Monoclonal Antibodies to Yeast Poly(A) Polymerase (PAP) Provide Evidence for Association of PAP with Cleavage Factor I†

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ABSTRACT: Purified yeast poly(A) polymerase (PAP) was used to produce monoclonal antibodies which recognize the enzyme in immunoblots. Epitope mapping using truncated forms of PAP and cyanogen bromide cleavage products revealed two classes of antibodies. One class (N-term) recognizes an eptiope in the first 100 amino acids, and a second class (C-term) is specific for a determinant located in the last 20 amino acids of PAP. These C-terminal 20 amino acids can be removed without affecting the nonspecific poly(A) addition activity of the purified enzyme. Neither antibody inhibits the nonspecific poly(A) polymerase activity or the sequence-specific activity observed in processing extracts. The antibodies show species specificity and cannot recognize mammalian, *Xenopus*, or vaccinia PAP. The C-term antibodies can deplete PAP from yeast whole cell extracts, resulting in loss of poly(A) addition activity. This immunodepletion also causes a reduction in the cleavage activity which can be restored by addition of yeast cleavage factor I [CF I; Chen, J., & Moore, C. (1992) *Mol. Cell Biol. 12*, 3470−3481], a factor needed for both the cleavage and poly(A) addition reactions. This demonstrates that a complex of PAP and CF I exists in extracts in the absence of ATP or exogenous RNA substrate. The monoclonal antibodies against yeast PAP will be a useful tool for further study of factors required for yeast mRNA 3′ end processing.

All mRNAs in yeast cells are polyadenylated at their 3′ ends (Jackson & Standart, 1990). These poly(A) tails are 50−60 nucleotides in length and are thought to be involved in stability and translation. The poly(A) tail is added to the 3′-OH generated after endonucleolytic cleavage at the poly(A) site of the primary transcript. This process occurs in the nucleus, and the use of alternative poly(A) sites can affect the kind and availability of mRNAs derived from some genes (Manley & Proudfoot, 1994).

Saccharomyces cerevisiae has been a very useful organism for the study of many aspects of mRNA metabolism including transcription (Sawadogo & Sentenac, 1990), splicing (Guthrie, 1991), transport from the nucleus to the cytoplasm (Amberg et al., 1992), translation (Linder & Prat, 1990), and mRNA stability (Sachs, 1993). The formation of mRNA 3′ ends has been investigated in yeast with the aid of the in vitro system developed by Butler and Platt (1988). Recently, Chen and Moore (1992) separated and identified four factors which are required for cleavage and polyadenylation in yeast. One of these components is the poly(A) polymerase (PAP)[1] which has been purified (Lingner et al., 1991b) and cloned (Lingner et al., 1991a). The yeast gene encoding PAP (*PAP1*) is essential (Lingner et al., 1991a), and a temperature-sensitive mutation in this gene has been isolated (Patel & Butler, 1992).

Human and bovine PAP cDNAs have also been cloned (Thuresson et al., 1994; Raabe et al., 1991; Wahle et al., 1991) and show 47% identity with the yeast PAP, primarily in the N-terminal 400 amino acids of the protein (Lingner et al., 1991a). Mammalian PAP contains a ribonucleoprotein (RNP)-type RNA binding domain (RBD) which is found near the amino-terminal end. Raabe et al. (1994) showed that mutations in the RBD region of bovine PAP cause a reduction of polyadenylation activity. This reduction can be relieved by higher primer concentrations, suggesting a role for the RBD domain in primer binding. The yeast PAP contains an RBD domain in the N-terminal region as well (Lingner et al., 1991a). Bovine PAP also contains a region necessary for catalytic activity adjacent to the RBD domain (Raabe et al., 1994). In addition, bovine PAP contains two nuclear localization signals (NLS) required to direct PAP to the nucleus. Interestingly, amino acids in one NLS are also required for AAUAAA-dependent polyadenylation (Raabe et al., 1994). Finally, bovine and human PAP have serine/threonine-rich C-terminal regions which are phosphorylated (Thuresson et al., 1994; Raabe et al., 1994). Little else is known about the domains involved in ATP binding, polymerization activity, and interaction with other factors involved in 3′ end processing.

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[1] Abbreviations: PAP, poly(A) polymerase; CF, cleavage factor; CPSF, cleavage/polyadenylation specificity factor; PABII, poly(A) binding protein II; PF I, polyadenylation factor I; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; PEG, poly(ethylene glycol); TCA, trichloroacetic acid; PMSF, phenylmethanesulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PVDF, poly(vinylidene difluoride); IPTG, isopropylthio β-D-galactoside. GST, glutathione *S*-transferase.

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The principal function of PAP is the synthesis of mRNA poly(A) tails. PAP can also add poly(A) tails nonspecifically to the 3′-OH of any RNA. This reaction is enhanced by Mn^{2+}, which increases the affinity of the enzyme for the primer (Wahle, 1991). In the presence of other factors, PAP adds poly(A) only to the 3′-OH generated after cleavage of the primary transcript. These interactions are best understood for mammalian PAP. This enzyme on its own has a highly distributive mode of action, which changes to a processive reaction in the presence of additional factors. One of these is the cleavage and polyadenylation specificity factor (CPSF), which is a multisubunit RNA binding factor that recognizes the AAUAAA sequence on the pre-mRNA and functions in cleavage and in poly(A) addition (Bienroth et al., 1991; Murthy & Manley, 1992). Poly(A) addition by purified PAP and CPSF is also stimulated by the poly(A) binding protein II (PABII), which recognizes the first 10−12 adenylate residues and then increases the rate of extension (Sachs & Wahle, 1993). Cleavage of mammalian pre-mRNAs requires CPSF and three additional factors, a cleavage stimulation factor (CstF) and two cleavage factors (CF1 and CF2) (Manley & Proudfoot, 1994). Mammalian PAP is also required for efficient cleavage of many pre-mRNAs (Manley & Proudfoot, 1994; Sachs & Wahle, 1993). The activity of mammalian PAP can also be negatively modulated by the U1 snRNP A protein (U1A). This protein autoregulates the synthesis of its own mRNA by inhibiting poly(A) addition activity. This inhibition requires the carboxyl-terminal region of mammalian PAP (Manley & Proudfoot, 1994). PAP is also involved in the extension of the poly(A) tails of specific cytoplasmic mRNAs which are translated during oocyte maturation (Bilger et al., 1994).

In yeast, cleavage of the pre-mRNA requires two cleavage factors (CF I and CF II), while poly(A) addition requires CF I, PAP, and an additional polyadenylation factor (PF I) (Chen & Moore, 1992). Since CF I is required for both reactions, its function is probably analogous to that of mammalian CPSF.

To study functional regions of PAP and its interactions with other proteins, reagents such as monoclonal antibodies are required. In this paper, we present the isolation and characterization of monoclonal antibodies which recognize epitopes in the N- and C-terminal regions of PAP. Antibodies which recognize the C-terminal region of PAP can deplete PAP from yeast whole cell extracts, but neither antibody can inhibit poly(A) addition activity. Extracts depleted of PAP are also defective in cleavage activity, which can be restored by addition of the CF I factor. This suggests a stable interaction between the yeast PAP and CF I.

MATERIALS AND METHODS

Reagents. Sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE) prestained protein standards were obtained from Amersham and New England Biolabs. Unstained markers were from New England Biolabs. Acetylated bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), and yeast tRNA were from Sigma. Standards for protein concentration were from Sigma or from Bio-Rad. Protease inhibitors were from Boehringer-Mannheim. Mice were from Jackson Laboratories. Materials used for chromatography were purchased from the indicated vendors and prepared according to the manufacturer's instructions.

Poly(A) Polymerase Assays. Poly(A) polymerase was assayed for its ability to add poly(A) to the 3′ end of yeast tRNA in the presence of Mn^{2+} as described by Chen and Moore (1992). Reactions were conducted in 50 μL containing 20 mM Tris-HCl, pH 8.0, 2% poly(ethylene glycol) (PEG) 8000, 0.1 mM ATP, 20 mM creatine phosphate, 5 μg of yeast tRNA, 0.1 μL of [α-^{32}P]ATP (10 mCi/mL), and 0.5−10 μL of fraction or extract containing PAP. The reaction mixtures were incubated for 30 min at 30 °C and stopped by addition of 50 μL of 20 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.2 M NaCl, 0.4% SDS, and 0.4 mg/mL BSA. Nucleic acids were precipitated with 1 mL of ice-cold 10% trichloroacetic acid (TCA) and incubation on ice for 10 min. The samples were then applied to Whatman CF/A glass fiber filters. These filters were then washed with 5% TCA and then with 95% ethanol and allowed to dry, and the radioactivity was measured by Cherenkov counting. The assay reaction for fractions from the Mono S column contained 0.8 mg/mL acetylated BSA and did not contain creatine phosphate.

Cleavage and Polyadenylation Assays. Cleavage and poly(A) addition assays were conducted with a radiolabeled yeast *Gal7* substrate, described by Chen and Moore (1992), which contains 161 nucleotides upstream and 133 nucleotides downstream of the poly(A) site. Processing reactions were carried out in 12 μL containing 10 nM ^{32}P-labeled pre-mRNA, 2 mM ATP, 20 mM creatine phosphate, 1 mM magnesium acetate, 75 mM potassium acetate, 2% PEG 8000, and 2 μL of extract. Reconstitution reactions contained 2 μL of PAP-depleted extract and the amounts of PAP and/or CF I described in the figure legends. Reactions mixtures were incubated at 30 °C for 20 min, and stopped with proteinase K and SDS as described by Chen and Moore (1992). After extraction with phenol/chloroform/isoamyl alcohol (24:24:1), $^1/_{20}$th of the reaction products was fractionated on 5% polyacrylamide/8.3 M urea gels and visualized by autoradiography. Poly(A) addition reactions using whole cell extracts were conducted with the *Gal7* substrate precleaved at the poly(A) site (Chen & Moore, 1992) with the same conditions described above in the presence of 1 mM $MnCl_2$.

Proteins. The protein concentration in PAP-containing fractions was determined by the method of Bradford (1976) or from SDS−PAGE gels stained with Coomassie (Lingner et al., 1991b) using serum albumin as standard. To measure the concentration of purified antibody preparations, bovine plasma γ-globulin (Bio-Rad) was used as standard.

Buffers. Buffer 1: 0.2 M Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 μM pepstatin A, and 0.6 μM leupeptin. *Buffer 2*: 20 mM Tris-HCl, pH 8.0. *Buffer 3*: 30 mM potassium phosphate, pH 6.80. *Buffer 4*: 10 mM potassium phosphate, pH 6.80, no EDTA. *Buffer 5*: 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)−KOH, pH 7.0. Buffers 2−5 also contained 0.2 mM EDTA, 10% glycerol, and 0.5 mM dithiothreitol (DTT) unless indicated otherwise. The concentration of KCl or potassium phosphate in different fractions was measured with a conductivity meter calibrated with standards of known salt concentration containing all components in the buffer.

Purification of Yeast PAP. All procedures were done at 0−4 °C, and all fractions were frozen in liquid nitrogen and stored at −70 °C. *S. cerevisiae* 1097/930 (gift from C.

Guthrie) was grown as described by Chen and Moore (1992). The packed cells (100 g of cells from 32 L in culture) were washed twice in water, resuspended at 2 mL/g of cells in buffer 1, and lysed with glass beads as described by Klekamp and Weil (1982). PMSF and mercaptoethanol were added to 1 mM; the lysate was adjusted to 0.5 M KCl, and slowly stirred for 30 min. The extract was cleared by centrifugation at 23000*g* (Sorvall GSA rotor) for 30 min followed by 200000*g* (Beckman Ti50.2 rotor) for 2 h and then dialyzed for 6 h at 4 °C against 4 L of buffer 2 containing 40 mM KCl, 20% glycerol, 1 mM PMSF, 2 μM pepstatin A, and 0.6 μM leupeptin. All buffers used in the chromatographic steps described contained 1 mM PMSF, 2 μM pepstatin A, and 0.6 μM leupeptin, except in the case of the Mono S column. The yeast extract was diluted with buffer 2 to reach the conductivity equivalent to 40 mM KCl and 10% glycerol, and loaded on a DEAE-Sephacel column (Pharmacia, 5 × 46 cm in two applications) equilibrated with buffer 2 containing 40 mM KCl. The column was washed with 2 volumes of this same buffer, and proteins were eluted with a 2 L gradient from 40 to 500 mM KCl in buffer 2. The PAP activity eluted at 80 mM KCl. Fractions containing PAP were pooled, adjusted to 30 mM potassium phosphate, and loaded on a phosphocellulose column (Whatman, 1.5 × 22.6 cm) equilibrated with buffer 3 containing 80 mM KCl. The column was washed with 2 column volumes of this same buffer, and proteins were eluted with a 10 column volume gradient from 30 to 400 mM potassium phosphate in buffer 3 (with 80 mM KCl). Fractions containing PAP, which eluted at 300 mM potassium phosphate, were pooled, dialyzed against buffer 4 for 3 h at 4 °C, and applied to a 2.5 mL hydroxyapatite column (Bio-Rad HT 1 × 3 cm) equilibrated in buffer 4. After the column was washed with 2 column volumes of this same buffer, proteins were eluted with a 10 column volume gradient from 10 to 400 mM potassium phosphate in buffer 4. PAP eluted at 300 mM potassium phosphate. The pooled fractions from this column were dialyzed against buffer 5 containing 50 mM KCl for 2 h at 4 °C and applied to a 1 mL Mono S FPLC column (Pharmacia, run at 0.5 mL/min) equilibrated in the same buffer. After the column was washed with 5 mL of starting buffer, it was developed with a 40 mL gradient from 50 to 500 mM KCl in buffer 5, as used by Lingner et al. (1991b). PAP eluted at 250 mM KCl. This material was used for primary injections in three mice.

The PAP used for second and third booster injections was purified from yeast whole cell extracts with cleavage and polyadenylation activities as described by Butler and Platt (1988) and modified by Chen and Moore (1992). These extracts were fractionated on DEAE-Sephacel and phosphocellulose columns as described above. The hydroxyapatite step was skipped, and the sample was directly applied to the Mono S column also as described above.

Purification of Recombinant PAP. PAP used for the fourth booster and final injections as well as for hybridoma supernatant screening was obtained in recombinant form using the overexpression system described by Lingner et al. (Lingner et al., 1991a; Lingner & Keller, 1993). Extract (510 mg of protein) was prepared from a 3 L culture of *Escherichia coli* as described (Lingner et al., 1991a; Lingner & Keller, 1993), diluted to 100 mM KCl with buffer 2, and applied to a 70 mL DEAE-Sephacel column. All PAP activity appeared in the flow-through fraction (118 mg), which was adjusted to 30 mM potassium phosphate and loaded on a 8 mL phosphocellulose column equilibrated with buffer 3 with 80 mM KCl. The column was washed and developed as described above. The recombinant PAP eluted at 300 mM potassium phosphate. Fractions containing PAP (26 mg) were applied to a 1 mL Mono S FPLC column which was run as described above. PAP activity eluted as the major protein peak at 250 mM KCl (8 mg). Some of this material was further purified by using heparin−Sepharose chromatography. PAP from Mono S (0.78 mg) was diluted twice in buffer 5 and applied to a 1 mL Hi-Trap heparin column (Pharmacia) equilibrated with buffer 5 containing 125 mM KCl. After the column was washed with this same buffer, PAP was eluted with a gradient from 125 to 500 mM KCl in buffer 5. PAP (0.7 mg) eluted at 350 mM KCl.

Production of Monoclonal Antibodies to PAP. For primary injections, 10 μg of PAP purified from yeast and 90 μg of KLH carrier in 0.1 mL were added to 0.1 mL of 10% aluminum potassium sulfate (Schick et al., 1987). The pH was adjusted to 7 with 1 N NaOH. After being mixed for 20 min at 25 °C, the mixture was centrifuged at 8000*g* for 10 min. The pellet was washed in phosphate-buffered saline (PBS), resuspended in saline containing heat-killed *B. pertussis* (4×10^6 cells), and injected intraperitoneally into three adult female Balb/c ByJ mice.

Booster injections of PAP purified from yeast contained PAP (10 μg) and complete Freunds' adjuvant. Booster injections with recombinant PAP contained 18 μg of PAP (in 0.125 mL of PBS) mixed with 0.125 mL of Titermax adjuvant (Bennett et al., 1992). All boosts were intraperitoneal injections. All final injections were made 3 days before fusion and contained 12.5 μg of recombinant PAP in 0.25 mL of PBS injected intraperitoneally.

The immune response was monitored by immunoblot analysis using PAP as antigen and dilute serum from tail bleeds as probes. Preimmune serum from the same mice was used as negative control. Mice that gave a good immune response were sacrificed, and their spleen cells were fused with either X63-Ag8.653 (Kearney et al., 1979) or SP2/0 (Shulman et al., 1978) myeloma cells using poly(ethylene glycol) (PEG) according to standard methods (de St. Groth & Scheidegger, 1980; Galfre & Milstein, 1981). After fusion, the cells were incubated overnight at 37 °C in a T-75 flask and then seeded into 96-well microtiter dishes containing 0.1 mL (per well) of peritoneal macrophages prepared from Balb/c ByJ mice as described (de St. Groth & Scheidegger, 1980). Media used to select hybridomas contained RPMI 1640 supplemented with 25 mM HEPES, 0.7 mM L-glutamine, 100 units of penicillin G/mL, 100 μg/mL streptomycin sulfate, 0.05 mM β-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 15−20% CPSR-3 serum (Sigma), 0.1 mM hypoxanthine, and 1 μg/mL azaserine (Foung et al., 1982).

Fusions were tested for PAP-reacting antibodies by a dot-immunoblotting assay described below (Hawkes et al., 1982). Immunoreactivity was confirmed by using immunoblots of PAP electrophoresed in SDS−PAGE gels. In all blotting assays, monoclonal antibodies produced against the papilloma E2 protein (a gift of Dr. Elliot Androphy, Tufts University) were used as negative controls. Hybridomas producing antibodies which reacted with PAP were cloned twice by limited dilution (Harlow & Lane, 1988). The antibodies are of the IgG_1 isotype, as determined by an

enzyme-linked immunosorbent assay (ELISA) with a mouse IgG isotyping kit (Sigma).

Electrophoresis and Immunoblots. SDS−polyacrylamide gels and immunoblots were done essentially as described by Zhelkovsky and Moore (1994). Briefly, proteins were separated in SDS gels (Laemmli, 1970), soaked in transfer buffer (25 mM Tris/192 mM glycine and 20% methanol) for 25 min, and transferred to poly(vinylidene difluoride) (PVDF Immobilon P membranes, Millipore) according to the method of Towbin et al. (1979). Detection of proteins in SDS gels was done by silver staining (Gottlieb & Chavko, 1987). Nonspecific protein binding sites in the membranes were blocked with a buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20 (TBST), and 10% nonfat dry milk for 1 h at 25 °C. When less background was desired, the blots were treated with 10% fetal calf serum and 0.2% Tween 20 in PBS overnight (Zhelkovsky & Moore, 1994). Blotted proteins were reacted with antibody-containing serum (diluted 1:500 to 1:1000 in TBST) or hybridoma supernatant (diluted 1:10 to 1:100 in TBST) for 1−2 h at 25 °C. After being washed in TBST, the blots were treated with an alkaline phosphatase-conjugated anti-mouse IgG secondary antibody (Promega). Immunoreactive peptides were visualized by reaction with the alkaline phosphatase substrates 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitrotetrazolium blue (Promega) in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, and 5 mM $MgCl_2$ (Blake et al., 1984; McGadey, 1970).

Dot-Immunoblotting Assay. To detect PAP-specific antibodies in hybridoma supernatants, a modification of the method of Hawkes et al. (1982) was used. Recombinant PAP (60−100 ng in 30 μL of immunoblotting transfer buffer with 10% methanol) was applied to PVDF membranes using a mini filtration apparatus. Following blocking of nonspecific protein sites as described above, the filters were reintroduced in the mini filtration apparatus in their original position over a sheet of Parafilm. After assembly, the wells of the apparatus were filled with 50 μL of diluted hybridoma supernatant in contact with the membrane and incubated for 1 h at room temperature. Liquid was removed from the wells, and they were washed 3 times in TBST buffer. The assembly was dismantled, and the membranes were washed and treated with secondary antibody and alkaline phosphatase substrates as described above.

Epitope Mapping Using Truncated PAPs. Deleted recombinant PAPs were generated by mutagenesis of the wild-type *PAP1* gene in the expression vector pJPAP1 (Lingner et al., 1991a; Lingner & Keller, 1993). Three deletions were created using available restriction sites. The first one (Δ1) eliminated amino acids 217−467 (*Apa*I to *Stu*I). The second deletion lacks amino acids 2−100 (*Sac*I to *Eco*RV). In the third deletion, amino acids 549−568 are missing (*Bgl*II to *Pvu*II). All constructions were made in plasmid pJPAP1 and the DNAs used to transform the expression strain BL21DE3-(pLysS) (Studier, 1991). *E. coli* cells expressing the different PAPs were grown as described by Lingner et al. (Lingner & Keller, 1993) with induction with IPTG for 2 h at 37 °C. Extracts used for immunoblotting were prepared by lysing cells in SDS. To measure PAP activity, extracts were prepared by first washing the cells in PBS containing 1 mM PMSF, resuspending them in 10 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Nonidet P-40, 0.25 M KCl, 0.5 mM DTT, 1 mM PMSF, 2 μM pepstatin A, and 0.6 μM leupeptin) per liter of cell culture, and freezing in liquid nitrogen. The cells were then thawed and lysed by incubation at 30 °C for 20 min. The extract was then cleared by contrifugation at 213000*g* (Beckman TL 100.2 rotor) for 20 min at 4 °C.

Epitope Mapping Using CNBr-Generated Fragments of PAP. Purified recombinant PAP was cleaved by treatment with CNBr as described by Nikodem and Fresco (1979). Eighty micrograms of PAP was first dialyzed against 5 mM acetic acid for 4−5 h and then lyophilized. The protein was then resuspended in 40 μL of 70% formic acid, and 0.2 mg of fresh sublimated CNBr was added in 4 portions every 1 or 2 h and then incubated overnight in the dark. The reaction was stopped by dilution in water followed by two rounds of lyophilization. Peptides were resuspended in water and kept frozen. Peptides generated by CNBr cleavage of PAP were fractionated in 16.5% acrylamide gels prepared with the Tris/Tricine/SDS gel system described by Schagger and von Jagow (1987) and modified by Plaug et al. (1989). Peptides fractionated in these gels were transferred to a PVDF membrane, stained with Coomassie blue, or used for immunoblotting as described above.

Purification of IgG Monoclonal Antibodies. For the production of ascites fluid, hybridomas were injected into Balb/c ByJ mice primed 1 week earlier with Pristane (Sigma) (Harlow & Lane, 1988). Two weeks later, ascites fluid was collected by tapping twice. Immunoglobulins (IgGs) were purified from either ascites fluid or culture supernatants by protein A−Sepharose as described by Harlow and Lane (1988). For ascites fluid, the antibodies were first precipitated by addition of 45% ammonium sulfate followed by resuspension and dialysis in PBS. For application to a protein A−Sepharose (Zymed) column, the antibody solutions were adjusted to 3 M NaCl and 0.1 M sodium borate, pH 8.9. Unbound proteins were removed with this same buffer and then with 3 M NaCl and 10 mM sodium borate, pH 8.9. The C-term class of antibodies was eluted with 0.1 M glycine hydrochloride, pH 2.5. The N-term class of antibodies was eluted with 0.1 M glycine hydrochloride, pH 2.2. Eluted IgGs were immediately neutralized with 0.1 volume of 1 M Tris-HCl, pH 9.5, then dialyzed against PBS, and concentrated in Millipore GL micro concentrators.

Immunodepletion of PAP from Yeast Whole Cell Extracts. First, antibodies were attached to Protein G Gamma-Bind Plus Sepharose beads (Pharmacia); 0.3 mL beads were drained and mixed with 0.2 mL of Gamma-Bind buffer (10 mM sodium phosphate, pH 7.0, 0.15 M NaCl, 10 mM EDTA, and 0.5 mg/mL acetylated BSA) containing 15 μg of purified antibodies or 0.3 mL of hybridoma supernatant. Negative controls included no antibodies and antibodies against β-galactosidase (Sigma). Antibodies and beads were incubated overnight at 4 °C with gentle mixing. The beads were then transferred to disposable columns (Bio-Rad) and washed twice with AB buffer (20 mM Tris-HCl, pH 7.8, 0.125 M NaCl, 1 mM PMSF, and 0.1 mM EDTA). Each column was loaded with 0.2 mL of solution containing 0.1 mL of yeast whole cell extract (11 mg/mL) and 0.1 mL of AB buffer. The loaded columns were stirred gently with a pipet tip and kept at 4 °C for 10−15 min. Unbound material was collected by gravity flow and added back to the column. This process was repeated 3 times. The final column flow-through was dialyzed against 1 L of buffer 2 containing 50

mM KCl, 1 mM PMSF, 0.6 μM leupeptin, and 2 μM pepstatin A, for 2 h at 4 °C. This material was assayed for cleavage and polyadenylation with precursor RNA containing the *Gal7* poly(A) site, as described above. Assays for poly(A) addition were done with precleaved *Gal7* precursor described by Chen and Moore (1992) in the presence of 1 mM $MnCl_2$. These extracts were also analyzed by immunoblots to determine the extent of PAP depletion. For reconstitution experiments, purified recombinant PAP was added to the extracts depleted of PAP as well as partially purified CF I from yeast. The details of the CF I purification will be described in a separate manuscript.

RESULTS

Purification of PAP. By using a combination of chromatographic methods, PAP was isolated from yeast extracts to near-homogeneity, resulting in a 13 000-fold purification relative to the crude extract. A major band of 63 kDa in size was obtained which migrates slightly faster than BSA on SDS−PAGE gels (not shown). This is in agreement with the purification of Lingner et al. (1991b). The PAP purification scheme was modified later by first precipitating proteins with 40% ammonium sulfate and omitting the hydroxyapatite step, resulting in an 11 000-fold purification.

Due to the low abundance of PAP obtained from yeast, it was necessary to purify recombinant PAP using the T7 expression system in *E. coli* described by Lingner et al. (Lingner et al., 1991a; Lingner & Keller, 1993) to obtain the quantities needed for screening hybridoma cell supernatants. Three liters of culture yielded approximately 8 mg of recombinant PAP.

Production of Monoclonal Antibodies to PAP. From 3 separate immunization and fusion procedures, a total of 16 stable monoclonal hybridoma cell lines producing antibody to yeast PAP were isolated. Of these, 13 gave a stronger response and were chosen for further characterization. Because the secondary antibody used in the screening of hybridoma supernatants was anti-mouse IgG, only lines producing IgG antibodies were detected. Antibodies produced by all 13 cell lines recognize PAP in immunoblot experiments.

Mapping the Monoclonal Antibody Epitopes on PAP. To localize epitopes on PAP recognized by the monoclonal antibodies, two approaches were used. In the first approach, three deletions were created directly in the gene coding for the yeast PAP (Figure 1A). In the second, purified PAP was digested with CNBr to generate different peptides (Figure 1B) which were separated by electrophoresis, transferred to a PVDF membrane, and tested for immunoreactivity with the different hybridoma supernatants. Both approaches revealed two classes of monoclonal antibodies. One class (N-term) recognizes the N-terminal region of PAP, and a second class (C-term) recognizes the C-terminal region of PAP.

(A) Analysis of PAP Deletions. Three deleted forms of PAP were generated as shown in Figure 1A. The first deletion eliminated 40% of the internal region of PAP (amino acids 217−467). The second truncated PAP lacks amino acids 2−100 at the N-terminus, and the third is missing amino acids 549−568 at the C-terminus. Extracts were prepared from *E. coli* cells expressing the wild-type and truncated PAPs and assayed for Mn^{2+}-dependent poly(A) addition to tRNA as described under Materials and Methods. Extracts from cells expressing deletions 1 and 2 had no detectable poly(A) addition activity, except for that endogenous to *E. coli* (Cao & Sarkar, 1992). This endogenous activity is present in extracts from cells expressing vector without the *PAP1* gene and is 200-fold less than that of cells expressing wild-type yeast PAP. Extracts from cells expressing deletion 3 retained 100% of the poly(A) addition activity of extracts prepared from cells expressing wild-type PAP. This result indicates that regions of PAP located in amino acids 217−467 and in 2−100 are required for poly(A) addition activity. The results also indicate that amino acids 549−568 at the C-terminus of PAP are not required for poly(A) addition to tRNA.

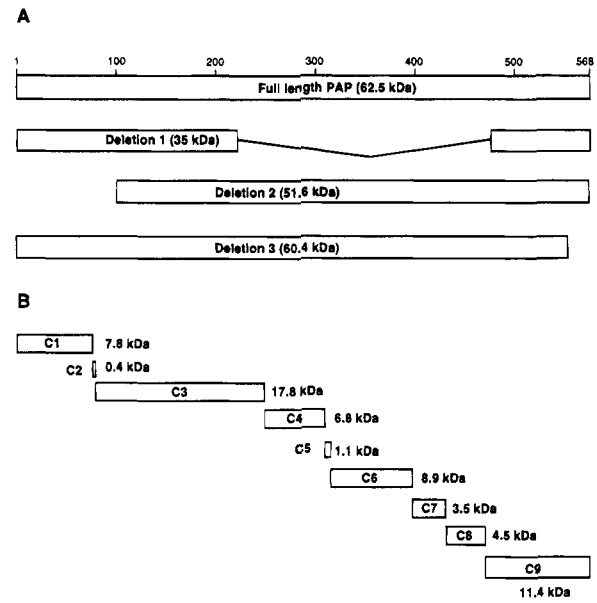

FIGURE 1: Mapping of epitopes on PAP recognized by the monoclonal antibodies. (A) Diagram of deletions created in the PAP protein by mutagenesis of the *PAP1* gene and expression in *E. coli*. The scale represents the number of amino acids. (B) Diagram of the nine peptides (C1−C9) generated by CNBr cleavage of purified recombinant PAP. The molecular masses of the fragments are calculated from the number of amino acids.

Both the N-term and C-term antibodies recognize the wild-type PAP in immunoblots (Figure 2, lanes 5 and 10). No reaction was observed with monoclonal antibodies directed to the papilloma E2 protein (not shown). The monoclonal antibodies are PAP-specific because they do not recognize BSA (not shown) and do not recognize *E. coli* proteins from extracts derived from cells expressing vector lacking the *PAP1* gene (Figure 2, lanes 6 and 11).

Immunoblot analysis of extracts from *E. coli* expressing recombinant PAP with deletion 1 showed that both the N-term and the C-term class of monoclonal antibodies recognize the truncated PAP of 35 kDa (Figure 2, lanes 2 and 7). This result suggests that the epitopes recognized by these antibodies must be located in either the first 216 amino acids of PAP (1−216) or the 102 amino acids at the C-terminal end (467−568).

Immunoblot analysis of extracts from *E. coli* expressing PAP deletion 2 showed that antibodies from 6 of the 13 hybridoma lines tested (N-term) could no longer recognize the 51.6 kDa truncated PAP which lacks 100 amino acids at the N-terminus (for example, Figure 2, lane 3). However, antibodies from the other seven hybridoma lines (C-term)

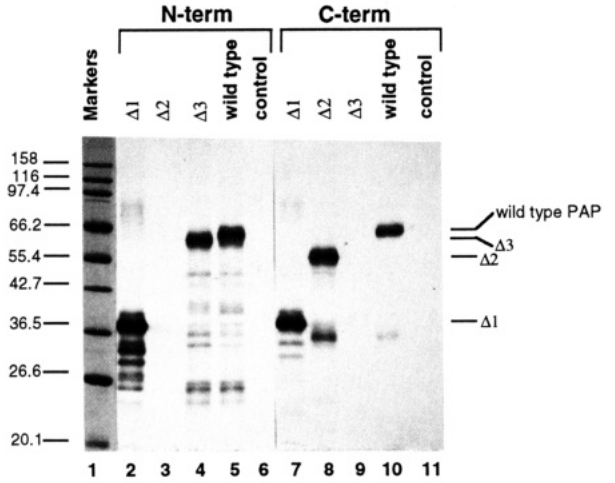

FIGURE 2: Immunoblot analysis of extracts from *E. coli* cells expressing wild-type and truncated forms of PAP. Extracts from cells expressing wild-type PAP (lanes 5 and 10), PAP deletion 1 (lanes 2 and 7), PAP deletion 2 (lanes 3 and 8), PAP deletion 3 (lanes 4 and 9), and vector without PAP (lanes 6 and 11) were separated in a SDS−polyacrylamide gel, transferred to PVDF, and immunostained with dilutions of the N-term class of antibodies (lanes 2−6) or the C-term class of antibodies (lanes 7−11). Lane 1 contains blotted protein markers stained with Coomassie blue. The sizes of protein molecular mass markers are given in kilodaltons.

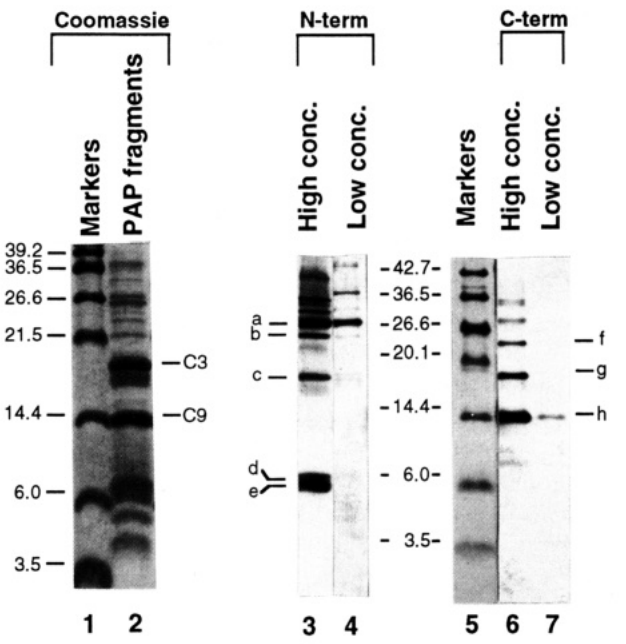

FIGURE 3: Blot analysis of PAP fragments generated with CNBr. Purified recombinant PAP was digested with CNBr; fragments were separated on Tris/Tricine/SDS gels and blotted onto a PVDF membrane as described under Materials and Methods. Lanes 1 and 5 contain molecular mass markers (sizes are indicated in kilodaltons); lanes 2, 3, 4, 6, and 7 contain PAP fragments. Lanes 1, 2, and 5 were stained with Coomassie blue. Lanes 3 and 4 were immunostained with N-term antibodies. Lanes 6 and 7 were immunostained with C-term antibodies. In lanes 3 and 6, more antigen (50 times) was loaded on the Tricine/SDS gel as compared to lanes 4 and 7.

still recognize PAP with deletion 2 (for example, Figure 2, lane 8). These results with the first two deletions indicate that two classes of antibodies are being produced by the hybridoma lines.

To more precisely map the location of the epitope recognized by the C-term class of antibodies, deletion 3 was generated. Immunoblot analysis of extracts from *E. coli* cells expressing this deletion of PAP showed that this class of antibodies cannot recognize this 60.4 kDa truncated PAP (Figure 2, lane 9), but that the N-term class of antibodies can (Figure 2, lane 4). This result indicates that the epitope recognized by the C-term class of antibodies is located in the 20 amino acids (549−568) at the C-terminal end of PAP.

(B) CNBr Cleavage at Methionines. Cleavage of purified PAP at methionine residues by CNBr yields nine peptides as predicted from the amino acid sequence. The size and location within PAP of these fragments (designated C1−C9) are shown in Figure 1B. These peptides can be separated using high-resolution protein gels (Figure 3, lane 2). Major peptides detected are one of 17.8 kDa (C3, amino acids 76−238), one of 11.4 kDa (C9, corresponding to the C-terminal peptide, amino acids 464−568), a group of peptides of 7−9 kDa in size, and some smaller peptides of 6 kDa and below. Larger peptides can also be seen which correspond to partial CNBr digestion products. In this type of gel, the 14.4 kDa protein marker (lysozyme) has a faster electrophoretic mobility (11 kDa) than that predicted from its size.

When blots containing the separated peptides were treated with supernatants from the different hybridoma cell lines, again two classes of antibodies were detected. The first class of antibodies (N-term) does not recognize any of the CNBr-generated fragments of sizes 18 kDa and below (Figure 3, lane 4). This suggests that the epitopes for this class of antibodies were destroyed by CNBr cleavage. However, the N-term antibodies recognize a 26 kDa band generated by partial digestion of PAP by CNBr (Figure 3, lane 4, band a). These same antibodies did not recognize PAP with deletion 2 (see above), which lacks the first 100 amino acids of PAP. This region spans CNBr fragments C1, C2, and C3, whose combined size is 26 kDa (Figure 1B) and most likely corresponds to the peptide detected by these antibodies. When the immunoblot was repeated using more antigen (Figure 3, lane 3), the N-term class of antibodies recognizes additional fragments of 25 kDa (band b), 18 kDa (band c), a doublet of 8 kDa (bands d and e), as well as the 26 kDa peptide (band a). The 8 kDa doublet probably represents fragment C1, and a combination of C1 and C2, respectively, and the 18 kDa peptide corresponds to the size of the C3 fragment or the C2 and C3 fragments together. The 25 kDa fragment corresponds to the combined size of fragments C2, C3, and C4 together. The C2 peptide is only four amino acids long and was probably lost during electrophoresis and blotting. The reaction with these additional peptides is weak and is only detected with larger amounts of antigen. These data suggest that important determinants of the epitope recognized by the N-term class of antibodies must be located in a region spanning the C1−C2 junction, and the C2−C3 junction. The precise boundaries of this epitope have not been determined.

The second class of antibodies (C-term) only detects the 11.4 kDa C-terminal peptide C9 (Figure 3, lane 7, band h). These same antibodies did not detect PAP with deletion 3 (see above), but did detect PAP with deletion 1 and deletion 2, which contain the region of PAP corresponding to the 11.4 kDa peptide (Figure 1). This band is not detected with the N-term antibodies (Figure 3, lanes 3 and 4). When the blot was repeated with more antigen, a ladder of partial products of CNBr digestion is detected by the C-term antibodies (Figure 3, lane 6, bands f, g, and higher).

However, the antibodies do not detect any peptides smaller than C9 (band h), which gives the strongest reaction. This result confirms that the epitope for this class of antibodies is located near the C-terminus.

The data obtained by immunoblot analysis with truncated forms of PAP and with the CNBr fragments indicate that two classes of antibodies were present in the hybridoma collection. The N-term class of antibodies recognizes a region of PAP located in the first 100 amino acids, and the C-term class of antibodies recognizes an epitope located in amino acids 547−568 of PAP.

Effect of the Antibodies on PAP Activity. The antibodies were purified in order to test if they could inhibit the catalytic activity of PAP. Large-scale production of antibodies was first attempted by inducing ascites tumors in mice by peritoneal injection of hybridoma cells. Only hybridomas secreting the C-term class of antibodies produced soft-fluid tumors in the mice with high yields of antibodies in ascitic liquid. Hybridomas secreting the N-term antibodies produced only solid tumors which attached to the intestine of the mice without producing much fluid, and in this case cell culture supernatants were used as the starting material for purification. Both classes of antibodies did not bind protein A−Sepharose with high affinity in low salt, but did bind the column with high salt and alkaline pH, confirming that they belong to the IgG_1 subclass as determined by the isotyping kit (Harlow & Lane, 1988). This purification method produced antibodies of high concentration (0.8 mg/mL) and highly pure as determined by SDS−PAGE stained with silver (not shown).

Purified monoclonal antibodies were mixed with pure recombinant PAP, and the mixture was tested for poly(A) addition activity using tRNA and 1 mM Mn^{2+}. Even when the antibodies were present in molar excess to the PAP, no inhibition of activity was observed with either class of antibody. This result indicates that antibody binding to the PAP epitopes does not affect poly(A) addition activity. Antibodies were also added to yeast whole cell extracts with an estimated molar excess of 10/1 over PAP. The amount of PAP in yeast whole cell extracts was estimated by comparing the signal obtained in immunoblots with that obtained with recombinant PAP of known concentration. After preincubation of the extract with antibodies for 1 h, no effect on the cleavage or poly(A) addition activity was observed (data not shown). This observation suggests that the antibodies are not interfering with the association of PAP with the other factors in the extract involved in sequence-specific polyadenylation.

Species-Specificity of the Antibodies. The monoclonal antibodies against the yeast PAP were tested for their ability to recognize PAP from other sources. Bovine PAP (the N-terminal 184 amino acids which include the RBD domain and part of the proposed polymerase module, a gift to Tobias Raabe and James Manley) (Raabe et al., 1994) was not recognized in immunoblots by either class of monoclonal antibodies. The PAP in HeLa cell nuclear extracts was also not recognized by the antibodies. The same result was observed with recombinant *Xenopus* PAP expressed as a GST fusion (a gift of F. Gebauer and J. Richter) (Gebauer & Richter, 1994, personal communication) or with PAP in extracts from *Xenopus* oocytes and fertilized eggs. This is not surprising since the *Xenopus PAP* gene was cloned based on homology to mammalian PAP (Gebauer & Richter, 1994, personal communication). Finally, purified vaccinia PAP (a gift of Paul Gershon and B. Moss) (Gershon et al., 1991) was not recognized by antibodies directed against the yeast PAP. This result is also not unexpected since there is no amino acid sequence homology between any of the vaccinia PAP subunits and the yeast PAP (Gershon et al., 1991; Lingner et al., 1991a). The C-term antibodies recognize a unique polypeptide in plant extracts which have polyadenylation activity (Li & Hunt, 1994, personal communication).

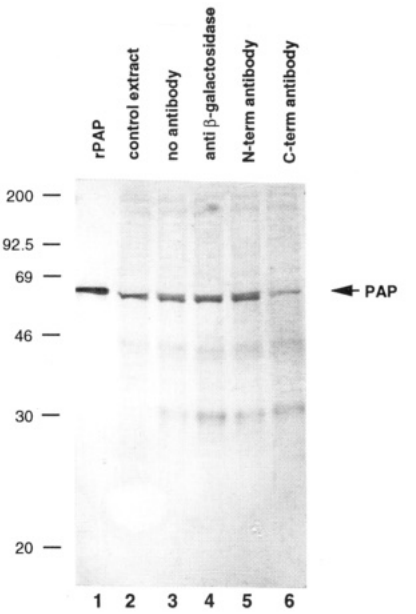

FIGURE 4: Immunoblot analysis of yeast whole cell extracts treated with different antibodies. Samples containing purified recombinant PAP (lane 1), untreated yeast whole cell extract (lane 2), extract depleted with protein G−Sepharose beads alone (lane 3), extract depleted with beads containing anti-β-galactosidase antibodies (lane 4), extract depleted with beads containing N-term antibodies (lane 5), and extracts depleted with beads containing C-term antibodies (lane 6) were fractionated on a 10% SDS−PAGE, blotted onto a PVDF membrane, and immunostained with anti-yeast PAP (N-term) antibodies. The sizes (in kilodaltons) of protein molecular mass markers run in the blot are indicated.

Immunodepletion of PAP from Yeast Whole Cell Extracts. The monoclonal antibodies against PAP were also used to remove PAP from yeast whole cell extracts and examine the effects of this immunodepletion on the cleavage and poly(A) addition reactions. The antibodies are specific for PAP and detect only a single band in yeast whole cell extracts (Figure 4, lane 2). Immunoblot analysis of extracts depleted with different antibodies shows that only the C-term class of antibodies was able to remove most of the PAP from the extract (Figure 4, lane 6). Protein G beads alone, antibodies against β-galactosidase, and the N-term class of antibodies were not able to remove PAP from the extract (Figure 4, lanes 3−5). The same result was observed with recombinant PAP mixed with nonfat dry milk proteins at the same concentration as in the extract, and only the C-term class of antibody was able to remove PAP from the mixture of proteins in solution (not shown).

In the presence of manganese poly(A) polymerase loses its specificity for mRNA precursors (Lingner et al., 1991b; Wahle, 1991). Extracts depleted with different antibodies were tested for this nonspecific poly(A) addition activity using labeled precleaved *Gal7* RNA as a substrate. All extracts, except those depleted using the C-term antibody,

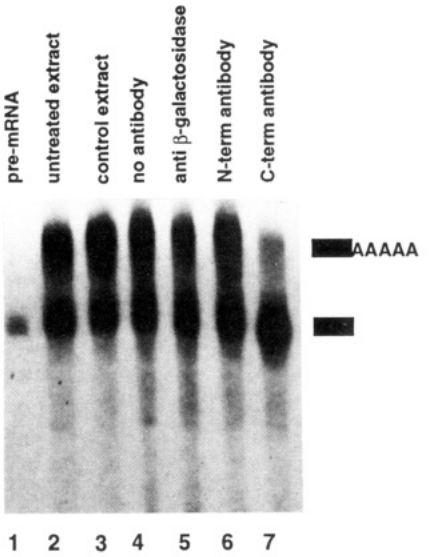

FIGURE 5: Nonspecific PAP activity in extracts treated as described in Figure 4. Extracts were tested for PAP activity in the presence of Mn^{2+} and radiolabeled precleaved *Gal7* transcript. The reaction products were separated in a 5% acrylamide/8.3 M urea gel and visualized by autoradiography. Unreacted precursor, lane 1. Reaction products generated with untreated extract, lane 2. Extract diluted with AB buffer and dialyzed, lane 3. Extract depleted with beads alone, lane 4; anti-β-galactosidase antibodies, lane 5; N-term antibodies, lane 6; and C-term antibodies, lane 7. The schematic drawings at the right of the figure represent from bottom to top: unreacted precleaved substrate and polyadenylated product.

retained their original level of PAP activity (Figure 5, lanes 2−6). Extracts depleted with the C-term antibody have significantly reduced activity (Figure 5, lane 7). This residual activity indicates that some PAP still remains in this extract, in agreement with the immunoblot data.

Immunodepletion of PAP from extracts with the C-term class of antibodies caused also a reduction in the cleavage and poly(A) addition activities with full-length *Gal7* pre-mRNA. (Figure 6, lane 7) as compared with extracts depleted with beads alone (lane 4), anti-β-galactosidase antibodies (lane 5), or the N-term antibodies (lane 6). The immunodepletion with C-term antibodies did not add any inhibitors to the extract, because addition of untreated extract reconstitutes activity (not shown). Addition of purified recombinant PAP to the extracts immunodepleted with the C-term antibodies did not restore 3′-end processing activity (Figure 6, lanes 8 and 9). Even if PAP is added in a 500-fold molar excess, compared to the original amount of PAP present in extracts, there is no restoration of cleavage or polyadenylation (not shown). These results suggest that the depletion removed not only PAP but also some component in the extract required for cleavage/polyadenylation. Chen and Moore (1992) have shown that in yeast the cleavage reaction requires fractions containing the CF I and CF II factors. Addition of a partially purified CF I fraction restores 3′-end processing activity to extracts depleted of PAP (Figure 6, lanes 10−12). CF I alone exhibits no processing activity (Chen & Moore, 1992). The majority of the processed precursor is polyadenylated, presumably by the residual PAP left in the extracts, since we cannot detect PAP in CF I containing fractions in immunoblots (not shown). However, there is accumulation of cleaved, but unpolyadenylated products in the reaction (Figure 6, lanes 10−12). These cleavage products can be chased to polyadenylated species by addition of PAP in combination with CF I, reconstituting

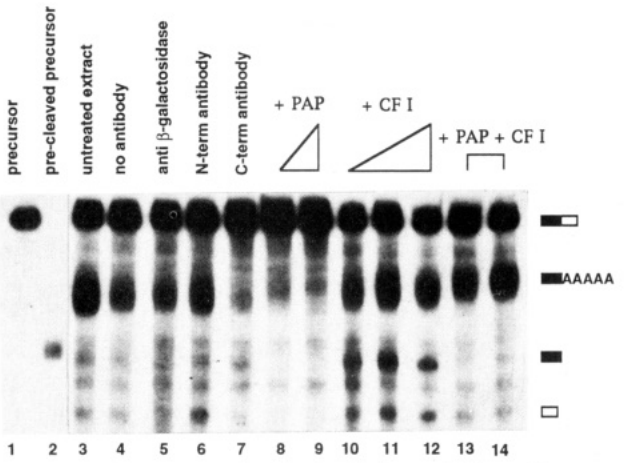

FIGURE 6: Cleavage and polyadenylation activities of extracts treated with different antibodies and supplemented with additional factors. Extracts depleted for PAP as described under Materials and Methods were assayed for cleavage and poly(A) addition using full-length radiolabeled *Gal7* transcript. The reaction products were separated in a 5% acrylamide/8.3 M urea gel and visualized by autoradiography. Unreacted full-length precursor, lane 1. Unreacted precleaved substrate, lane 2. Reaction products generated with extract diluted wth AB buffer and dialyzed, lane 3. Extract depleted with beads alone, lane 4; anti-β-galactosidase antibodies, lane 5; N-term antibodies, lane 6; and C-term antibodies, lane 7. Reaction products of extracts immunodepleted with the C-term antibodies, supplemented with purified recombinant PAP (7.5 ng, lane 8; 37.5 ng, lane 9). Reaction products of PAP-depleted extracts supplemented with partially purified CF I fraction (0.25 μL, lane 10; 0.5 μL, lane 11; 1 μL, lane 12). Reaction products of PAP-depleted extracts supplemented with both recombinant PAP and CF I (37.5 ng of PAP and 0.5 μL of CF I, lane 13; 37.5 ng of PAP and 1 μL of CF I, lane 14). The schematic drawings at the right of the figure represent from top to bottom: precursor RNA, cleaved and polyadenylated RNA, 5′ cleavage product (or precleaved substrate), and 3′ cleavage product.

the full reaction as in untreated extracts (Figure 6, lanes 13 and 14). Addition of a CF II containing fraction to PAP-depleted extracts did not restore cleavage/polyadenylation activity (not shown). These results suggest that a stable complex containing PAP and CF I exists in the extract and can be removed by depletion with the C-term class of antibodies.

DISCUSSION

We have used purified yeast PAP to prepare two classes of PAP-specific IgG_1 monoclonal antibodies which recognize different epitopes in the PAP protein. Both classes of antibodies can detect the PAP protein in immunoblot analysis. The antibodies which recognize the C-terminal region of PAP can deplete it from yeast whole cell extracts. Removal of PAP from extracts causes a decrease in the cleavage and poly(A) addition activities which are reconstituted by adding back CF I factor. This indicates that a stable complex between CF I and PAP exists in extracts in the absence of ATP or exogenous RNA substrate.

The N-term class of antibodies recognizes an epitope in a region located in the first 100 amino acids of PAP. This epitope is destroyed by cleavage with CNBr at the methionines located at positions 72 and 76. This is a region located between the two parts of the proposed RNA binding domain found in yeast PAP (Lingner et al., 1991a). The C-term class of antibodies recognizes an epitope located in amino acids 549−568 located at the C-terminal end of the protein.

The last 20 amino acids of PAP, where the epitope recognized by the C-term antibodies is located, is a region of the yeast PAP not conserved in either bovine or human PAP (Lingner et al., 1991a; Thuresson et al., 1994). This is in agreement with the observation that antibodies against the yeast PAP did not recognize mammalian or *Xenopus* PAP. The most antigenic epitopes might be expected to be found in regions of the yeast PAP not conserved in mammalian PAP.

A recent report by Thuresson et al. (1994) reported that HeLa cell extracts contain multiple forms of PAP, some of which are phosphorylated. In addition, three different cDNAs encoding PAP were isolated from a bovine library (Raabe et al., 1991; Wahle et al., 1991). In yeast extracts, only one form of PAP is detected by immunoblot analysis with either type of antibody. This correlates with the observation of only one *PAP* gene in yeast without alternative splicing or polyadenylation of transcripts (Lingner et al., 1991a). In addition, if there are posttranslational modifications, all of the PAP must be modified.

The C-term antibodies adsorbed to protein G−Sepharose can deplete PAP from yeast whole cell extracts or from solution while the N-term antibodies cannot do either. The N-term antibodies do bind efficiently to protein G-Sepharose as determined by analysis of the material bound to the beads (not shown). The N-term antibodies cannot deplete PAP even when adsorbed to protein A−Sepharose beads via a secondary anti-mouse IgG antibody. It is possible that the epitope recognized by the N-term antibodies is not accessible unless PAP is slightly denatured or attached to a membrane.

The observation that the antibodies can precipitate PAP or remove it from extracts suggests the usefulness of these antibodies in identifying other yeast proteins that interact with PAP and take part in the cleavage or polyadenylation reactions. These could be components of the CF I factor identified by Chen and Moore (1992). Extracts depleted of PAP are also deficient in cleavage activity, which can be reconstituted by addition of partially purified CF I. This result indicates that PAP and CF I exist in a stable complex in the extract, which is removed by the antibodies. This complex does not require ATP or exogenous RNA substrate. In addition, components of the CF II or PF I factors do not appear to be part of this complex, as it is not necessary to add these back to restore activity. Chen and Moore (1992) showed that CF I is required for poly(A) addition in the presence of PAP and PF I. However, CF I also participates in the cleavage reaction with CF II, and it is not surprising that removing CF I affects the cleavage reaction as well. The yeast CF I activity has an analogous function to that of the mammalian cleavage/polyadenylation specificity factor (CPSF) (Bienroth et al., 1991; Murthy & Manley, 1992). CPSF and PAP form a complex on the pre-mRNA which is detectable in gel mobility shift assays (Bienroth et al., 1993; Murthy & Manley, 1992). The interaction of either CPSF or PAP with substrate RNA is enhanced when both factors are present. However, a direct interaction between CPSF and PAP in the absence of RNA has not been demonstrated. It is likely that CF I, with the help of PF I, recruits the yeast PAP to the cleaved pre-mRNA to guarantee specific poly(A) addition.

The C-term antibodies do not inhibit the nonspecific poly(A) addition activity of PAP, suggesting that the carboxyl-terminal region of PAP where the epitope resides is not directly involved in a catalytic function. This is consistent with the observation that this domain of yeast PAP is dispensable for nonspecific polyadenylation. Because the C-term antibodies can recognize this epitope of PAP in extracts, this region is also probably not involved in the association of PAP and CF I. Further in vitro and in vivo studies will be necessary to determine whether this domain of PAP participates in other functions, such as interactions with other factors or in nuclear localization.

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