this eluate did not change before and after immunodepletion

TABLE I
Purification of PAN activity

The PAB1-Sepharose column is not included in the table due to the small amounts of protein recovered.

Step	Total protein	Total units[a]	Specific activity	Recovery	Purification
	mg	*pmol/mg*	*units/10 mg*	%	*-fold*
S100	12,195	720	0.59		1
60% $AmSO_4$	4,600	7,900	17		29
DEAE	756	6,850	117	100	198
Q-Sepharose	88	4,500	489	49	829
P-cellulose	25	9,100	3,640	100	6,170
Poly(U)	0.34	1,900	55,900	22	94,750

[a] Enzyme units are as defined in Ref. 1.

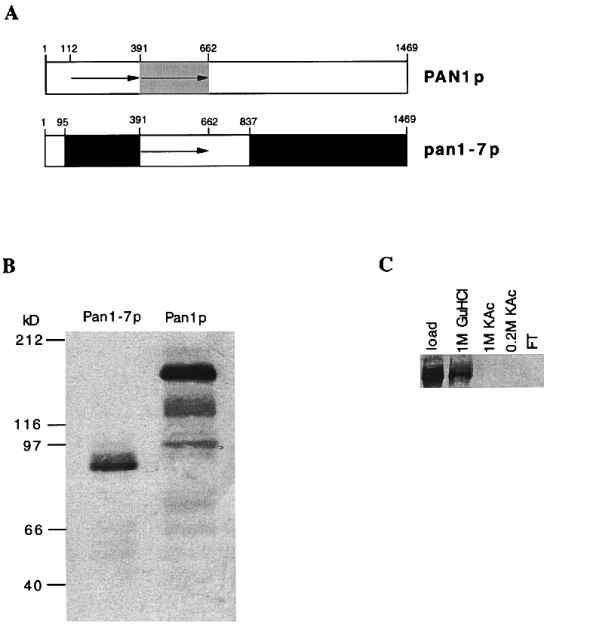

FIG. 2. **Production of Pan1p antibodies using recombinant Pan1p antigen.** *A*, schematic diagram of the Pan1p. The two repeated NH_2-terminal domains are indicated by the *arrows*. The position of the overproduced protein fragment is indicated by shading in the full-length protein. A diagram of the truncated Pan1p, *pan1–7p* is shown below, with the regions of the protein that are deleted highlighted in *black*. Amino acid numbers are listed on top. *B*, Western analysis of Pan1p in crude extracts from wild type (*Pan1p*) and Pan1p truncation (*pan1-7p*) containing cells. Immunocomplexes were visualized by luminescence as described under "Materials and Methods." The position of the molecular weight markers are indicated to the left. *C*, a Pan1p fragment co-purifies with PAN activity on Pab1p-Sepharose. An equal percentage of the indicated column eluates from Fig. 1*B* were separated by SDS-PAGE. Pan1p was visualized by Western analysis.

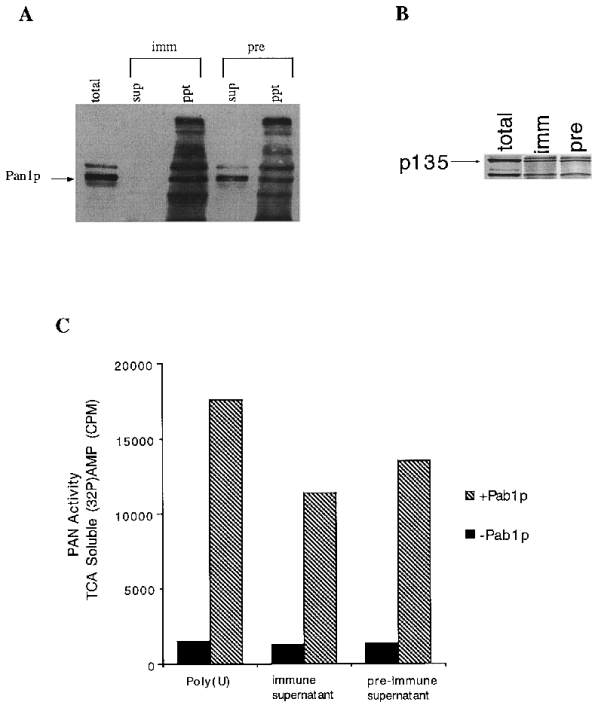

FIG. 3. **Pan1p is not required for PAN activity.** *A*, immunodepletion of Pan1p from the poly(U)-Sepharose eluate. Following removal of preimmune (*pre*) or immune (*imm*) serum antibody complexes by protein A-Sepharose absorption, the residual supernatants (sup) and immunoprecipitates (*ppt*) were boiled in SDS and resolved by SDS-PAGE. Pan1p was visualized by Western analysis. A sample of the starting material (*total*) is shown for comparison. *B*, the amount of visible 135-kDa protein after immunodepletion of Pan1p remains unchanged. Proteins in the residual supernatants described in *A* were resolved by SDS-PAGE, and then visualized by silver staining. The location of the p135 protein is indicated by an *arrow*. The protein found above the 135-kDa band is introduced when serum is added to the sample. *C*, the amount of soluble PAN activity is unchanged after immunodepletion of Pan1p. The supernatants from the immunoprecipitates described in *A*, as well as the starting sample, were assayed for PAN activity. Activity is shown as the total amount of soluble radioactivity released in a PAN assay (as described under "Materials and Methods").

(Fig. 3*B*). Furthermore, the amount of PAN activity in the supernatant from the immunodepleted material was nearly identical to that found in the supernatant from the preimmune control sample (Fig. 3*C*). These data provide definitive evidence that Pan1p is not required for the PAN enzymatic activity, and that the predominant 135-kDa protein purified in the large scale procedure is not Pan1p. However, these data cannot rule out the possibility that Pan1p is associated with the PAN enzyme since it is present in substoichiometric amounts in our preparation. Therefore its immunodepletion would not be expected to remove the PAN enzyme not bound to it.

The Predominant 135-kDa Protein Is Pan2p, a Novel Protein That Is Not Essential for Yeast Cell Viability—In order to identify the gene encoding the major 135-kDa protein co-purifying with the PAN activity (Pan2p), this protein was isolated from SDS-polyacrylamide gels and partially digested with trypsin. Following their purification by high performance liquid chromatography, six peptide fragments were successfully microsequenced. A search of yeast sequence obtained in one of our laboratories (M. Rieger) as part of the yeast genome sequencing project revealed a predicted open reading frame of 126,958 daltons that contained all of the sequenced peptides (Fig. 4). This open reading frame shows no significant homology to other proteins in the sequence data bases. Interestingly, the single peptide sequence previously reported to be present in the Pan1p preparation but not found in the Pan1p open reading frame (see "Materials and Method"s of Ref. 1) was also found in Pan2p's predicted protein sequence (*boxed region* in Fig. 4). This both confirms the presence of Pan2p in the original PAN preparations, and shows that despite an extensive degree of purification these preparations were impure.

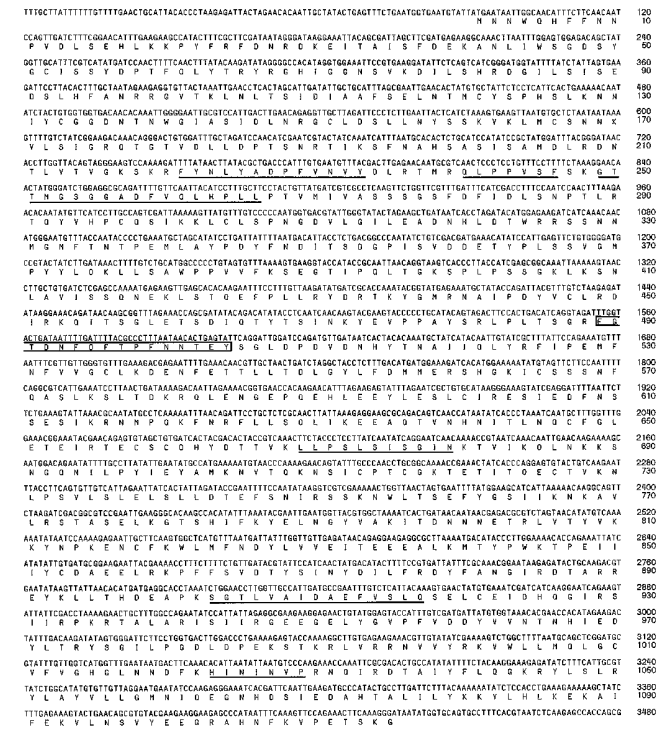

FIG. 4. **Nucleotide and predicted amino acid sequence of the *PAN2* gene.** *Underlined* regions of the open reading frame indicate the peptide sequences dermined by protein sequencing. The *boxed* region indicates the peptide fragment identified in a previous PAN preparation (1). The Genbank accession number for *PAN2* is U39204.

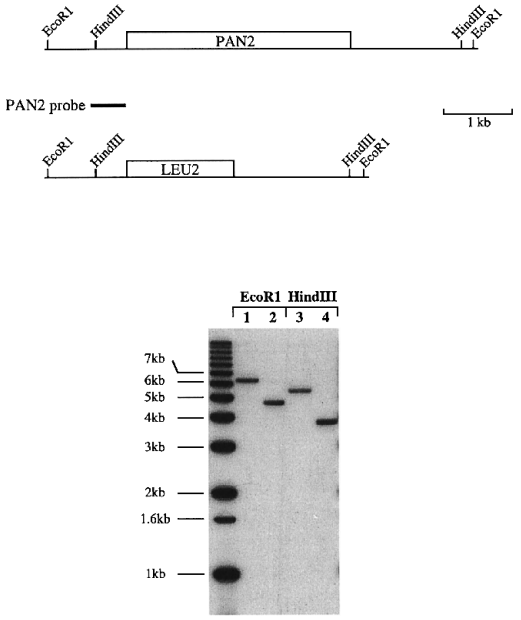

FIG. 5. **Southern blot for *PAN2* confirms disruption of the *PAN2* gene in strain YAS1837.** A 450-nucleotide radioactive *PAN2* probe was hybridized to a Southern blot containing yeast genomic DNA digested with the indicated enzymes. *Lanes 1* and *3*, wild type strain (YAS306); *lanes 2* and *4*, *PAN2* deleted strain (YAS1837). Molecular size markers (kb) are indicated. The diagram indicates the relative positions of the restriction sites, the placement of the *LEU2* marker in the *PAN2* gene, as well as the position of the *PAN2* probe.

A Southern blot of yeast DNA using a *PAN2* probe containing 450 nucleotides of sequence just 5′ to the open reading frame indicated *PAN2* is not a duplicated gene in the yeast genome (Fig. 5 and data not shown). Low stringency hybridization of this blot with the DNA spanning the entire *PAN2* open reading frame failed to reveal cross-reacting species (data not shown), thereby indicating the absence of *PAN2* homologues in the genome.

The deletion of the *PAN2* gene from the yeast genome by targeted gene disruption revealed Pan2p is not essential for yeast cell viability, as haploid cells deleted for the open reading frame were viable and diploids heterozygous for the *PAN2* deletion yielded viable haploid progeny after meiosis (Fig. 5). A yeast strain containing the deleted *PAN2* gene grew at approximately the same rate as the isogenic wild type strain, and did not show a formamide, cold-, or heat-sensitive growth phenotype. Furthermore, overexpression of Pan2p by placing its gene under the control of the GAL1 promoter did not severely restrict cell growth.

Deletion of PAN2 from the Yeast Genome Results in Abnormal Poly(A) Tail Lengths in Vivo and the Absence of Pab1p-stimulated PAN Activity in Vitro—The lack of a requirement for Pan2p for cell viability allowed for a direct investigation of its role in poly(A) nuclease function. As previously reported, the deletion of the *PAB1* gene from yeast results in mRNAs with an abnormally large amount of long poly(A)-tailed mRNAs (4). Because poly(A) tails can be visualized directly on polyacrylamide gels, this phenotype is easily measurable. As would be expected for inactivation of a Pab1p-stimulated PAN, deletion of the *PAN2* gene also led to the appearance of an abnormally large amount of long poly(A) tailed mRNAs in yeast (Fig. 6*A*, *lanes 1* and *2*). The degree of accumulation of these species was very similar to that seen for Pab1p mutations (data not shown). This poly(A) tail accumulation was due to the absence of the Pan2p, and not to an indirect effect of changing the chromosomal region by deleting the *PAN2* gene, since the presence of a plasmid expressing the *PAN2* gene relieved this accumulation phenomenon (Fig. 6*A*, *lanes 3–6*). Note that short poly(A) tails are still found in the *PAN2* disrupted strain, suggesting that enzymes other than the Pab1p-stimulated PAN can destroy poly(A) tails *in vivo*.

The appearance of long poly(A) tails in a *PAN2* mutant is consistent with but does not prove that Pan2p is required for PAN activity. For instance, mutations in Pan1p also affected poly(A) tail metabolism (1), but as shown above Pan1p is not required for PAN catalytic activity. In order to directly show that a deletion of *PAN2* results in the ablation of PAN activity, crude extracts from various yeast strains were examined (Fig. 6*B*). As previously reported (1), S100 extracts from wild type strains showed a stimulation of PAN activity when recombinant Pab1p was added to them (Fig. 6*B*, *column 3*). This activity was due to the Pab1p-stimulated PAN and not a general ribonuclease since only versions of Pab1p that stimulate purified PAN (1) stimulated this activity (data not shown). Consistent with the interpretation that Pan2p is required for PAN activity, we found that an extract prepared from a strain missing Pan2p did not contain a Pab1p-stimulated PAN (Fig. 6*B*, *columns 2* and *3*). As with the *in vivo* poly(A) tail phenotype, this deficiency of PAN activity was restored in extracts from cells containing the deleted version of *PAN2* in the genome and expressing the *PAN2* gene on a plasmid (Fig. 6*B*, *columns 4* and *5*).

DISCUSSION

The large scale purification of PAN from yeast extracts reported here allows for the production of almost 50 μg of pure protein from approximately 1.5 kg of yeast cell paste. The purified material is highly enriched for three polypeptides with apparent molecular masses of 135, 110, and 76 kDa. The 135-kDa Pan1p fragment in this preparation is not required for PAN activity. The novel 135-kDa protein Pan2p also found in this preparation is required for PAN activity, as shown by both

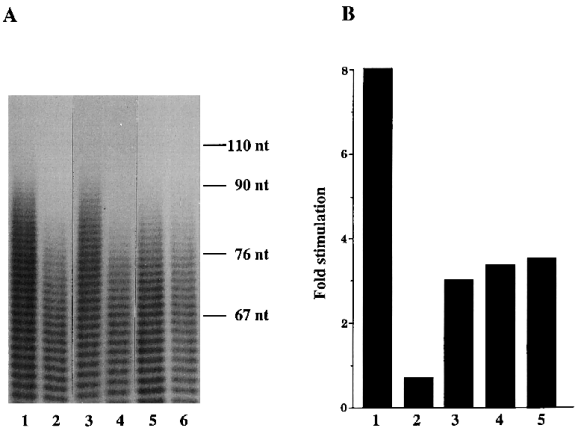

FIG. 6. **Disruption of the *PAN2* gene results in the accumulation of long poly(A) tails *in vivo* as well as the loss of Pab1p-stimulated PAN activity in crude extracts, both of which can be rescued by overexpressing Pan2p.** *A*, poly(A) tails in mRNA preparations were end labeled with [^{32}P]cytidine 3′,5′-bis(phosphate), resolved on a 12% polyacrylamide-7 M urea gel, and visualized as described previously (1). *Lane 1*, *PAN2* deleted strain (YAS1837); *lane 2*, wild type strain (YAS306); *lane 3*, *PAN2* deleted strain containing the *PAN2* ORF under the control of the GAL1 promoter (YAS1838) grown in glucose medium; *lane 4*, wild type strain containing the *PAN2* ORF under the control of the GAL1 promoter (YAS1839) grown in glucose medium; *lanes 5* and *6*, same strains as in *lanes 3* and *4*,respectively, but grown in galactose medium. Molecular size markers (*nt*.) are indicated. *B*, S100 extracts were prepared and 5 μg of protein from each extract was assayed for PAN activity in the presence and absence of recombinant Pab1p as described under "Materials and Methods." The data are expressed as the ratio of the stimulation of PAN activity by Pab1p over the amount of PAN activity found in the absence of Pab1p addition. *Column 1*, partially purified PAN fraction; *column 2*, *PAN2* deleted strain containing the *PAN2* ORF under the control of the GAL1 promoter (YAS1838) grown in glucose medium; *column 3*, wild type strain containing the *PAN2* ORF under the control of the GAL1 promoter (YAS1839) grown in glucose medium; *columns 4* and *5*, same strains as in *columns 2* and *3*, respectively, but grown in galactose medium.

in vivo and *in vitro* assays.

Although Pan1p is not required for enzymatic activity, it remains to be resolved whether it is associated with the PAN enzyme. The persistent co-purification of a proteolytic fragment of Pan1p provides some evidence for this possibility. Furthermore, the identical chromatographic properties of the 135-kDa Pan1p proteolytic fragment and the PAN enzymatic activity on Pab1p-Sepharose, in combination with the similarities of the loss of function phenotypes of Pan1p and Pab1p in yeast (1), also make it likely that Pan1p is involved in some aspect of Pab1p-dependent metabolism. That only a proteolytic fragment of Pan1p is found to co-purify with the enzyme suggests that the full-length protein is either incapable of such a putative association, or that only the fragmented protein remains tightly bound to the PAN enzyme throughout the procedure. Co-immunoprecipitations studies on Pan1p using antibodies against either Pan2p or possibly the p76 polypeptide will be invaluable in determining whether Pan1p is associated with these proteins.

Both the small and large scale purification procedures for PAN resulted in the isolation of proteins with apparent molecular masses of 135 and 76 kDa. The cloning and disruption of the *PAN2* gene confirms the 135-kDa proteins involvement in PAN function. The cloning and mutagenesis of the gene encoding the 76-kDa protein that co-purifies with Pan2p will determine whether it too is needed for PAN function. Once this is known, a detailed investigation into the role of the Pab1p-stimulated PAN in yeast mRNA translation and degradation can be initiated.

Because poly(A) tail shortening is still occurring in strains lacking the Pab1p-stimulated enzyme, we assume there are other poly(A) nucleases in the yeast cell. Pan2p may not be essential because of the existence of these other deadenylases. The biochemical purification of these deadenylases from a strain lacking Pan2p could be one way to identify them. Alternatively, a genetic approach using synthetic lethality as a tool to identify proteins with overlapping functions could lead to their gene isolation. Once these other enzymes are identified, it should be possible to examine the effects of disrupting poly(A) tail degradation on mRNA metabolism.

Poly(A) tail removal from mRNA probably results in decreases in translation and a stimulation of degradation. As a result, it is anticipated that cellular control of gene expression could be exerted by regulating the poly(A) nucleases. Furthermore, the existence of sequences within the 3′-untranslated region of mRNAs that control each of these processes raises many questions about their mechanism of action. It is anticipated that studies using a combination of biochemistry and genetics, as highlighted in this work, will lead to the discovery of regulatory proteins for deadenylation, and an elucidation of the mechanisms by which mRNA sequences determine the level of an mRNA's expression.

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