We have isolated cDNA clones encoding a novel RNA-binding protein that is a component of a multisubunit poly(A) polymerase from pea seedlings. The encoded protein bears a significant resemblance to polynucleotide phosphorylases (PNPases) from bacteria and chloroplasts. More significantly, this RNA-binding protein is able to degrade RNAs with the resultant production of nucleotide diphosphates, and it can add extended polyanucleotides on to RNAs using ADP as a donor for adenylation of 5'-ends of RNAs. These activities are characteristic of PNPase. Antibodies raised against the cloned protein simultaneously immunoprecipitate both poly(A) polymerase and PNPase activity. We conclude from these studies that PNPase is the RNA-binding cofactor for this poly(A) polymerase and is an integral player in the reaction catalyzed by this enzyme. The identification of this RNA-binding protein as PNPase, which is a chloroplast-localized enzyme known to be involved in mRNA 3'-end determination and turnover (Hayes, R., Kudla, J., Schuster, G., Gabay, L., Maliga, P., and Gruissem, W. (1996) EMBO J. 15, 1132–1141), raises interesting questions regarding the subcellular location of the poly(A) polymerase under study. We have reexamined this issue, and we find that this enzyme can be detected in chloroplast extracts. The involvement of PNPase in polyadenylation in vitro provides a biochemical rationale for the link between chloroplast RNA polyadenylation and RNA turnover which has been noted by others (Lisitsky, I., Klaff, P., and Schuster, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13398–13403).

Polynucleotide Phosphorylase Is a Component of a Novel Plant Poly(A) Polymerase

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Polynucleotide phosphorylase is a component of a novel plant poly(A) polymerase that consists of two distinct components, both of which were required for activity. One of these (termed PAP-I in previous papers) copurified with a 43-kDa polypeptide that could be recognized by monoclonal antibodies raised against the yeast poly(A) polymerase (12). The other (PAP-III) included one or more RNA-binding proteins of between 100 and 105 kDa in size; only RNAs associated with these proteins could be polyadenylated by this enzyme (13). Here, we describe the isolation of cDNAs encoding these poly(A) polymerases. Analysis of these clones suggests that these RNA-binding proteins are related and are in activity polynucleotide phosphorylase. In addition, we report that the poly(A) polymerase under study can be found in chloroplasts. These findings raise interesting questions regarding the interplay between RNA polyadenylation and turnover in chloroplasts.

MATERIALS AND METHODS

Isolation of cDNA Clones—PAP-III, purified through the Mono Q stage (12, 13), was separated by SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore) by electroblotting and the resulting polypeptides visualized by staining briefly with Coomassie Brilliant Blue. The PAP-III polypeptides (13) were excised and submitted to the University of Kentucky Macromolecular Structure Analysis Facility for proteolytic digestion with endo-LysC and NH2-terminal peptide sequencing. The resulting sequences were used to design degenerate oligonucleotides intended to amplify the coding segments for the corresponding peptides. These oligonucleotides (5'-CGCTGAGACIA-RIGCYTICAYTCT-3' and 5'-CGGATCCGTIGARIGGICARGA-3', where I is inosine, R is purine, and Y is pyrimidine) were used to amplify products from a pea cDNA library. These products were cloned (after digestion with BamHI and EcoRI) into appropriately digested pBluescript (Stratagene) and the inserts of different recombinants sequenced. Clones whose sequence matched those predicted from the peptide sequences were used to probe the same cDNA library, and several near full-length clones were identified. These were sequenced using the deoxy-aid method (15).

Assays—The assay for nonspecific plant poly(A) polymerase activity has been described elsewhere (12, 13). In this study, bovine serum albumin was omitted from the polyadenylation reactions, and poly(A) was added to a final concentration of 3.33 mM/ml except where otherwise indicated. Unless noted otherwise PAP-I and PAP-III were purified through the Mono Q steps (12). For the experiment in Fig. 4, reactions were supplemented with the concentrations of unlabeled ADP indicated.

To determine ADP-dependent adenylation, labeled RNA (RNA 3, described in Li et al. (13), in a total volume of 2 μl, mixed with 5 μl of PAP-III; the concentrations of labeled RNAs in these reactions were about 40 nM. After 30 min at 30°C, 5 μl of buffer I (12) and a revised polynucleotide polymerase reaction mix (167 mM Tris-HCl, pH 8.0, 267 mM KCl, 3.33 mM MgCl2), 0.33 mM EDTA, 3.33 mM dithiothreitol, 0.67% Nonidet P-40, and 3.33 ADP) were added. Reactions were incubated for 60 min at 30°C, terminated by extraction with phenol and chloroform, and RNAs were recovered by precipitation with ethanol and separated on 6% sequencing gels. Electrophoresis was carried out for a

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF010578.

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PNPase, polynucleotide phosphorylase; GST, glutathione S-transferase.
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Fig. 1. p98 is similar to chloroplast and bacterial PNPases. The deduced amino acid sequence of p98 (noted here as PAP-III; GenBank accession number AF010578) is shown on the top line. The similarity with the spinach 100 ribonucleoprotein (cpPNP; 23) and E. coli PNPase (ecPNP; 19) is shown as well. Homology was determined with ClustalW 1.6. Positions of amino acid identity or chemical similarity are shown as capital letters and dissimilar positions as lowercase letters. The peptides derived from purified PAP-III from which NH2-terminal sequences were obtained are underlined. The domain of the E. coli PNPase which is homologous to the RNA binding domain of ribosomal protein S1 (19, 20) is double underlined.

Period of time appropriate for the resolution of small RNAs; under these conditions, larger RNAs failed to resolve well but instead were apparent as an apparently homogeneous band. This property of our separations helps in quantitating these reactions because the signals corresponding to polyadenylated RNAs were localized in a small band rather than dispersed (as would be the case if more extending runs were conducted). Gels were visualized by autoradiography and quantitated using a Molecular Dynamics PhosphorImaging System.

Phosphate-dependent RNA breakdown was assayed as above, except ADP was replaced with 0.25 mM sodium phosphate, pH 7.4.32P-Labeled RNA (rbcS-wt, derived from a NsiI-HaeIII fragment of the pea rbcS-E9 gene polyadenylation signal (16); 5,000–10,000 cpm, corresponding to approximately 1 pmol, was used per reaction) was preincubated with PAP-III for 15 min at 30 °C to permit binding of RNA to PAP-III (for details of the labeling and purification of the RNA, see Li et al. (13)). The PAP reaction mix, containing phosphate instead of ADP or ATP, was then added, and reactions were incubated for 60 min at 30 °C (well within the period during which the quantity of ADP increases in a linear manner). After halting reactions with an equal volume of phenol/chloroform, 1–4 ml aliquots of each reaction were applied to polyethyleneimine-cellulose thin layer chromatography plates. The plates were developed in 0.3M LiCl and visualized by autoradiography. One ml each of 25 mM AMP, ADP, and ATP was applied as a standard (visualized by irradiating plates directly with ultraviolet light). The radioactivity in intact RNA (which remains at the origin) and ADP were quantitated using a Molecular Dynamics PhosphorImaging System.

Preparation of Antibodies and Immunoprecipitations—The complete p98 coding region was cloned into pGEX-2T or pGEX-3X (Amersham Pharmacia Biotech) to produce protein for antibody production. The appropriate fragment was produced by polymerase chain reaction with suitable restriction sites and reading frame adjustments incorporated into the primers. Escherichia coli carrying the recombinant plasmid was induced with isopropyl 1-thio-D-galactopyranoside, and protein was purified by affinity chromatography using glutathione-Sepharose 4B, as recommended by the manufacturer (Pharmacia). Affinity-purified fusion proteins were eluted from the affinity matrix with SDS-PAGE sample buffer (0.1M Tris-HCl, pH 6.8, 2.9 M b-mercaptoethanol, 4% SDS, 0.2% bromphenol blue, 20% glycerol), separated by SDS-PAGE, and transferred to nitrocellulose membranes by electroblotting. The fusion proteins were visualized by staining briefly with Coomassie Brilliant Blue, excised, and the nitrocellulose was dissolved in dimethyl sulfoxide. These preparations were used to inject rabbits (200 mg/injection); the schedule for injection and other details have been described...
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Polynucleotide Phosphorylase—Previously, we reported that preparations of PAP-III contained a group of polypeptides between 100 and 105 kDa in size, one or more of which could be cross-linked to exogenous RNAs and to the products of polyadenylation (13). Moreover, an association of substrate RNAs with these polypeptides was necessary for polyadenylation to occur (13). Accordingly, we obtained NH₂-terminal sequences from proteolytic fragments derived from these polypeptides. Because it was not possible to resolve the two or three individual species noted earlier (13), we isolated these polypeptides in a single sample for peptide sequencing. Five peptide sequences were obtained, and this information was used to isolate corresponding cDNA clones. The longest cDNA (pQL105a) contained a single open reading frame capable of encoding a polypeptide of 897 amino acids (termed hereafter as p98), with a predicted size of about 98 kDa (Fig. 1). Because this open reading frame contained all of the peptides identified by NH₂-terminal sequencing of endo-

Preparation of Extracts and Immunoblot Analysis—Pea chloroplasts were isolated as described by Orozco et al. (18), and extracts were prepared by suspending chloroplasts in a lysis buffer (62.5 mM Tris-HCl, pH 7.5, and 2 mM MgCl₂) and removing insoluble debris by centrifugation. Chloroplast extracts typically had protein concentrations between 1 and 5 mg/ml. These extracts were fractionated and assayed for poly(A) polymerase activity as described (12). Alternately, chloroplast extracts were evaluated by immunoaffinity analysis (15), using appropriate antisera.

Denatured extracts from pea leaves and roots, and extracts from isolated nuclei, were prepared essentially as described by Yang and Hunt (17). These extracts were examined by immunoblot analysis using the antisera described in the text and figure legends.

For immunoblots, proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane using a Trans-Blot Cell (Bio-Rad) following the manufacturer’s recommendations. Filters were then washed and probed with antibodies (at dilutions of 1/500 to 1/1000) as described elsewhere (15, 17).

RESULTS

PAP-III is Similar to Bacterial and Chloroplast Polynucleotide Phosphorylase—Previously, we reported that preparations of PAP-III contained a group of polypeptides between 100 and 105 kDa in size, one or more of which could be cross-linked to exogenous RNAs and to the products of polyadenylation (13). Moreover, an association of substrate RNAs with these polypeptides was necessary for polyadenylation to occur (13). Accordingly, we obtained NH₂-terminal sequences from proteolytic fragments derived from these polypeptides. Because it was not possible to resolve the two or three individual species noted earlier (13), we isolated these polypeptides in a single sample for peptide sequencing. Five peptide sequences were obtained, and this information was used to isolate corresponding cDNA clones. The longest cDNA (pQL105a) contained a single open reading frame capable of encoding a polypeptide of 897 amino acids (termed hereafter as p98), with a predicted size of about 98 kDa (Fig. 1). Because this open reading frame contained all of the peptides identified by NH₂-terminal sequencing of endo-

The sequence similarity of p98 to PNPase suggested a biochemical similarity as well. Consequently, we assayed purified PAP-III for phosphate-dependent breakdown of RNA (with a subsequent production of NDPs) and for the extension of RNA substrates using NDPs as donors for nucleotide units, two activities associated with PNPase enzymes (22). After incubation of PAP-III with inorganic phosphate and RNA that had been labeled with [γ-32P]ATP, labeled ADP could be detected (Fig. 2A); this ADP formation was dependent on the presence of phosphate. In addition, incubation of labeled RNA with PAP-III in the presence of ADP resulted in a substantial elongation of the labeled RNA (Fig. 2B). These results indicate that PAP-III has biochemical activities similar to those of a PNPase.

Several attempts were made to isolate and characterize a GST-p98 fusion protein in a soluble form from extracts prepared from E. coli. However, the fusion protein (as well as thrombin-treated protein) produced could not be eluted from the glutathione-Sepharose affinity matrix under non-denaturing conditions, thereby precluding a direct assay of the fusion protein. Thus, as an alternative, we raised antibodies against the gel-purified fusion protein and examined the ability of these antibodies to immunoprecipitate PAP-III and PNPase activities. After affinity purification, these antibodies specifically recognized a group of polypeptides that copurified with PAP-III activity (Fig. 3). The mobilities of this group of polypeptides were similar to those present in purified PAP-III (15), and this was consistent in these gels with the predicted size (98 kDa; Fig. 3).

Subsequently, a partially purified preparation of this poly(A) polymerase (through the heparin-Sepharose step; this preparation contains PAP-I and PAP-III (see Ref. 12)) was treated sequentially with affinity-purified anti-p98 antibodies and protein A-Sepharose. After pelleting and washing the protein A-Sepharose, proteins that were bound to the pellets were as-
Poly(A) polymerase or PAP-III from different stages of purification were shown to the Pase activity as the percent of label in RNA converted to ADP in the lane 4 poly(A) polymerase. Lane 4, 7 µg of DEAE-purified poly(A) polymerase. Lane 5, 85 µg of whole leaf extract. The positions of size standards are shown to the right of the blot.

### TABLE I
**Anti-PAP-III antibodies immunoprecipitate PAP-III and PNPase activity**

Partial purified PAP (after the heparin-Sepharose step (12)) was treated with antibody raised against a PAP-III-GST fusion protein and affinity purified using the same protein. Complexes containing this antibody were adsorbed to protein A-Sepharose, collected by centrifugation, washed extensively, and assayed for PAP-III activity (defined as PAP-I-stimulated poly(A) polymerase activity) and PNPase activity (here, phosphate-dependent ADP formation from RNAs prepared with labeled ATP). The immunoprecipitate was compared with purified PAP-III (after the Mono Q step (12)) and with a mock immunoprecipitation in which antibody was omitted. PAP activity is represented as cpm incorporated from [γ-32P]ATP into poly nucleotide in 120 min, and PNPase activity as the percent of label in RNA converted to ADP in the same time.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PAP activity</th>
<th>PNPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ PAP-I</td>
<td>- PAP-I</td>
</tr>
<tr>
<td>Immunoprecipitate</td>
<td>8649</td>
<td>138</td>
</tr>
<tr>
<td>Purified PAP-III</td>
<td>15,854</td>
<td>503</td>
</tr>
<tr>
<td>Control (no Ab)</td>
<td>147</td>
<td>0</td>
</tr>
</tbody>
</table>

![Graph showing ADP concentration vs. enzyme activity](image.png)

**FIG. 4. ADP does not inhibit ATP-dependent adenylation by PAP-I + PAP-III.** Increasing concentrations of ADP were added to standard polyadenylation reactions containing 10 µl of Mono Q-purified PAP-I and 0.4 (squares), 1 (diamonds), or 2.5 (circles) µl of PAP-III. Reactions were terminated after 120 min and incorporation of label into product determined as described under “Materials and Methods.”

### TABLE II
**Poly(A) polymerase activity in chloroplast extracts**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity without polyA</th>
<th>Activity with polyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE enzyme</td>
<td>0.016</td>
<td>0.025</td>
</tr>
<tr>
<td>Chloroplast extract</td>
<td>0.025</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Activity observed in the presence of added poly(A) (a) was only then only polyadenylation observed here or previously. Activity observed in the presence of added poly(A) was available as a primer for the polymerase.

The Poly(A) polymerase under Study Is a Chloroplast Enzyme—The realization that p98 is a PNPase raises some important questions regarding the polyadenylation activity observed with the combination of PAP-I + PAP-III. In particular, it is possible the observed activity (12, 13), which was measured as incorporation of label from [α-32P]ATP into long molecules, might be the result of the action of a PNPase using trace amounts of [α-32P]ADP. This ADP might be present from a contaminant in commercial [α-32P]ATP preparations or might be produced by the action of unanticipated ATPases, nucleases, or other contaminants. To evaluate this, the effect of added unlabeled ADP on the incorporation of label from [α-32P]ATP was examined. As seen in Fig. 4, relatively low concentrations of unlabeled ADP had a modest stimulatory effect on the incorporation of label from ATP into long molecules. Because the lowest concentration of ADP added in this experiment (0.02 mM) was at least 10-fold greater than possible trace contamination of [α-32P]ADP in the preparations of [α-32P]ATP we use (labeled ADP was not detectable by thin layer chromatography), we conclude that ADP present in the substrate mixture is not responsible for the ATP-dependent polyadenylation observed here or previously. In addition, because unlabeled ADP concentrations comparable to the ATP concentrations (0.25 mM) used in our experiments had no effect on activity (Fig. 4), we conclude that conversion of ATP to ADP is not responsible for the observed ATP-dependent polyadenylation.

The Poly(A) polymerase under Study Is a Chloroplast Enzyme—The above results suggest that the poly(A) polymerase we have characterized (12, 13) may be located in the chloroplast (from which other plant PNPases have been isolated (23)). To test this, we prepared extracts from purified pea chloroplasts and assayed these for poly(A) polymerase activity. Using...
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our standard assay, we could detect activity that, like the enzyme isolated whole cell extracts, did not require the addition of exogenous RNAs (Table II). This lack of dependence on exogenously added RNA is one of the hallmarks of PAP-III isolated from whole leaf extracts (13) and is the result of the association of RNA with PAP-III during purification. The specific activity in crude chloroplast extracts was comparable to that after fractionation of whole cell extracts on DEAE-Sepharose (Table II), which suggests that poly(A) polymerase activity is enriched through the purification of chloroplasts. Fractionation of the chloroplast extracts on DEAE-Sepharose, heparin-Sepharose, and Mono Q columns yielded results identical to those obtained with whole cell extracts: fractions analogous to PAP-I and PAP-III could be obtained (not shown). These observations support the hypothesis that the novel poly(A) polymerase we have studied is a chloroplast-localized enzyme.

This possibility was tested further by examining chloroplast extracts for the presence of p98 by immunoblotting. Because poly(A) polymerase is typically presumed to be a nuclear enzyme, pea nuclear extracts were also examined. As seen in Fig. 5, p98 is readily detectable in chloroplast extracts. In contrast, this protein was absent from nuclear extracts (Fig. 5). Probing identical blots with antibodies against U1 small nuclear ribonucleoprotein (snRNP; a nuclear marker) and the large subunit of ribulose-1,5-bisphosphate carboxylase (a chloroplast marker) indicated that the chloroplast and nuclear extracts were free from appreciable cross-contamination (Fig. 5). In addition to these results, p98 could not be detected in roots (not shown). These observations are all consistent with the hypothesis that PAP-III is a chloroplast enzyme.

DISCUSSION

Several results, from this and previous studies, support our conclusion that PAP-III consists at least in part of PNPass. Before, we had noted the presence, in highly purified preparations of PAP-III, of a group of approximately 100–105-kDa polypeptides (13). One or more of these polypeptides could be cross-linked to the labeled products of polyadenylation, indicative of a close association with the poly(A) polymerase reaction. Moreover, there was a requirement that substrate RNAs for the poly(A) polymerase be bound to one or more of these proteins (15). In the present study, we have isolated cDNA clones based on peptide sequences obtained from these RNA-binding polypeptides. Antibodies raised against the product of the polypeptide encoded by the p98 clone recognize polypeptides of about 100 kDa (Fig. 3), and these antibodies immunoprecipitate PAP-III activity efficiently (Table I). These observations indicate that the encoded protein (p98) is a component of PAP-III. The identity of p98 with PNPass is indicated by the extensive amino acid sequence similarity with known PNPasses (Fig. 1) and by the copurification (Fig. 2) and coimmunoprecipitation (Table I) of PNPass and PAP-III activities.

PNPass has been identified previously in chloroplasts (23), thus suggesting a chloroplast localization for the poly(A) polymerase we have characterized. Two lines of evidence presented here support this hypothesis. First, poly(A) polymerase activity is present in chloroplast extracts (Table II), and the activity that can be purified from such extracts is biochemically indistinguishable from the activity that has been purified from whole leaf extracts (Table II). Second, p98 is present in chloroplast extracts (Fig. 5). We have not been able to detect this polypeptide in root extracts (data not shown), an observation that suggests that p98 is not present in mitochondria or other subcellular compartments that are present in root cells. However, we cannot rule out the presence of multiple locations for p98 in photosynthetic tissues, or in highly specialized cell types in roots.

p98 is very similar to that of a previously described spinach PNPass (23), but these sequences diverge at their NH2 and COOH termini. The NH2-terminal divergence might be expected in a chloroplast transit peptide. The COOH terminus of p98 retains the ribosomal protein S1-related RNA binding domain found in bacterial PNPasses, whereas the spinach PNPass lacks most of this domain (Fig. 1). Because p98 serves as an RNA-binding cofactor in the reaction catalyzed by the chloroplast poly(A) polymerase (13), the COOH-terminal sequence differences raise the interesting possibility that plants may possess more than one form of PNPass, only some of which are suitable as partners for polyadenylation.

PNPass is an important part of the RNA turnover machinery in bacteria (11, 24, 25), acting to degrade RNAs in a 3′→5′ direction via its phosphorolytic activity. In chloroplasts, polynucleotide phosphorylase is associated with a complex that helps to determine mRNA 3′-ends and can also degrade RNAs in vitro (23). Poly(A) tracts can destabilize RNAs in chloroplast extracts, and chloroplast RNAs of a transient (and presumably unstable) nature in vivo also may have polyadenylate tracts at their 3′-ends (8, 9). Our results provide a possible link between polyadenylation and increased RNA turnover in chloroplasts. This follows from the observation that only those RNAs that are associated with p98 (which the present study indicates is identical to PNPass) can be polyadenylated by the poly(A) polymerase we have characterized (13); because p98 is also PNPass, our results indicate that, in vitro, the products of polyadenylation by a chloroplast poly(A) polymerase are necessarily associated with an enzyme known to be involved in RNA degradation.

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