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Nuclear and Chloroplast Poly(A) Polymerases from Plants Share a Novel Biochemical Property

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Poly(A) polymerases are centrally involved in the process of mRNA 3′ end formation in eukaryotes. In animals and yeast, this enzyme works as part of a large multimeric complex to add polyadenylate tracts to the 3′ ends of precursor RNAs in the nucleus. Plant nuclear enzymes remain largely uncharacterized. In this report, we describe an initial analysis of plant nuclear poly(A) polymerases (nPAPs). An enzyme purified from pea nuclear extracts possesses many features that are seen with the enzymes from yeast and mammals. However, the pea enzyme possesses the ability to polyadenylate RNAs that are associated with polynucleotide phosphorylase (PNP), a chloroplast-localized enzyme involved in RNA turnover. Similar behavior is not seen with the yeast poly(A) polymerase (PAP). A fusion protein consisting of glutathione-S-transferase and the active domain of an *Arabidopsis*-encoded nuclear poly(A) polymerase was also able to utilize PNP, indicating that the activity of the pea enzyme was due to an interaction between the pea nPAP and PNP, and not to other factors that might copurify with the pea enzyme. These results suggest the existence, in plant nuclei, of factors related to PNP, and an interaction between such factors and poly(A) polymerases. © 2000 Academic Press

The polyadenylation of RNA is a nearly ubiquitous posttranscriptional modification in biology. In eukaryotes, poly(A) tails play important roles in mRNA translation and turnover (1), and may also be directly involved in the movement of RNA from the nucleus to the cytoplasm (2). In prokaryotes (3) and chloroplasts (4, 5), poly(A) tails are signals for accelerated turnover of the attendant RNA. Poly(A) tracts are added to RNAs by characteristic enzymes, termed poly(A) polymerases (PAPs). These enzymes share a fundamental activity, namely the ability to add polyadenylate tracts to the 3′ ends of suitable substrate RNAs. However, PAPs from different sources differ significantly in terms of the scope of their activities *in vivo* as well as their amino acid composition and sequence. Thus, the enzymes involved in mRNA polyadenylation in the nucleus of eukaryotes interact with and are regulated by an evolutionarily conserved apparatus that limits polyadenylation to the 3′ ends of suitably processed RNA polymerase II transcripts (6). The complete scope of physiological substrates for bacterial PAPs has not been defined, nor has a clear understanding of the means by which these enzymes are regulated *in vivo* been arrived at (3). However, in terms of amino acid sequence, these enzymes (two of which have been described) are quite different from the enzymes that polyadenylate RNAs in eukaryotic nuclei (7, 8).

In plants, polyadenylation of the "eukaryotic" and "prokaryotic" types are both known to occur. Thus, nucleus-derived RNA polymerase II transcripts are polyadenylated in a process that is controlled by distinctive polyadenylation signals (9). As is the case in other eukaryotes, the poly(A) tract on these mRNAs is important for the translation of these mRNAs in the cytoplasm (10). In contrast, in chloroplasts, polyadenylation is a signal for accelerated RNA turnover (4, 5). PAPs that may correspond to the chloroplast and nuclear enzymes have been identified and characterized, to various extents, from several plant species (9). One chloroplast enzyme is novel in its requirement for an RNA-binding cofactor that is identical to polynucleotide phosphorylase (PNP; 11, 12). However, much re-

Abbreviations used: PNP, polynucleotide phosphorylase; PAP, poly(A) polymerase; Hepes, *N*-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediamine tetraacetic acid; SDS–PAGE, electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate; nt(s), nucleotide(s); rps1, protein 1 of the *E. coli* 30S ribosomal subunit.

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TABLE 1

Oligonucleotide Primers Used for PCR and Sequencing

Primer designation	Sequence
5′13	AGATCTAGCTATGGGATCACGGAGCCA
5′16	AGATCTATGGTGAGTACTCAACAACGCACG
5′15	AGTGGTATCACTACTAGTGGGACTCCTCAGATT
3′2	TCGTGGGTCCCAAACAGGAAA
3′8	AGATCTCAGCCTCAACTTCCACGTTAGG
3′11	AGATCTGAGATCATGATTTGCATAAACCAA

mains unknown about the characteristics of nuclear and chloroplast PAPs in plants.

In this report, we describe initial studies of plant nuclear PAPs (nPAPs). Specifically, we present a confirmed cDNA sequence of an *Arabidopsis* gene the product of which is related to mammalian and yeast PAPs. In addition, we describe the raising of antibodies that recognize the product of this cDNA, and the use of these antibodies to monitor the partial purification of a PAP from pea nuclei. We find that this enzyme possesses a nonspecific PAP activity that is similar to that described for the PAPs from other eukaryotic organisms. Notably, however, we find that the nuclear enzyme is capable of using PNP, a chloroplast-localized enzyme, as an RNA-binding cofactor. In this respect, the nuclear enzyme shares a distinctive feature with the chloroplast-localized PAP. These results suggest the existence, in the nuclear compartment, of a factor with properties similar to PNP, and raise the possibility that the nPAP may play roles in aspects of nuclear RNA metabolism in addition to its well-known function in the formation of translatable mRNAs.

METHODS

Isolation and characterization of plant-derived cDNAs related to mammalian and yeast PAPs. Arabdiopsis sequences that were related to mammalian and yeast PAPs were identified using the BLAST algorithm (30). *Arabidopsis* cDNAs were isolated by PCR or RT/PCR, using the oligonucleotides listed in Table 1. Amino acids 22–603 were amplified using the 5′13 and 3′8 oligonucleotides. Amino acids 1–300 were amplified using the 5′16 and 3′2 oligonucleotides. Amino acids 561–795 were amplified using the 5′15 and 3′11 oligonucleotides. These 3 fragments were amplified in a continuous (1-step) reverse transcriptase PCR (RT-PCR) reaction. The 50 μl reaction mixture contained: 350 mM Tris–HCl (pH 8.8), 250 mM KCl, 2.5 mM DTT, 6 mM $MgSO_4$, 0.5% Triton X-100, 1 mM deoxynucleotide triphosphates, 30 U Rnase inhibitor (Eppendorf), 100 U SuperScript RT II (GibcoBRL), 1 U Taq DNA polymerase, 5 pmol of each primer, and 500 ng *Arabidopsis* RNA. PCR reactions were run at 50°C for 30 min, 95°C for 2 min, then 35 cycles of 95°C for 30 s, 50°C for 45 s, 72°C for 90 s, and one step at 72°C for 7 min. RT-PCR products of the expected sizes (1779 bp, 921 bp, and 705 bp respectively) were reamplified using the same PCR protocol (95°C for 2 min, then 35 cycles of 95°C for 30 s, 50°C for 45 s, 72°C for 90 s, and one step at 72°C for 7 min) and subcloned into the EcoRV site of pGEM-T Easy vector (Promega). DNA sequencing was done by automated sequencing (Perkin & Elmer, ABI Prism 310 Genetic Analyzer) using a BigDye Terminator Cycle Sequencing Ready Reaction kit (ABI Prism).

For these manipulations, total RNA was isolated from *Arabidopsis* plants using the TRIZOL Reagent according to the manufacturer's instructions (GIBCO BRL).

Production of proteins in E. coli. The part of the *Arabidopsis* nPAP cDNA encoding amino acids 21 through 603 was cloned into pGEX-2T (Pharmacia) so as to yield a glutathione-S-transferase-nPAP fusion protein. The recombinant pGEX-2T derivatives containing the NIa VPg and proteinase coding sequences were introduced into *E. coli* BL21 [F^-, ompT, hsdS (r_B^-,m_B^-), gal]. Overnight cultures of bacterial cells were used to inoculate 200 ml LB medium at a ratio of 1:20. These cultures were incubated at 37°C until the cell density reached A_{600} 0.7–0.9. IPTG was then added to a final concentration of 1 mM. After 4 h of induction at 37°C, cells were pelleted and resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF). For subsequent stages, cells and extracts were kept on ice. Cells were broken by sonication. Cell debris was removed by centrifugation at 18,000*g* for 10 min. Then glutathione Sepharose beads, which had been thoroughly washed with lysis buffer, were added to the supernatant. The mixture was incubated for 0.5 h with gentle agitation. The glutathione Sepharose beads were then collected by centrifugation at 500*g* for 5 min. The beads were washed three times with 10 volumes of lysis buffer. Proteins bound to the beads were eluted with glutathione elution buffer (10 mM reduced glutathione, 50 mM Tris–HCl, pH 8.0), and dialyzed overnight in dialysis buffer (40 mM KCl, 25 mM HEPES-KOH, pH 7.9, 0.1 mM EDTA, 1 mM PMSF, 10% glycerol, 6 mM 2-mercaptoethanol).

GST fusion proteins were further purified by chromatography on MonoQ columns. The affinity-purified, dialyzed fusion protein was loaded at 0.5 ml/min onto a MonoQ HR5/5 column (Pharmacia) that had been equilibrated with dialysis buffer. The column was developed with a linear gradient (10 ml) of 40 mM to 500 mM KCl in dialysis buffer. Fractions of 0.5 ml were collected, and 20 μl of each fraction were evaluated by immunoblotting, using antibodies specific for GST. Fractions containing the GST-nPAP fusion protein were pooled and used for the characterizations.

Purification of poly(A) polymerase. Pea nuclei and nuclear extracts were prepared according to the method described by Yang and Hunt (13). Nuclear extracts were examined by immunoblot analysis to verify that they were free of chloroplast contaminants; these characterizations have been described in detail elsewhere (12, 14). Nuclear extracts were dialyzed against Buffer I (40 mM KCl, 25 mM Hepes-KOH, pH 7.9, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10% glycerol and 5 μg/ml of each of leupeptin, chymostatin, and antipain) prior to DEAE chromatography. 12 mg of nuclear protein was loaded onto a 50 ml DEAE-Sepharose column that had been equilibrated with Buffer I. The column was washed at 0.5 ml/min with 70 ml of Buffer I and proteins retained in the column were eluted with a 70 ml gradient (40–500 mM) of KCl in Buffer I. The column was then washed with 50 ml of Buffer I containing 500 mM KCl. 4 ml fractions were collected and 20 μl of each fraction were assayed for PAP activity. Fractions containing PAP activity were pooled, dialyzed against Buffer I, and loaded onto a heparin-Sepharose column at a flow rate of 0.5 ml/min. The column was washed with 12 ml of Buffer I and bound proteins eluted with a 30 ml gradient of 40–500 mM KCl in NEB. 1.5 ml fractions were collected and 20 μl assayed for activity. Active fractions were pooled, dialyzed against Buffer I, and loaded at 0.25 ml/min onto a Mono-Q (Pharmacia) column that had been equilibrated with Buffer I. This column was developed with a 20 ml gradient of KCl (40–500 mM) in Buffer I and 1 ml fractions collected. 10–20 μl of each fraction were assayed (without prior dialysis) for PAP activity. Active fractions were pooled and used without further treatment; a typical yield was about 50 μg of protein (between 20 and 40 μg/ml), and specific activities were on the order of 200 pmol AMP incorporated min^{-1} mg^{-1} protein.

The two components of the chloroplast PAP, the PAP and PNP, were purified from extracts prepared from the leaves of young pea

TABLE 2

Properties of the Nuclear PAP

Divalent metal[1]	Label[1]	[KCl][1]	Relative activity (%)[2]
Mn (0.5 mM)	α-^{32}P-ATP	40 mM	100
Mg (0.5 mM)	α-^{32}P-ATP	40 mM	134
EDTA (0.5 mM)	α-^{32}P-ATP	40 mM	0.02
Mn (0.5 mM)	α-^{32}P-3′-dATP	40 mM	0.09
Mn (0.5 mM)	α-^{32}P-GTP	40 mM	0.17
Mn (0.5 mM)	α-^{32}P-ATP	120 mM	112
Mn (0.5 mM)	α-^{32}P-ATP	360 mM	23.3
Mn (0.5 mM)	α-^{32}P-ATP	1080 mM	$<$0.02

[1] Variations on the standard assay used to characterize the nPAP. Under standard conditions, assays included 0.5 mM $MnCl_2$, 40 mM KCl, and 0.5 mM labeled ATP. Changes from these conditions are as noted.

[2] Activity was measured as described under Methods, using 10 μl (ca. 400 ng of protein) of MonoQ-purified enzyme. For each treatment, activity was normalized with the activity obtained under standard conditions being set as 100%.

seedlings as described previously (15). The RNA content of the purified PNP preparations was determined by measuring the nucleic acids recoverable from these preparations (11) by UV spectroscopy. The yeast PAP was purchased from US Biochemicals; the activity of this enzyme in the buffers used for the assay of the chloroplast and plant nuclear PAPs was confirmed prior to the studies described here.

Poly(A) polymerase assay. Unless otherwise indicated, the fraction of interest (5–20 μl) was brought to a total volume of 50 μl with Buffer I without the protease inhibitors. This was mixed with 9 μl of PAP reaction mix (167 mM Tris–HCl, pH 8.0, 267 mM KCl, 3.33 mM $MnCl_2$, 0.33 mM EDTA, 3.33 mM DTT, 0.67% [v/v] nonidet-P40, 1.28 mg/ml bovine serum albumin, 3.33 mM ATP, and 1–2 μCi of α-^{32}P-ATP). Poly(A) or PNP were added as indicated in the text; for standard conditions (used for the studies in Table 1), the PAP reaction mixture contained 3.33 mg/ml poly(A) (average length = 400 nts [16]; from ICN). Reactions were incubated at 30°C for 2 h and then stopped by extraction with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). 20 μl of the aqueous phase were spotted on a 1 cm^2 piece of DE-81 filter paper (Whatman), the filters washed five times in 5% Na_2HPO_4, and the incorporated radioactivity was measured by liquid scintillation spectrometry.

Immunoblot analysis. For immunoblots, proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane using a Trans-Blot Cell (Bio-Rad Laboratories) following the manufacturer's recommendations. Filters were then washed and probed with antibodies (at dilutions of 1/500 to 1/1000) as described elsewhere (12, 14).

RESULTS

A Partially-Purified Plant nPAP Can Use a Chloroplast-Localized Enzyme as an RNA-Binding Cofactor

In previous work, we described the purification and characterization of a novel PAP that is situated within chloroplasts (11, 12, 14, 15). In the course of this work, and of parallel studies with PAPs from pea nuclear extracts, we made observations (detailed later in this study) that suggested some biochemical similarities between the nuclear and chloroplast enzymes. Accordingly, we set out to compare and contrast some of the properties of these two enzymes. For this, a purification of the nuclear enzyme was necessary. The strategy used was similar to that described for the chloroplast enzyme: prepare a suitable extract (from isolated nuclei) and fractionate it on DEAE-Sepharose, heparin-Sepharose, and MonoQ columns. Importantly, these extracts were free from detectable chloroplast-derived contamination (12, 14). This enzyme was also free from nuclease and ATPase activity by this stage of purification (not shown) and thus suited for the characterization that follows. (It should be noted that attempts to further purify this enzyme have been largely unsuccessful, possibly because this enzyme may be part of a multimeric complex, as is the case for the yeast PAP [17].)

A summary of some of the properties of the partially-pure nPAP is given in Table 2. This enzyme was equally active with Mg^{2+} or Mn^{2+} as a divalent metal ion, similar to the yeast enzyme (18) but in contrast with the mammalian enzyme (19). No activity was detected when labeled ATP was replaced with labeled 3′-dATP, indicative of a polymerase (as opposed to a terminal transferase-like activity). Very little activity could be detected when labeled ATP was replaced with labeled GTP, a property which contrasts this enzyme from the chloroplast PAP (4). The activity of the enzyme was optimal at KCl concentrations of approximately 100 mM, and was significantly inhibited by increasing ionic strength (Table 2).

Previously, we reported that the chloroplast PAP was poorly active when assayed using free RNA, in the absence of an additional RNA-binding factor (11). In contrast, the nuclear enzyme was able to utilize free poly(A) as a substrate (Table 2, Fig. 1). Under the conditions used here, half-maximal activity was observed at a concentration of poly(A) of approximately

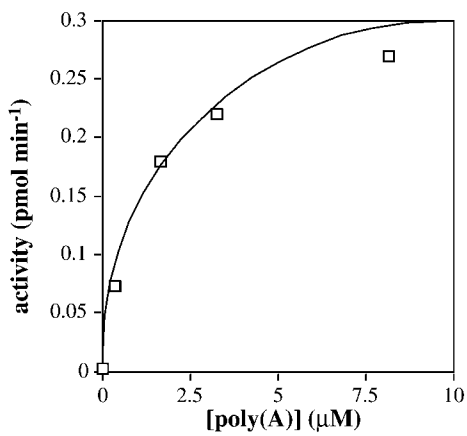

FIG. 1. Activity of the nPAP with different concentrations of poly(A). Activity was determined as described under Methods, using about 1 μg of MonoQ-purified enzyme for each reaction. The concentration of poly(A) noted here represents the approximate concentration of 3′ ends present in the reactions; this was calculated from the size distribution of poly(A) in the commercial preparations (see 16).

1.3 μM (or 0.13 mg/ml). This is in the same range (0.08–0.25 mg/ml) as apparent Km's reported for the maize, cowpea, mung bean, and wheat enzymes (summarized in Hunt and Messing [9]).

The chloroplast PAP we have characterized is distinctive in its requirement for an RNA binding cofactor, PNP (11, 12). This enzyme, as purified from pea leaves, is associated with RNAs that can serve as substrates for the chloroplast enzyme in the absence of any additional RNA (11, 15). As mentioned above, early work in this lab suggested that, like the chloroplast PAP, the nuclear enzyme could also use PNP as an RNA binding cofactor. This was examined in more detail (Fig. 2A). As shown, in the presence of a constant quantity of the nuclear enzyme, increasing quantities of PNP yielded increasing PAP activities. In these assays, no exogenous RNA was added; thus, the only RNAs available for the nuclear enzyme were those that copurify with PNP. Importantly, comparable PAP activity could be seen with PNP quantities that contained about 100-fold less RNA than was needed for comparable activity when assayed with free RNA (Fig. 2B). This suggests a positive role for PNP in this reaction. The interaction between the nPAP and PNP was specific, as no activity was seen when the plant enzyme was replaced by the yeast PAP (Fig. 2A).

These observations indicate that the nPAP can interact in a productive manner with PNP, a chloroplast-localized enzyme. This in turn suggests a certain structural similarity between the nuclear and chloroplast poly(A) polymerases. To explore this possibility, the nuclear enzyme was examined by immunoblot analysis using antibodies raised against the chloroplast enzyme. As shown in Fig. 3, the partially-purified nPAP possesses no polypeptides that are recognized by anti-PAP-I antibodies. This result indicates that any structural similarities that exist between the nuclear and chloroplast PAPs are not manifest as shared immunological epitopes.

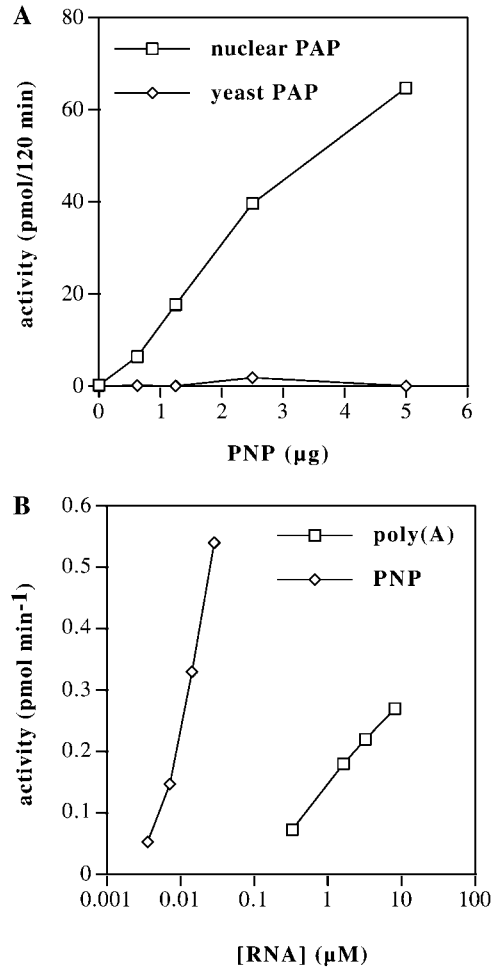

FIG. 2. Activity of the nPAP with PNP. (A) The activity of the pea nuclear and yeast PAP's using PNP as an RNA binding cofactor. Different quantities of PNP (purified as described [11, 12]) were added to a constant quantity of nPAP (activity with poly(A) as a substrate was about 30 pmol incorporated in 120 min) or yeast PAP (activity with poly(A) was about 120 pmol incorporated in 120 min) and PAP activity determined as described under Methods. Note that no exogenous RNA was added to reactions containing PNP. In the case of the yeast PAP, a similar curve is obtained when tenfold more enzyme is used. (B) Comparison of the activities of the nPAP using poly(A) or PNP as a source of RNA 3′ ends. The data shown in Figs. 1 and 2A were replotted to compare the relative effectiveness of poly(A) and PNP as RNA substrates for the nuclear PAP. The concentrations of RNA were estimated using the range distributions of RNA in each preparation (poly(A) as in Yang and Hunt [16] and PNP as in Li *et al.* [11]).

Recombinant Plant nPAP Can Use PNP as an RNA-Binding Cofactor

The utilization of PNP by the nPAP preparation from nuclear extracts may be due to a direct interaction between the nPAP and PNP (by analogy with the interaction observed between the cpPAP and PNP; Li *et al.*, 1998). Alternatively, it may be due to other, as yet unidentified factors that are present in the nPAP preparations. To distinguish between these possibilities, we set out to characterize recombinant nPAP isolated from suitably-programmed *E. coli.* This required the assembly of cDNAs encoding plant nPAPs. Perusal of the *Arabidopsis* databases revealed the existence of at least three possible nPAP genes, located on chromosomes II, III, and IV, respectively. Using RT/PCR (starting with total *Arabidopsis* RNA), cDNAs corresponding to the gene located on chromosome II were assembled. These cDNAs lacked complete 5′- and 3′-untranslated regions, but contained a single complete open reading frame. There were no obvious indications of alternative splicing, although the sensitivity of our detection methods (ethidium bromide staining) may not have been sufficient to reveal low-frequency events.

This cDNA encodes a single polypeptide, the amino acid sequences of which are shown in Fig. 4. The predicted polypeptide is 30% identical to the bovine PAP

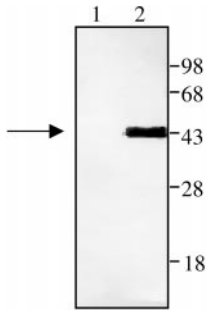

FIG. 3. nPAP is not immunologically related to the chloroplast PAP. 20 μl of the pea nuclear (lane 1) or 10 μl of the chloroplast (lane 2) PAP were separated by SDS–PAGE, transferred to nitrocellulose, and probed with antibodies raised against the chloroplast enzyme. The mobilities of protein size standards are shown to the right, and the mobility of the cpPAP is noted with an arrow on the left. The quantities of each enzyme analyzed yielded roughly equivalent activities when assayed with identical amounts of PNP.

over the entire length of the *Arabidopsis* protein, and 46% identical over the first 410 amino acids (which constitute the bulk of the conserved portion of the three PAPs; Fig. 4). The plant PAP is 23% identical to the yeast PAP, and 40% identical over the first 410 amino acids (Fig. 4). The extent of similarity with the bovine enzyme includes the catalytic core (amino acids 59–186 of the bovine enzyme; 27). However, the similarity did not include those parts of the bovine enzyme implicated in cell-cycle-related phosphorylation (amino acids 517–739 of the bovine enzyme; 29). The divergence of the C-termini of PAPs from different groups of organisms has been noted before (e.g., 27).

The results presented in Fig. 2 indicate that the partially-purified nPAP was able to utilize PNP as an RNA-binding cofactor. This property was examined accordingly with recombinant nPAP. For this, that portion of the chromosome II nPAP (amino acids 22 to 603) that was most similar to the bovine PAP was fused to the GST coding region of the plasmid PGEX-2T. This domain was chosen based on the knowledge that the portion of the chloroplast PAP that functions with PNP is coincident with that part of the enzyme that is by itself enzymatically active (14). Amino acids 22 to 603 of the *Arabidopsis* enzyme contain all of the conserved sequences present in the yeast and mammalian enzymes (Fig. 4), and corresponding portions of the yeast and mammalian enzymes have been shown to retain non-specific poly(A) polymerase activity (27–29).

The recombinant GST-nPAP was isolated from *E. coli* cells carrying the pGEX2T-nPAP clone by fractionating extracts on glutathione-Sepharose and chromatography on MonoQ columns. This protein was capable of polyadenylation when presented with poly(A) and labeled ATP (Fig. 5A), indicating that the truncated form chosen for this study retained nonspecific PAP activity. Interestingly, the apparent Km for the recombinant GST-nPAP was approximately 0.1 μM (Fig. 5A), about 13-fold lower than that seen with the enzyme purified from nuclear extracts (Fig. 1). This result indicates that the polypeptide encoded by the cDNA shown in Fig. 4 is a poly(A) polymerase, and is suggestive of possible differences in the activities of the recombinant enzyme compared with that isolated from nuclear extracts.

As shown in Fig. 5B, in the presence of a constant quantity of the GST-nPAP, increasing quantities of PNP yielded increasing PAP activities. As was the case in the experiment shown in Fig. 2, no exogenous RNA was added; thus, the only RNAs available for the nuclear enzyme were those that copurify with PNP (11). Moreover, the quantity of RNA present in the PNP preparation used in this experiment (estimated to be between 1 and 10 nM, based on the quantity of RNA that co-purified with PNP; 11) is more than ten-fold lower than the lowest poly(A) concentration at which significant activity could be detected (Fig. 5A). Thus, "release" of RNA from PNP is not a viable explanation for the PAP activity that is observed when the recombinant nPAP was assayed with PNP, in the absence of added RNA. This result indicates that the recombinant nPAP is able to utilize PNP as an RNA-binding cofactor, and argues against an involvement of other factors in the similar activity that is seen with the nPAP purified from nuclear extracts (Fig. 2).

DISCUSSION

Plant PAPs have been purified from a number of sources (9). These may be of chloroplast or nuclear origin, since RNA polyadenylation is known to occur in both compartments. One chloroplast PAP requires an additional RNA-binding cofactor, PNP, for activity (11, 12). This cofactor is PNP (12), a component of the RNA degradation machinery of the chloroplast (20). Interestingly, the nuclear enzyme can also utilize PNP as an RNA binding cofactor (Fig. 2), and has an apparent affinity for PNP-associated RNAs that is some two orders of magnitude greater than that seen for free RNA (Fig. 2B). This enhanced affinity is somewhat reminiscent of the effect of mammalian polyadenylation factors on the mammalian PAP; the apparent affinity of the latter for its RNA substrates is increased when combined with CPSF (21).

Our observations suggest the involvement of a PNP-like factor in the functioning of the nPAP. Since PNP can act *in vitro* as an RNA-binding cofactor for the PAP reaction (11), and since PNP possesses an rps1-type RNA binding domain (22), we would predict that such a factor would contain, and perhaps consist of, an rps1-type domain as well. Such a factor may be a functional analog of one or more of the factors that process and polyadenylate messenger RNAs in mammals and yeast. This would be consistent with the observation that the plant nPAP has a much greater apparent

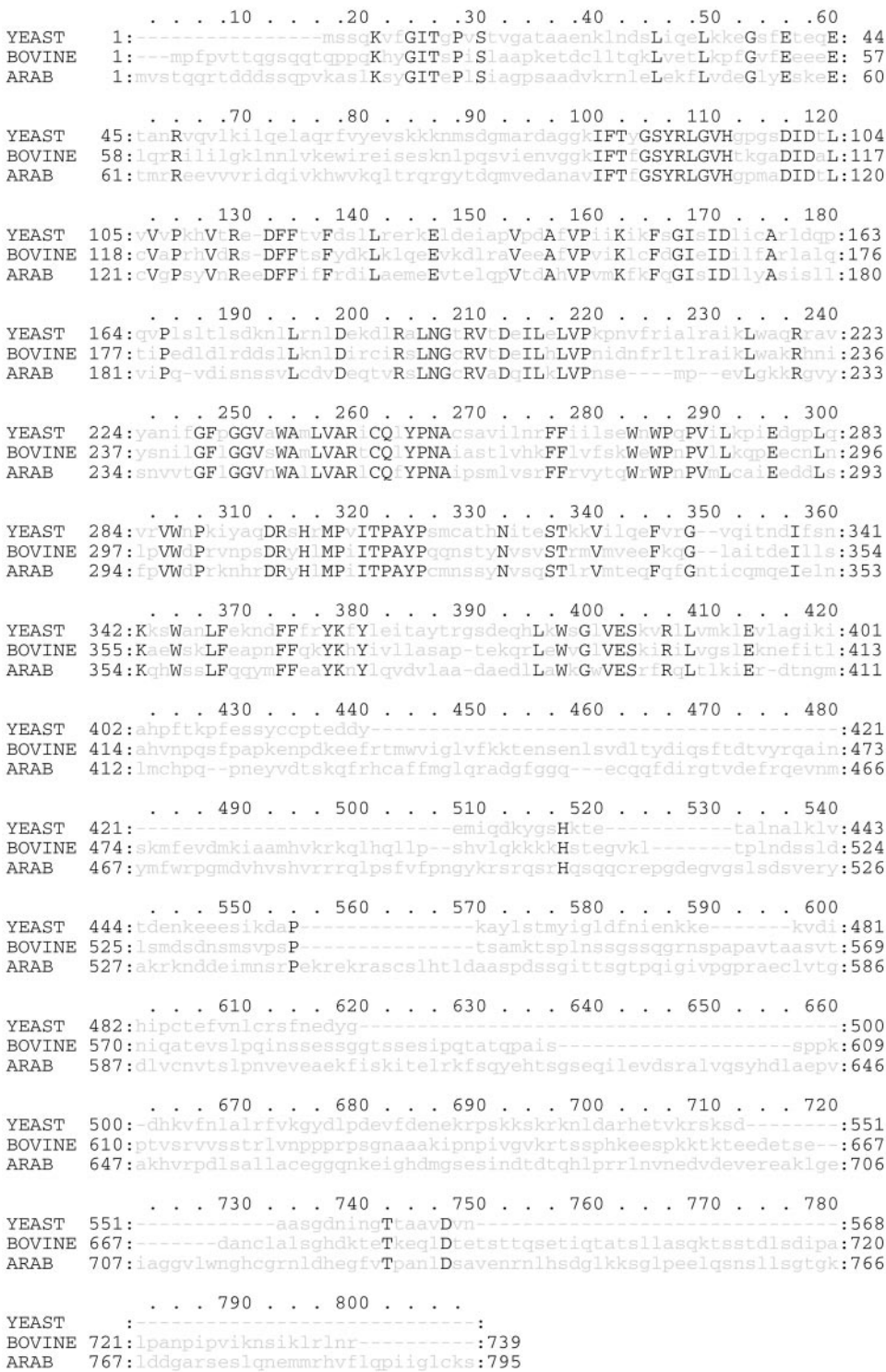

FIG. 4. Comparison of eukaryotic nPAPs. The amino acid sequence of the open reading frame encoded by the *Arabidopsis* cDNA (GenBank Accession No. AF255297) was compared with the bovine (SwissProt P2550) and yeast (SwissProt P29468) PAPs with ClustalW. The results were formatted using MacBoxshade. Residues of identity amongst the three proteins are denoted with black upper case letters, and other amino acids with gray lower case letters.

affinity for PNP-bound RNAs than for free RNA (Figs. 2 and 3). Alternatively, the PNP-nPAP interaction we report here may reflect a regulatory role not immediately associated with cleavage and processing of the primary transcript. Precedent for such a possibility exists with the case of the U1A protein-PAP in mam-

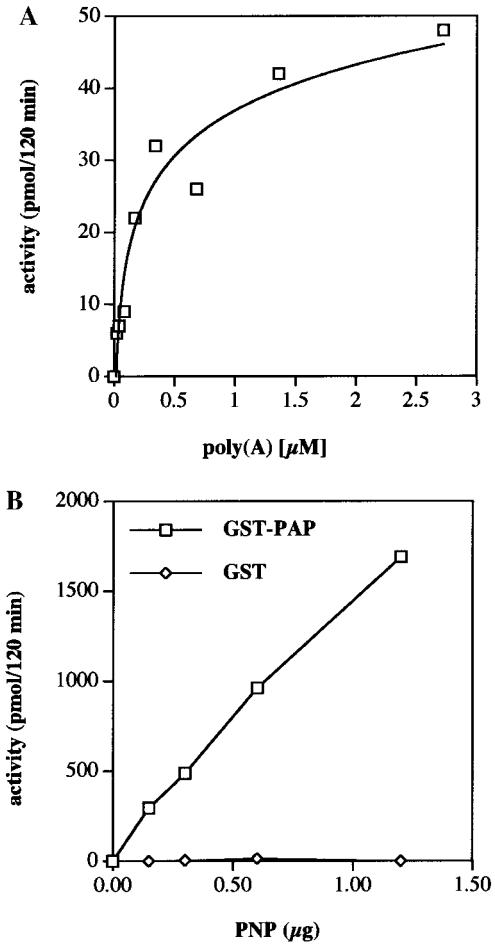

FIG. 5. Catalytic activities of a GST-nPAP fusion protein. (A) Activity as a function of increasing [poly(A)]. Activity was determined as described under Methods, using about 0.22 μg of MonoQ-purified GST-nPAP for each reaction. The concentration of poly(A) noted here represents the approximate concentration of 3′ ends present in the reactions; this was calculated from the size distribution of poly(A) in the commercial preparations (see 16). (B) Activity with PNP as an RNA-binding cofactor. Different quantities of PNP (purified as described [11, 12]) were added to 0.22 μg of nPAP and PAP activity determined as described under Methods. Note that no exogenous RNA was added to reactions containing PNP.

mals. In this instance, the U1A protein apparently is capable of regulating the polyadenylation of U1A-encoding mRNAs, through an interaction with the mammalian PAP (23, 24). Perhaps one or more rps1-containing proteins act in a similar manner to coordinate or otherwise regulate the PAP in plant nuclei. Interestingly, in this regard, rps1-type RNA binding domains are present in two proteins (Prp22p and Rrp5p) that are involved in RNA splicing in yeast (22).

These models focus on roles in pre-mRNA processing and 3′ end formation for the PAP-PNP interaction we document here. An alternate model may also be proposed, based on the known functions of PNP in bacteria and chloroplasts. In this model, the PAP-PNP interaction might reflect a role for the nPAP in RNA turnover in the nucleus. In bacteria and chloroplasts, PNP is a component of a larger complex that consists of several nucleases and ancillary factors (20, 25); this complex (termed the degradosome in the case of bacterial systems) functions in the turnover of RNA. Eukaryotic cells possess a complex (termed the exosome) that also functions in RNA turnover (26). This complex includes subunits that are homologous to several of the parts of the degradosome. While no homologues of PNP have been reported to be present in exosome preparations, exosomes do contain phosphorolytic 3′ → 5′ exonucleases that are related to *E. coli* RNAse PH, which is in turn distantly related to PNP. Thus, it is possible that the nPAP-PNP interaction noted here reflects the participation, in the plant nucleus, of the nPAP in nuclear RNA turnover. Further experimentation, especially studies focused on the identification of nuclear factors that can affect the nPAP-PNP interaction, is needed to resolve these issues.

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