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Recommended Citation
Boeck R., Tarun Jr. S., Rieger M., Deardorff J.A., Müller-Auer S., Sachs A.B. (1996) The yeast Pan2 protein is required for poly(A)-binding protein-stimulated poly(A)-nuclease activity. Journal of Biological Chemistry 271: 432-438. doi: 10.1074/jbc.271.1.432
The Yeast Pan2 Protein Is Required for Poly(A)-binding Protein-stimulated Poly(A)-nuclease Activity*

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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-kDa 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 laevis 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) tail plays a role in translation. It has 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 (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

¹The 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.

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‡ Supported by a post-doctoral fellowship from the Swiss National Science Foundation.

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1 Tarun, S., and Sachs, A. B. (1995) Genes & Dev. 9, 2997–3007.
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<sub>b</sub> = 1) harvested from a 200-liter culture of YPD (12) were washed twice in 1 l of buffer A (50 mM Tris, pH 7.4, 2 mM MgAc, 14 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5% 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 previously (1). Briefly, up to 10 µl of protein sample was mixed with 1 µl of 200 ng/ml recombinant Pan1p and incubated for 15 min on ice. 5 µl of a cold RNA mixture containing 2 µg of RNA and 5000 cpm of homogeneously labeled 32P-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 MgCl₂, 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 µl of Amcasul (5 M NaHCO₃, 0.5 M NaCl, pH 8.3) to resin overnight 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 AmSO₄ 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 was added to 60% final concentration with slow stirring over 30 min.

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 AmSO₄ 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 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 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 was added to 60% final concentration with slow stirring over 30 min.
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 0-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 ng of the 38-kDa purified PAN1 fusion protein was purified from 3 liters of bacteria harvested at A600 = 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.

Polymerase chain reaction—Pan1p was detected by Western analysis of crude extracts from 0.3 OD600 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 175 volts/cm in transfer buffer (10% methanol, 10% TBE). 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 then washed 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 end mixing for 90 min at 4 °C, the suspension was centrifuged for 30 s at 14,000 g and 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 Pan2p gene was disrupted in the yeast strain YAS306 (MATα 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. The amplified fragment was digested with three restriction enzymes (OAS181, Bgl II, and EcoRI) and inserted into the PstI site of plasmid pAS135. The resulting plasmid pAS135-PAN2URACEN was transformed into the yeast strain YAS1838 (MATα pAS464) by LiAc transformation (1). To test for disruption of the PAN2 gene, 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 (BioRad) 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'-GAAGATCTACCGGCTTCTTCTGATCACTT-3') and OAS189 (5'-CTCGAGCCGTCGCTGTTAAT-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-32P]dCTP using random hexamer priming. The membrane was incubated for 14 h at 65 °C in 1 ml EDTA, 0.5 M NaPO4, pH 7.2, 7% SDS containing 2 × 105 cpm mini Pan2 probe and then washed in 1 ml EDTA, 40 mM NaPO4, 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. 1A 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 bums 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 resin, and the vast majority of it eluted between 150 and 200 mM potassium acetate (Fig. 1A). 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. 1B, 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 protein phosphorylase a, and that the Zeta-Probe membrane (BioRad) using standard techniques. The Pab1 probe used for the Southern analysis consisted of a 0.45-kb DNA fragment, amplified using 20 µM OAS188 (5'-GAAGATCTACCGGCTTCTTCTGATCACTT-3') and OAS189 (5'-CTCGAGCCGTCGCTGTTAAT-3'), corresponding to 450 nucleotides upstream of the Pab1 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-32P]dCTP using random hexamer priming. The membrane was incubated for 14 h at 65 °C in 1 ml EDTA, 0.5 M NaPO4, pH 7.2, 7% SDS containing 2 × 105 cpm mini Pan2 probe and then washed in 1 ml EDTA, 40 mM NaPO4, 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).
The large scale purification is quite good, although it is 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. 2A) 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. 2B. 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 1:1,000. 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. 2C). 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. 3A). However, the amount of the 135-kDa protein detected by silver staining in this eluate did not change before and after immunodepletion.
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. 3C). The data provide definitive evidence that Pan1p is not required for the PAN enzymatic activity, and that the predominant 135-kDa protein purified by the immunodepletion procedure is not Pan1p. However, these data cannot rule out the possibility that Pan1p is associated with PAN enzymes since it is present in substoichiometric amounts in our preparations. 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 databases.

### Table I

| Step | Total protein (mg) | Total units | Specific activity (units/10 mg) | Recovery (%) | Purification -fold |
|------|-------------------|-------------|-------------------------------|-------------|-------------------|
| S100 | 12,195            | 720         | 0.59                          | 60%         | 1                 |
| 60% AmSO₄ | 4,600          | 7,900       | 117                          | 100         | 198               |
| DEAE | 756               | 6,850       | 489                          | 49          | 829               |
| Q-Sepharose | 88           | 4,500       | 3,640                        | 100         | 6,170             |
| P-cellulose | 25            | 9,100       | 55,900                       | 22          | 94,750            |
| Poly(U) | 0.34             | 1,300       |                               |             |                   |

*Enzyme units are as defined in Ref. 1.

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**Fig. 2.** Production of Pan1p antibodies using recombinant Pan1p antigen. **A,** schematic diagram of the Pan1p. The two repeated NH₂-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. **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 Pab1-Sepharose. An equal percentage of the indicated column eluates from Fig. 1B 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”).
Pan2p, and not to an indirect effect of changing the chromosomal region by deleting 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 gene grew at approximately 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. 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 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. 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. 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. 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. 6A, 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. 6B). As previously reported (1), S100 extracts from wild type strains showed a stimulation of PAN activity when recombinant Pab1p was added to them (Fig. 6B, 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. 6B, 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. 6B, 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. 4.** Nucleotide and predicted amino acid sequence of the PAN2 gene. Underlined region of the open reading frame indicates the peptide sequences derived by protein sequencing. The boxed region indicates the peptide fragment identified in a previous PAN preparation (1). The Genbank accession number for PAN2 is U35204.
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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.

Acknowledgment—We thank Christine Brown for valuable advice and comments on the manuscript.

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