We have purified a novel factor (PAP-III) that is a component of a multisubunit poly(A) polymerase from pea seedlings. This factor consists of one or more polypeptides with molecular masses of about 105 kDa and of a population of associated RNAs that can serve as substrates for polyadenylation. When these RNAs are separated from the 105-kDa polypeptides, polyadenylation becomes dependent upon exogenously added RNA. This RNA-dependent activity does not require the presence of a polyadenylation signal in the substrate, indicating that the activity under study is a nonspecific polyadenylation activity. One or more of the 105-kDa polypeptides could be cross-linked to the products of polyadenylation labeled with [α-32P]ATP and to exogenously added labeled RNAs. Cross-linking of the 105-kDa polypeptides to the products of polyadenylation was not affected by the presence of exogenously added competitors, whereas cross-linking to exogenous RNAs was diminished by excesses of RNA homopolymers. Exogenous RNAs could be polyadenylated by the combination of PAP-I + PAP-III, and this activity was diminished if the binding of the exogenous RNAs to PAP-III was prevented. We conclude from these studies that PAP-III is an RNA binding protein, that polyadenylation by the poly(A) polymerase occurs while the substrate RNAs are associated with this protein, and that the pea poly(A) polymerase can only polyadenylate those RNAs that are associated with PAP-III.

Messenger RNA 3'-end formation in eucaryotes is an RNA processing event involving an endonucleolytic cleavage at the polyadenylation site, followed by the addition of a polyadenylate tract to the 3' end of the processed RNA (1). Characteristic poly(A) polymerses are responsible for the addition of the polyadenylate tracts to the 3' ends of mRNAs in mammalian and yeast cells. The purified calf thymus enzyme consists of a mixture of polypeptides ranging between 57 and 60 kDa (2, 3). This enzyme can polyadenylate RNAs nonspecifically (e.g. independent of the presence of a polyadenylation signal) in the presence of Mn2+ (3) and acts in concert with other factors to process polyadenylate precursor mRNAs in a poly(A) signal-dependent manner (4). The purified yeast enzyme is a 63-kDa polypeptide that can also polyadenylate RNAs nonspecifically (5); with this enzyme, Mn2+ and Mg2+ can satisfy the divalent metal cation requirement. The yeast enzyme is one of several factors required for poly(A) signal-dependent polyadenylation of precursor mRNAs (6).

The mammalian and yeast enzymes share substantial sequence similarity (2, 7) and act in concert with an apparatus that has certain shared components as well as significant differences. However, these poly(A) polymerases are substantially different, in terms of sequence and activities, from viral (8) and bacterial (9) poly(A) polymerases. Thus, while poly(A) polymerases may be widespread in biology (10), it is likely that different classes of organisms possess poly(A) polymerases with significantly different characteristics.

We have previously described a novel plant poly(A) polymerase that consisted of two separable components (11). One of these components (PAP-I) included a polypeptide that was immunologically related to the yeast poly(A) polymerase. The other component (PAP-III) copurified with, or included, a population of RNA-protein complexes, the RNAs of which could serve as substrates for the reconstituted poly(A) polymerase. In the previous study, we were not able to determine the precise function (if any) of these RNA-binding proteins in the polyadenylation reaction. For example, it was possible that the catalytic component of the pea poly(A) polymerase fortuitously copurified with these RNA-protein complexes, and that these complexes were not important for polyadenylation per se. Alternatively, it was possible that the RNA-protein complexes were an integral part of the pea poly(A) polymerase.

Here, we describe the further purification and characterization of PAP-III. We find that this factor consists largely or solely of one or more 105-kDa RNA-binding proteins. Our studies indicate that the reaction catalyzed by the pea poly(A) polymerase occurs while the substrate RNAs are associated with these RNA-binding proteins. Moreover, they indicate that the pea poly(A) polymerase requires that substrate RNAs be bound to these proteins to be polyadenylated.

MATERIALS AND METHODS

Plant Materials—Garden pea (Pisum sativum cv. Laxton Progress) seed was obtained from Kentucky Garden Supply (Lexington, KY). Seeds were sown in the greenhouse and harvested after 2–3 weeks of growth, when plants had two to three extended open trifoliates.

RNA Substrates—Poly(A), average length ~300 nucleotides, was obtained from ICN Biochemicals. Poly(G), poly(U), and poly(C) (average lengths of ~10,000 nucleotides) were obtained from Boehringer Mannheim.

Four RNAs containing different parts of the pea rbcS-E9 3' region (12) were used in these studies (see Fig. 1). Two of these RNAs (RNAs 1 and 4 in Fig. 1) were prepared directly from PCR-amplified templates. To prepare these templates, 50 ng of the appropriate primer (one of which contained a T7 RNA polymerase promoter), approximately 50 ng of a plasmid containing the wild-type rbcS-E9 polyadenylation signal (pGB1B) (13), 5 μl of 10 × Taq DNA polymerase buffer (1 × = 10

3 The abbreviations used are: PCR, polymerase chain reaction; hnrNP, heterogeneous ribonucleoprotein; PAGE, polyacrylamide gel electrophoresis; FUE, far-upstream element; NUE, near-upstream element.
Poly(A) Polymerase-associated RNA-binding Protein

mTris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and 0.01% gelatin) were brought to a volume of 50 μl with H₂O and Taq DNA polymerase (1 unit) was added. DNAs were amplified (35 cycles, each consisting of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C), the PCR products were extended at 72°C for 5 min and then purified by electrophoresis in agarose gels and elution from ion exchange paper. Approximately 1 μg of PCR product was used in in vitro transcription reactions using the Ampliscribe T7 transcription kit (Epigenetech). To produce a third RNA (RNA 2 in Fig. 1), the linker substitution mutant rbs120/101 (13) was digested with BstEII and approximately 1 μg of digested plasmid was used in in vitro transcription reactions as described above.

To produce a fourth RNA (RNA 3 in Fig. 1), a Taq-Xbali fragment from a linker substitution mutant (rbs120/101 in Mogen et al. (13)) was cloned into PBPluescript. The resulting plasmid was digested with BstEII, and approximately 1 μg of digested plasmid was used in in vitro transcription reactions as described above.

To make labeled RNA, 100 μCi of [α-32P]ATP (3000 Ci/mmol) were added, and reactions were performed as directed by the manufacturer. After phenol/chloroform extraction and ethanol precipitation, RNAs were taken up in 50 μl of TE and separated from unincorporated label using Select-Dr(R) spin columns (5 Prime -3 Prime, Inc., Boulder, CO). Poly(A) Polymerase Reactions—The nonspecific assay used to assay plant poly(A) polymerases has been described elsewhere (11). When indicated, poly(A) was added to a final concentration of 3.33 mg/ml and unlabeled rbs5-derived RNAs to a final concentration of 2.5 μM. For some studies, labeled RNAs were preincubated with PAP-III before polyadenylation reactions were initiated. For these reactions, labeled and competitor RNAs, in a total volume of 2 μl, were 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 each of buffer I (40 mM KCl, 25 mM Hepes-KOH, pH 7.9, 0.1 mM EDTA, 1 mM diethothretol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 5 μg/ml of each of leupepin, chymostatin, and antipain), a revised poly(A) polymerase reaction mix (167 mM Tris-HCl, pH 8.0, 267 mM KCl, 3.33 mM MgCl₂, 0.33 mM EDTA, 3.33 mM diethothretol, 0.67% Nonidet P-40, 12.8 mg/ml bovine serum albumin, and 3.33 mM ATP), and PAP-I 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. The position of polyadenylated RNAs on these gels was confirmed by comparing reactions done with unlabeled ATP and unlabeled 3′-dATP. Gels were visualized by autoradiography, and reactions were quantitated using a Molecular Dynamics Phosphorimager system.

Analysis of Endogenous RNAs—Endogenous RNAs present in the various poly(A) polymerase preparations were characterized as described elsewhere (14). Briefly, RNAs were isolated by extraction with phenol/chloroform/isoamyl alcohol and precipitation with ethanol, end-labeled with purified yeast poly(A) polymerase, separated on 6% sequencing gels, and visualized by autoradiography.

Purification of PAP-III and Removal of RNA—Poly(A) polymerases were isolated from green leaves of young plants as previously described (14). In control, whole cell extracts were fractionated on DEAE-Sepharose (Sigma) and heparin-Sepharose (Pharmacia Inc.) columns. The heparin-Sepharose flow through, which had a majority of the poly(A) polymerase activity, was loaded onto a MonoQ HR5/5 column (Pharmacia). This column was developed with a linear gradient and analyzed as described elsewhere (11). Fractions corresponding to PAP-I and -II (11) were pooled, divided into 100-μl aliquots and stored at -80°C. PAP-III preparations at the stage, termed in this report as MonoQ-PAP-III, had protein concentrations of between 50 and 250 μg/ml. For further purification of PAP-III, the MonoQ fractions that had PAP-III activity were pooled and loaded onto a Superose 6 (HR10/30) sizing column in buffer I. The column was developed with buffer I, and 1-ml fractions were collected. The same column used for PAP-III was calibrated with standards, ranging from 13.7 to 2000 kDa (Pharmacia), to estimate the size of PAP-III.

To remove tightly bound RNAs from PAP-III, preparations from the Superose 6 column were treated with 0.5% sodium deoxycholate (Sigma) (14) at room temperature for 10 min. This was mixed with an equal volume of DEAE-cellulose (Sigma, equilibrated in buffer I without proteinase inhibitors). After 10 min at room temperature, the mixture was spun for 1 min in a microcentrifuge, the pellet was washed with two volumes of buffer I, and the proteins were eluted with buffer III (buffer I containing 0.5M instead of 0.04 M KCl). In some experiments, RNAs that were not tightly associated with RNA-binding proteins were removed by treatment with micrococcal nuclease prior to chromatography on Superose 6 columns. 500 μl of MonoQ-purified PAP-III were brought to 1 mM CaCl₂, and incubated with 300 units/ml micrococcal nuclease (Boehringer Mannheim). After 30 min at 30°C, EGTA was added to a concentration of 1 mM, and the mixture was directly applied to a Superose 6 column. The column was developed as described above. Aliquots of the resulting fractions were assayed for poly(A) polymerase activity. RNAs that were not accessible to micrococcal nuclease were recovered and analyzed as described in the preceding sections. In addition, aliquots were examined by SDS-PAGE and staining with Coomassie Brilliant Blue (15)

UV Cross-linking—Two UV cross-linking assays were performed. To cross-link the products of poly(A) polymerase reactions to proteins present in the reaction mixtures, 1 μl of unlabeled competitor RNA (when appropriate) and 20 μl of MonoQ PAP-I and -III were mixed with 10 μl of MonoQ PAP-I and 9 μl of a modified poly(A) polymerase reaction mix (with 0.33 mM ATP, at a specific activity of 300–600 mCi/mmol), and brought to a final volume of 50 μl with buffer I. After incubating at 30°C for 20 min, the reaction was transferred to 500 mM Eppendorf tube caps and irradiated under UV light (UV Stratallinker-1800, Stratagene; 254 nm, 1.2 × 10⁻¹ J for 10 min). The mixture was then transferred to a new tube, RNAse A (U. S. Biochemical Corp.), 4.6 units/μg was added to a final concentration of 1 μg/ml, and the samples were incubated for 30 min at 37°C. Finally, 12 μl of Laemmli loading buffer (5 ×) was added, and the samples were boiled for 10 min and separated on 12% SDS-polyacrylamide gels (15). After drying, gels were visualized by autoradiography and quantitated using a Molecular Dynamics Phosphorimager system.

To study the binding of labeled RNAs to polypeptides in PAP-III, labeled RNAs and unlabeled RNAs were preincubated with PAP-III in reaction mixtures, 1 μl of unlabeled competitor RNA was added, and reactions were performed as directed by the manufacturer. After phenol/chloroform extraction and ethanol precipitation, RNAs were taken up in 50 μl of TE and separated from unincorporated label using Select-Dr(R) spin columns (3 Prime, Inc., Boulder, CO). Purification of PAP-III—We have described a poly(A) polymerase from pea seedlings that could be resolved into at least two components, PAP-I and PAP-III. PAP-III included a population of RNAs associated with RNA-binding proteins; these RNAs could serve as substrates for the reconstituted enzyme. To better understand the nature of PAP-III, this factor was further purified by chromatography on a Superose 6 column. PAP-III activity eluted from these columns with an apparent molecular mass of about 330 kDa (data not shown). At this stage of purification, the reconstituted enzyme remained free of an absolute dependence on exogenous RNA for activity, although activity could be stimulated between 1.2- and 3-fold by added poly(A), depending on the preparation (data not shown). This observation suggested that RNA remained associated with PAP-III activity after this stage of purification.

To directly test this possibility, Superose 6 fractions containing the peak of PAP-III activity were analyzed by SDS-PAGE and was tested for the presence of RNA, as described under "Materials and Methods." The results of these experiments, shown in Fig. 2, indicate that a population of RNAs copurifies, along with two or more polypeptides of about 105 and 25 kDa, with PAP-III activity. Moreover, the resistance of these RNAs to endogenous nucleases present in crude extracts and to added micrococcal nuclease (11) suggests that they are tightly associated with an RNA-binding protein present in these preparations.

The Pea poly(A) Polymerase Does Not Require a Polyadenylation Signal in the Substrate RNA—As the pea poly(A) polymerase was not dependent upon exogenous RNA, it was difficult to determine whether this enzyme required a polyadenylation signal in the substrate RNA. We thus set out to remove the RNA from PAP-III. For this, MonoQ-purified PAP-III was treated with 0.5% deoxycholate, and proteins were separated from endogenous RNAs by chromatography on DEAE-cellulose. When this preparation was tested with added poly(A), substantial poly(A) polymerase activity could be detected (Table I). The extent of stimulation by exogenous RNA (~90-fold) was about 70 times greater than that seen with typical PAP-III prepara-
Table I

| RNA         | Activity with PAP-III | Activity with stripped PAP-III | dpm |
|-------------|-----------------------|--------------------------------|-----|
| None        | 143,978               | 694                            |     |
| Poly(A)     | 181,389               | 62,207                         |     |
| RNA 1       | 61,148                | 11,611                         |     |
| RNA 2       | 68,314                | 29,577                         |     |


tions (Table I). Thus, this treatment apparently removed the bulk of the endogenous RNA from PAP-III, rendering the reconstituted enzyme largely dependent upon added RNA for activity. However, this treatment was also deleterious to enzyme activity; typically, the specific activity of PAP-III after treatment with deoxycholate and subsequent purification was only 10–20% of that of preparations before such treatments (this is reflected in the differences in activity between the MonoQ and stripped PAP-III in Table I).

Using the RNA-depleted PAP-III, poly(A) polymerase activity was measured with one of three additional exogenously added RNAs (shown in Fig. 1). One RNA (RNA 1 in Fig. 1) was designed to be an authentic, precleaved polyadenylation substrate; it contained sequences between 1 and 220 nucleotides upstream from the principal polyadenylation site in the rbcS-E9 gene, including the FUE and NUE that control this site (13). Another RNA (RNA 2), contained sequences between -235 and -120 nucleotides upstream from the principal rbcS-E9 polyadenylation site; this RNA lacks the FUE and NUEs that play a role in mRNA 3'-end formation in this gene, and thus served as a nonspecific RNA. Each of these RNAs was found to be competent substrates (Table I); if anything, the nonspecific RNA was a better substrate than the precleaved RNA. Therefore, poly(A) polymerase activity does not require the presence of a polyadenylation signal in the substrate RNA.

Polyadenylated Endogenous RNAs Can Be Cross-linked to Polypeptides Present in PAP-III—One model for the action of the pea poly(A) polymerase holds that the polyadenylation reaction itself occurs while the substrate RNAs are associated with the polypeptide component of PAP-III. To test this hypothesis, we examined whether the products of polyadenylation could be cross-linked to any polypeptides in our poly(A) polymerase preparations. Polyadenylation reactions containing PAP-I, MonoQ PAP-III, and [α-32P]ATP were incubated for 30 min at 30°C. A 10-fold molar excess of unlabeled 3'-dATP was then added to stop the poly(A) polymerase reaction, and the mixtures were exposed to UV light for 10 min at room temperature. After treatment of the UV-irradiated samples with nuclease, proteins cross-linked to any labeled RNAs, which would only have arisen as a result of the polyadenylation reaction, were then identified by SDS-PAGE and autoradiography. One or more of the 105-kDa polypeptides shown in Fig. 2 were found to be labeled (Fig. 3, lane 1). To demonstrate that the observed labeling of the 105-kDa polypeptides was due to cross-linking to RNA and not ATP, the polyadenylation reaction was prevented by addition of 3'-dATP at the beginning of the polyadenylation reaction (Fig. 3, lane 3). In this case, no labeling could be detected (Fig. 3, lane 3), indicating the labeling of the 105-kDa polypeptides was due to cross-linking of RNA and not ATP. This cross-linking was somewhat diminished, but still substantial, when a ~100-fold weight excess of poly(A) was added at the time of UV cross-linking (Fig. 3, lane 2). This observation suggests that labeled RNAs in these experiments (the products of the poly(A) polymerase reaction) were generated while in close association with the 105-kDa polypeptide (and thus not available for competition with the poly(A)). However, the modest effect of poly(A) on UV cross-linking to the labeled products of polyadenylation may reflect an inability of poly(A) to bind to the 105-kDa polypeptides. To test this, labeled RNA 3 (Fig. 1) was added to MonoQ PAP-III in the absence or presence of different competing homopolymers, these mixtures incubated for 20 min at 30°C, and then exposed to UV light. Proteins cross-linked to the labeled RNAs were then identified by SDS-PAGE following RNase A treatment. The results of this experiment (Fig. 4) indicated that the 105-kDa polypeptides could be cross-linked to exogenously added RNAs, and that all the homopolymers tested (including poly(A)) could diminish the extent of UV cross-linking. Thus, poly(A) is able to bind to the 105-kDa polypeptides. Based on the experiments summarized in Figs. 3 and 4, we conclude that the products of polyadenylation are formed while in close association with the 105-kDa polypeptides present in PAP-III.

RNAs Must Be Associated with the RNP to Be Substrates for Poly(A) Polymerase—The experiment shown in Fig. 4 indicates that the poly(A) polymerase reaction occurs while the substrate RNAs are closely associated with the 105-kDa polypeptide components of PAP-III. To address the possible requirement of this association for the poly(A) polymerase reaction, the polyadenylation of a labeled RNA that had been preincubated with PAP-III, in the presence or absence of an excess of poly(A) known to diminish binding to PAP-III, was examined. Labeled RNA 4 (Fig. 1) was incubated with PAP-III in the absence (Fig. 5A, lane 1) or presence (Fig. 5A, lanes 2–5) of varying excesses of poly(A) for 30 min at 30°C. PAP-I and unlabeled ATP were then added, and polyadenylation reactions were incubated for 60 min at 30°C. In an additional sample (Fig. 5A, lane 6), labeled RNA 4 was incubated with PAP-III as in the sample in Fig. 5A, lane 1. However, the polyadenylation reaction was initiated by adding PAP-I, ATP, and an excess of poly(A) identical to that used in the sample in Fig. 5A, lane 5. This last sample should distinguish between effects of poly(A) on binding to PAP-III and the poly(A) polymerase reaction itself.

The results of this experiment indicated that labeled RNA 4 could serve as a substrate for the pea poly(A) polymerase (Fig. 5A, lane 1). Moreover, excesses of poly(A) that diminish binding of RNA 4 to PAP-III were seen to inhibit the polyadenylation of this RNA (Fig. 5A, lanes 2–5). Indeed, the profiles of inhibition
adenylation reaction was due to an inhibition of binding of RNA 5 of binding and polyadenylation by poly(A) were similar (Fig. 5). The position of the group of 105-kDa polypeptides referred to in the text is shown by the arrow at the right. The position of the group of 105-kDa polypeptides present in PAP-III. This conclusion is supported by the observation that poly(A) exerts its effect on the polyadenylation of RNA 4 only if added when the binding reaction (to PAP-III) is initiated, and not when added at the start of the polyadenylation reaction itself (compare lanes 5 and 6 in Fig. 5A, and the before and after columns in Fig. 5C). We conclude from this experiment that the polyadenylation of RNAs by the pea poly(A) polymerase requires that the substrate RNAs be bound to the 105-kDa polypeptides present in PAP-III.

**DISCUSSION**

In this study, we have characterized a novel factor (termed PAP-III) that acts as one component of a multisubunit plant poly(A) polymerase. This factor includes one or more of a group of 105-kDa polypeptides. As isolated from cell-free extracts, it also contains RNA that can serve as substrates for the poly(A) polymerase. Removal of this RNA renders the pea poly(A) polymerase dependent upon exogenously added RNA; however, substrate RNAs need not contain plant polyadenylation signals, indicating that the enzyme characterized here is a non-specific one. One or more of the polypeptide components of PAP-III can be cross-linked by UV light to the products of polyadenylation by poly(A) polymerase, indicating a close association of the polyadenylation reaction with this polypeptide. Exogenous RNAs could be polyadenylated by the combination of PAP-I + PAP-III, provided that they were able to bind to PAP-III. We conclude that PAP-III consists, at least in part, of an RNA-binding protein, that the reaction catalyzed by the pea poly(A) polymerase occurs while the substrate RNAs are closely associated with this protein, and that substrate RNAs must be bound to the RNA-binding protein in order to be polyadenylated.

Previously, we resolved the pea poly(A) polymerase into two distinct components (11): a 43-kDa polypeptide (PAP-I) that is immunologically related to the yeast poly(A) polymerase, and the RNA-binding protein described here. As stated above, PAP-I is a likely candidate for the pea analog of the other poly(A) polymerases. However, we cannot rule out the alternative that PAP-I is an accessory factor that activates a latent poly(A) polymerase activity present in PAP-III. Further work is needed to distinguish between these possibilities.

The requirement of the plant poly(A) polymerase described here that substrate RNAs be associated with RNA-binding pro-
of mammal, yeast, and I proteins distinguishes this enzyme from poly(A) polymerases of mammals (3), yeast (5), and Escherichia coli (9). However, vacu-20

cinia virus encodes a polypeptide (VP39) with properties similar to those of PAP-III. Like PAP-III, VP39 is a stimulatory protein, having been shown to bind to poly(A). However, VP55 can bind to itself polyadenylate free RNA, albeit inefficiently. Moreover, the purified vaccinia poly(A) polymerase (VP55 + VP39) is a rabbit-dependent enzyme, suggesting that VP39, or the combination of VP55 + VP39, does not retain RNAs through the course of purification of this enzyme. Nevertheless, these similarities are suggestive of functional similarity in the vaccinia and plant systems. As experiments dealing with the association of polyadenylated products with VP39 have apparently not been reported, it is difficult to determine how far back this analogy.

PAP-III may serve to present substrate RNAs to the poly(A) polymerase in a manner suitable for polyadenylation. In this sense, PAP-III may be considered to be functionally related to RNA binding proteins that are involved in general RNA metabolism in the nucleus (mammalian heterogeneous nuclear ribonucleoproteins, or hnRNPs, or the NAB proteins of yeast) (16–18). However, while there is evidence suggesting that certain hnRNPs (the C family) may facilitate mRNA polyadenylation in vitro (19), these proteins are not essential for the functioning of the purified polyadenylation apparatus (1). In contrast, the PAP-III is an essential component of the pea poly(A) polymerase.

PAP-III is not a polyadenylation specificity factor, as the experiment in Table I indicates. Its RNA binding properties indicate as well that it is different from PAB-II, a nuclear polyadenylate binding protein that is involved in polyadenylation (20). In particular, PAP-III displays little preference for any of the four homopolymers in RNA binding assays (Fig. 4), unlike PAB-II, which has a marked preference for poly(A) and poly(G) (20, 21).

From these various considerations, it is clear that PAP-III is a polyadenylation factor distinct from those identified in other organisms. Its obligatory role in nonspecific polyadenylation by the pea poly(A) polymerase indicates that plant poly(A) polymases may act in ways fundamentally different from poly(A) polymerases in other organisms. It is likely that further study of plant poly(A) polymerases will lend novel insight into the role of RNA handling by poly(A) polymerases. It is likely as well that a more detailed analysis of PAP-III will provide tools with which to address aspects of nuclear RNA metabolism in plants.

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