Poly(A) Polymerase and Poly(G) Polymerase in Wheat Chloroplasts

(ATP and GTP polymerization/RNA primers)

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ABSTRACT   Extracts of wheat chloroplasts contain a poly(A) polymerase which can polymerize AMP residues from ATP onto an RNA primer. Whole extracts of wheat leaves also contain another poly(A) polymerase which is present in much larger amount and is probably derived from the nuclei. Both polymerases can utilize as primer poly(A), poly(C), transfer RNA, and ribosomal RNA, but only the chloroplast polymerase can utilize poly(U) and poly(G). Both enzymes have a specific requirement for ATP. Extracts of wheat chloroplasts contain, in addition to the poly(A) polymerase, a poly(G) polymerase which can polymerize GMP residues from GTP onto primers such as poly(G), poly(A), or ribosomal RNA. The poly(G) polymerase cannot utilize ATP but can slowly polymerize CMP from CTP. When the two chloroplast polymerases are present together in an *in vitro* incubation with ATP plus GTP and poly(A), the polymerization product is a mixed poly(A,G) tract.

A poly(A) polymerase was discovered in animal tissues by Edmonds and Abrams in 1960 (1), but it was not until 1970 that the probable role of this enzyme emerged with the finding of poly(A) tracts on the 3' end of messenger RNAs (2–5). The animal poly(A) polymerase has been shown to be located in the cell nucleus (1, 6–9) where it may add poly(A) tracts to giant, heterogeneous, nuclear RNA molecules from which mRNAs bearing poly(A) are apparently released to pass into the cytoplasm (refs. 3–5, see ref. 10 for other references). This process of mRNA maturation involving poly(A) addition appears to occur generally in eukaryotes but not in bacteria (11, 12).

In many ways, protein synthesis in the organelles of eukaryotic cells resembles protein systhesis in bacteria. Evidence is appearing, however, that mRNA maturation in organelles is of the eukaryotic, and not the bacterial, type. A poly(A) polymerase has been found in mitochondria (13), and poly(A) tracts have been detected on mitochondrial mRNAs (14). In this paper, it will be shown that wheat leaf chloroplasts contain a poly(A) polymerase. Extracts of whole leaves exhibit two distinct poly(A) polymerases: the minor poly(A) polymerase of chloroplasts and a major poly(A) polymerase (compare refs. 15 and 16) which is probably of nuclear origin. In the course of this work, a new enzyme [a poly(G) polymerase] was discovered in the wheat leaf chloroplasts This paper reports the separation, identification, and properties of all three of these enzymes from wheat leaves: two poly(A) polymerases and a poly(G) polymerase.

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MATERIALS AND METHODS

Polynucleotide Primers. The ribosomal RNA (rRNA) was from yeast (17), and the tRNA was from wheat germ. Poly(A) was obtained from Miles Laboratories. Other synthetic polyribonucleotides were a gift of Dr. Leon Heppel. Calf-thymus DNA (Worthington) was alkali denatured before use.

Enzyme Preparation from Whole Leaves. Wheat seeds (*Triticum vulgare*) were soaked first in water, bathed in 1:20 diluted Chlorox, washed with sterile water, and germinated on sterile vermiculite wet with sterile water containing 100 μg of mycostatin per ml. The sterilized pans in which the seeds were germinated were covered with plastic film and kept at room temperature under 16 hr illumination per 24 hr. After 6–7 days the leaves were harvested, cut into small pieces with a razor blade, and chilled to 4°. All the following operations were conducted at 4°. The material was homogenized in a mortar for 10 min with 0.5 ml homogenizing mixture (15) per g of leaves. The homogenate was filtered through several layers of cheesecloth and then centrifuged at 78,000 × g for 3 hr to remove cell debris, organelles, and ribosomes. The supernatant was adjusted to 80% saturation by addition of solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 17,000 × g for 20 min and dissolved in a minimum volume of a buffer containing 50 mM Tris·HCl (pH 8), 0.1 mM EDTA, and 1 mM glutathione. This buffer was utilized throughout the enzyme preparation. The dissolved material was desalted with a column of Sephadex G-50 (15 cm × 1.5 cm). The high-molecular-weight material eluted with the void volume was fractionated by passage through DEAE-cellulose as shown in Fig. 1. After assay the peak fractions were pooled and stored at −70°.

Chloroplast Isolation. Prior to harvest, the seedlings were placed in the dark for 36 hr to deplete the leaves of starch. The excised leaves were quick frozen and freeze dried at −20° for 48 hr. The chloroplasts were then isolated by the nonaqueous technique of Charlton *et al.* (18). The enzyme from isolated chloroplasts was prepared by the method described for the leaves.

Enzyme Assays. The standard poly(A) polymerase reaction mixture (17) contained in 0.25 ml: 0.5 mM [³H]ATP (4 μCi/μmol), 50 mM Tris·HCl (pH 8), 5 mM mercaptoethanol, 1 mM $MnCl_2$, 250 μg of RNA primer, and enzyme. AMP incorporation was determined essentially as described by Twu and Bretthauer (19). Poly(G) polymerase was assayed by the same procedure substituting 0.5 mM [³H]GTP (4 μCi/μmol)
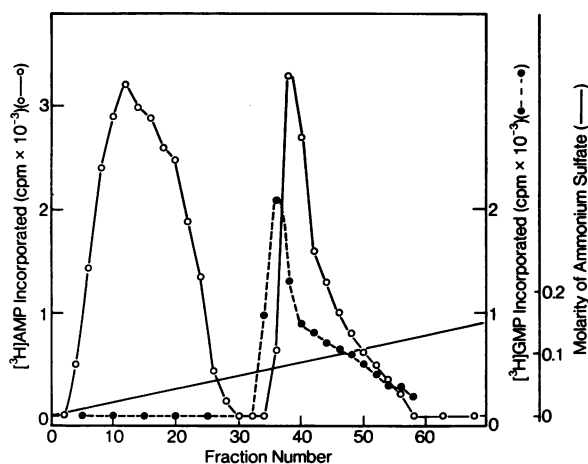
FIG. 1. DEAE-cellulose chromatography of a whole extract of wheat leaves. The sample was the total soluble fraction of leaf protein prepared as in *Materials and Methods*. The column (1.5 × 15 cm) was equilibrated with a buffer containing 50 mM Tris·HCl (pH 8), 0.1 mM EDTA, and 1 mM glutathione, and the sample was applied. Proteins were first eluted with 30 ml of the same buffer and then with a linear gradient (total volume 50 ml) from 0 to 0.3 M $(NH_4)_2SO_4$ in the same buffer. Each fraction was 0.5 ml. (O——O) Poly(A) polymerase and (●- - -●) poly(G) polymerase, each assayed with 1.0 mg/ml of poly(A) as primer, (——) molarity of $(NH_4)_2SO_4$ from measurement of conductivity of fractions. (The numbers on the ordinate have been multiplied by 10^{-3}.)
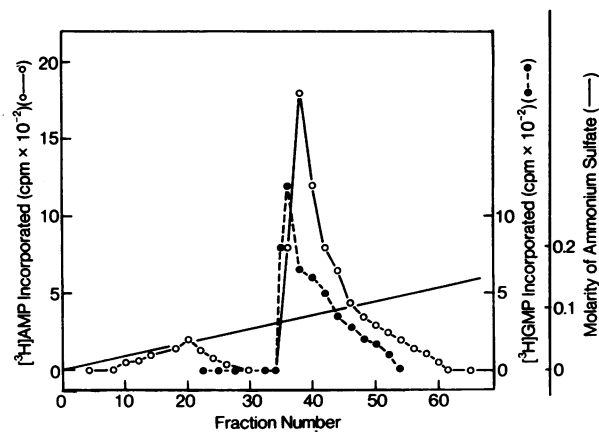

FIG. 2. DEAE-cellulose chromatography of an extract of isolated chloroplasts. The extract was prepared as described in *Materials and Methods*. Conditions for chromatography were as described in Fig. 1. (O——O) Poly(A) polymerase and (●- - -●) poly(G) polymerase, each assayed with 1.0 mg/ml of poly(A) as primer, (——) molarity of $(NH_4)_2SO_4$ from measurement of conductivity of fractions. (The numbers on the ordinate have been multipled by 10^{-2}.)

for the ATP. The ^{3}H-labeled samples were counted in a liquid scintillation counter (Packard) with an efficiency of 33%.

For the chain-length determinations, labeled poly(A) and poly(G) were synthesized in 5-fold standard reaction mixtures. After incubation the reaction mixture was precipitated with 2 volumes 5% trichloroacetic acid, washed with 5% trichloroacetic acid to remove the unreacted [^{3}H]ATP or [^{3}H]GTP, and finally hydrolyzed with 0.3 M KOH at 40° for 24 hr. The hydrolyzed samples were cochromatographed with standards of AMP and adenosine or GMP and guanosine on polyethyleneimine cellulose sheets (Brinkmann) with 0.4 M LiCl (20). The appropriate areas were cut out and counted in a scintillation counter. The ratio of total counts in nucleoside monophosphate and nucleoside divided by counts in nucleoside is equal to the chain length.

RESULTS

Presence of Two Poly(A) Polymerases and a Poly(G) Polymerase in Wheat Leaves. Chromatography of a crude wheat leaf extract on DEAE-cellulose showed the existence of two peaks of poly(A) polymerase activity (Fig. 1). The first peak eluted at a very low salt concentration, and the second eluted at about 0.1 M $(NH_4)_2SO_4$. The enzymes that eluted at low and high salt concentrations are referred to as poly(A) polymerase I and poly(A) polymerase II, respectively.

When the DEAE-cellulose column fractions were assayed using [^{3}H]GTP in place of [^{3}H]ATP, a peak of GMP-incorporating activity was detected which was close to, but definitely distinct from, the second peak of AMP-incorporating activity (Fig. 1). This assay was repeated with two other extracts. In each case, the peak of GMP-incorporating activity, which will be called poly(G) polymerase, preceded the

peak of poly(A) polymerase II. If we look at the ratio of GMP to AMP incorporation across these peaks in Fig. 1, we see a profound change in this ratio from infinity to 3.2 to 0.4. Such a marked change would not occur if both incorporations were due to a single enzyme.

Presence of Poly(A) Polymerase II and Poly(G) Polymerase in Wheat Chloroplasts. When the DEAE-cellulose chromatography was repeated on an extract of chloroplasts isolated from the wheat leaves by the nonaqueous method of Charlton *et al.* (18), a very active peak of poly(A) polymerase was found corresponding to polymerase II of the whole leaf extract and a very active peak of the poly(G) polymerase was also present (Fig. 2). Poly(A) polymerase I, which is the major peak in the whole leaf pattern, is present as a small peak in the chloroplast pattern probably due to contamination of the chloroplasts by cytoplasmic debris.

Primer and Substrate Specificities of the Two Poly(A) Polymerases. There is an absolute requirement for a primer by this type of preparation (compare Fig. 1) of poly(A) polymerase I (Table 1). The most active primers for this enzyme are tRNA and poly(A), which are much more active than rRNA or poly(C). Poly(G) and poly(U) cannot be utilized as primers by poly(A) polymerase I.

The activity of the poly(A) polymerase II preparation (compare Fig. 1) in the absence of primer varied from one preparation to another; the activity was sometimes as high as 10% of the value with added poly(A) (Table 1). When a preparation with such a blank value was preincubated with pancreatic RNase, polymerization was entirely dependent upon added primer, which suggests that, in the DEAE-cellulose chromatography, enzyme II had not been completely freed of endogenous RNA which could act as primer. The most active primers for enzyme II are tRNA, poly(A), and poly(U); rRNA, poly(C), and poly(G) can also be utilized as primers, but to a lesser extent.

Denatured DNA and oligo(dT) do not act as primers with either polymerase; in fact, they can inhibit the low blank activity of poly(A) polymerase II (Table 1).

TABLE 1. *Primer specificity of the two wheat poly(A) polymerases*

	AMP incorporated			
	Poly(A) Polymerase I†		Poly(A) Polymerase II‡	
Primer*	nmol AMP/ 100 µg protein in 30 min	% of the rate with poly(A)	nmol AMP/ 100 µg protein in 30 min	% of the rate with poly(A)
None	0	0	0.17	10
Poly(A)	0.89	100	1.72	100
Poly(U)	0	0	1.68	98
Poly(C)	0.20	22	1.32	77
Poly(G)	0	0	0.53	31
rRNA	0.36	40	1.06	62
tRNA	1.15	130	2.82	164
DNA	0	0	0	0
Oligo(dT)₉	0	0	0	0

* The source of each primer is given in *Materials and Methods.* Each primer was tested at a concentration of 1.0 mg/ml.

† The source of poly(A) polymerase I was the pooled fractions equivalent to fractions 4–25 in Fig. 1.

‡ The source of poly(A) polymerase II was the pooled fractions equivalent to fractions 33–50 in Fig. 1.

Some other properties of the two poly(A) polymerases are shown in Table 2. Synthesis of poly(A) by either enzyme requires the presence of a divalent cation. Both enzymes function optimally in the presence of 1 mM Mn^{++}. Enzyme II functions just as well with Mg^{++} at an optimum of 1 mM. With poly(A) polymerase I, Mg^{++} can only substitute poorly for Mn^{++}, even when the concentration is raised to 5 mM.

Inhibitors of transcription such as actinomycin D, α-amanitin, and DNase do not affect the polymerization reac-

TABLE 2. *Comparative properties of the two wheat poly(A) polymerases*

	Relative polymerization rate		
	Poly(A) polymerase I*	Poly(A) polymerase II*	
Assay conditions	+ poly(A) primer	+ poly(A) primer	+ poly(U) primer
Complete system, [³H]ATP	100	100	100
– Mn⁺⁺	0	0	—
– Mn⁺⁺, + 1 mM Mg⁺⁺	22	101	—
+ Actinomycin D (100 µg/ml)	98	99	—
+ α-amanitin (100 µg/ml)	97	103	—
+ DNase (100 µg/ml)	98	101	—
+ UTP (0.5 mM)	71	97	92
+ CTP (0.5 mM)	64	94	54
+ GTP (0.5 mM)	35	32	29
Complete system, [³H]UTP†	0	4	0
Complete system, [³H]CTP†	0	24‡	0
Complete system, [³H]GTP†	0	50‡	0

* The source of enzymes was the same as in Table 1.

† [³H]ATP omitted. Concentration and specific activity of substitute triphosphates were the same as those of [³H]ATP.

‡ Probably due to poly(G) polymerase (see Fig. 1).

TABLE 3. *Primer specificity of wheat poly(G) polymerase*

	GMP incorporated†	
Primer*	nmol GMP/100 µg protein in 30 min	% of the rate with poly(G)
None	0.07	2
Poly(A)	0.71	20
Poly(U)	0	0
Poly(C)	0	0
Poly(G)	3.6	100
rRNA	0.37	10
DNA	0	0
Oligo(dT)₉	0	0

* The source of each primer is given in *Materials and Methods.* Each primer was tested at a concentration of 1.0 mg/ml.

† The source of enzyme was the pooled fractions equivalent to fractions 33–50 in Fig. 1.

tion of either enzyme I or II (Table 2). With both enzymes, incorporation of labeled AMP is inhibited by each of the other nucleoside triphosphates, most strongly by GTP (Table 2).

Poly(A) polymerase I utilizes only ATP as substrate (Table 2). The preparation of poly(A) polymerase II used here (compare Fig. 1) when tested with a poly(A) primer gave significant incorporation of GTP and CTP (Table 2) due to the fact that it was contaminated with poly(G) polymerase which can utilize poly(A) as primer (see below). Since poly(G) polymerase cannot utilize poly(U) as primer, these substrate specificity tests on enzyme II were repeated using poly(U) as primer. The results presented in Table 2 show that poly(A) polymerase II has the same strict requirement for ATP that enzyme I has.

Primer and Substrate Specificities of Poly(G) Polymerase. The results in Table 3 show that this preparation of poly(G) polymerase has an almost complete requirement for primer. Poly(G) is the best primer of those tested; it is much better than poly(A) or rRNA. Poly(U), poly(C), DNA, and oligo-(dT) are inactive. Table 4 shows some other properties of the poly(G) polymerase. The polymerization of GMP requires

TABLE 4. *Properties of the wheat poly(G) polymerase*

Assay conditions	Relative polymerization rate with poly(G) primer*
Complete system, [³H]GTP	100
– Mn⁺⁺	0
– Mn⁺⁺, + Mg⁺⁺ (1 mM)	97
+ ATP (0.5 mM)	100
+ UTP (0.5 mM)	98
+ CTP (0.5 mM)	72
Complete system, [³H]ATP†	10‡
Complete system, [³H]UTP†	6
Complete system, [³H]CTP†	9

* The source of enzyme was the pooled fractions equivalent to fractions 33–50 in Fig. 1.

† [³H]GTP omitted. Concentration and specific activity of substitute triphosphates were the same as those of [³H]GTP.

‡ Probably due to poly(A) polymerase II (see Fig. 1).

TABLE 5. *Average chain length of polynucleotides synthesized in vitro*

| | | | Chain length obtained† | | |
| | | Incuba-tion time (min) | Poly(A) poly-merase I‡ | Poly(A) poly-merase II‡ | Poly(G) poly-merase‡ |
Primer*	Substrate				
Poly(A)	[³H]ATP	45	9	14	
Poly(A)	[³H]ATP	90	18	27	
rRNA	[³H]ATP	90	9	12	
Poly(G)	[³H]GTP	45			6
Poly(G)	[³H]GTP	90			10

* The source of each primer is given in *Materials and Methods*.

† Chain length determined as described in *Materials and Methods*. In all assays, enzyme protein was present at 0.5 mg/ml.

‡ Assayed with pooled fractions as indicated in Tables 1 and 3.

the presence of a divalent cation; Mg^{++} is as effective as Mn^{++}. Of the other three nucleoside triphosphates, only CTP gave significant inhibition. There is evidence that this inhibition is due to the fact that CTP is a competitive substrate, though much less active than GTP (Table 4); when the fractions obtained by DEAE-cellulose chromatography of a crude extract were tested for CMP incorporation as well as for GMP incorporation, it was found that the two curves were exactly superimposable (data not shown). Since UMP-incorporation activity in these fractions was very low, these fractions were not assayed for this activity. We, therefore, cannot say whether the low incorporation from UTP seen in Table 4 is due to the poly(G) polymerase. We attribute the AMP incorporation shown in Table 4 to the presence of poly-(A) polymerase II, since the first fraction having poly(G) polymerase activity shown in Fig. 1 does not show AMP incorporation.

The optimum pH for poly(G) polymerase was found to be rather broad, from pH 7.5 to 8.5.

Poly(G) polymerase is not inhibited at all by DNase, actinomycin D, or α-amanitin, which shows that the poly(G) polymerase is not dependent on an endogenous DNA template.

Products of the Three Polymerases. The rate of polymerization of AMP by the two poly(A) polymerases was linear for at least 90 min. A similar time curve was found for poly(G) polymerase.

The products formed from [³H]ATP or [³H]GTP after incubation with the appropriate enzymes were isolated by precipitation with 5% trichloroacetic acid and hydrolyzed with KOH to determine the chain length (see *Materials and Methods*). Table 5 shows that in each case the chain length of the product increased during the 90-min incubation. In the particular experiments shown, poly(A) polymerase I formed a product with 18 residues, poly(A) polymerase II a product with 27, and poly(G) polymerase a product with 10.

An experiment was done to determine whether a mixed poly(A,G) tract would be formed when the two chloroplast polymerases were present together in an incubation along with ATP and GTP. Labeled ATP and unlabeled GTP were employed, and after the polymerization reaction, the product labeled with AMP only was tested to determine whether the product would be rendered acid soluble by RNase T₁ which

TABLE 6. *Polymer produced by the combined action of poly(A) polymerase and poly(G) polymerase from chloroplasts*

| | Acid-precipitable [³H]AMP-labeled product after treatment indicated (cpm) | | |
Substrate	No nuclease	Pancreatic RNase	RNase T₁
[³H]ATP	1362	1292	1320
[³H]ATP + GTP	520	442	114

Using 5-fold standard reaction mixtures as described in *Materials and Methods*, two polymerization reactions were performed: one contained 0.5 mM [³H]ATP, the other contained 0.5 mM [³H]ATP and 0.5 mM unlabeled GTP. In both reactions, the primer was poly(A), and the reactions were catalyzed by a mixture of chloroplast poly(A) polymerase and poly(G) polymerase. After incubation, each reaction mixture was precipitated with 2 volumes ethanol. The insoluble material was dissolved in 10 mM Tris·HCl (pH 7.5), and aliquots of each reaction type were treated as indicated for 30 min at 37°, without nuclease, or with pancreatic RNase (100 µg/ml), or with RNase T₁ (100 units/ml).

is absolutely specific for cleavage at G residues. The product was found to be rendered largely acid soluble by this treatment (Table 6), showing that the A residues had been incorporated in the same tracts as G residues by the action of the two chloroplast enzymes.

DISCUSSION

In order to determine the cellular localization of the two wheat leaf poly(A) polymerases and the poly(G) polymerase, chloroplasts were isolated by the nonaqueous method of Charlton *et al.* (18) which includes centrifugation in a density gradient formed with carbon tetrachloride and hexane. Because of the marked density difference between chloroplasts, nuclei, and mitochondria, it is possible to prepare chloroplasts by this method which are free of the other organelles (21). Using this isolation procedure, it was established that the poly(A) polymerase II and the poly(G) polymerase are derived from the chloroplasts.

The major leaf poly(A) polymerase [poly(A) polymerase I] is not derived from chloroplasts, and there is every reason to assume that it is from the nuclei, as in the case of animal cells (1, 6–9). Because of the difficulty of isolating nuclei from wheat leaf cells, the nuclear localization could not be established experimentally in this study.

There are a number of significant differences between the two wheat poly(A) polymerases which prove that they are distinct enzymes. The two polymerases differ, of course, in their elution patterns on DEAE-cellulose, but, more significantly, they show different primer requirements. The chloroplast enzyme [poly(A) polymerase II] can utilize all four ribohomopolymers as primers, but poly(A) polymerase I cannot utilize poly(U) or poly(G).

When it was found that chloroplasts contain an enzyme for polymerizing GMP as well as one for polymerizing AMP, it seemed possible that these two enzymes could form mixed tracts on chloroplast mRNAs unless the enzymes were of the processive type. An experiment was therefore done in which ATP, GTP, poly(A), and the two chloroplast polymerases were incubated together. The label was present in the ATP. After the incubation for polymerization, the product was iso-

lated and treated with RNase T₁. The treatment solubilized most of the polymerized labeled AMP, which could only occur if the product was a mixed polymer of adenosines and guanosines. In a similar experiment (not shown) using unlabeled CTP with [³H]ATP, pancreatic RNase solubilized most of the labeled product, which indicates that it was a mixed tract of adenosines and cytidines. (The control in which CTP was omitted was not solubilized.) Thus, it was shown that the poly(A) and poly(G) polymerases are not processive enzymes.

These experiments with the two chloroplast polymerases lead to the conclusion that post-transcriptional tracts on chloroplast mRNAs may be mixed tracts with adenosines, guanosines, and cytidines which would be degraded by treatment with either RNase T₁ or pancreatic RNase. The composition of these tracts would be determined by the relative activities of the two polymerases and by the concentrations of ATP, GTP, and CTP in the chloroplasts.

The conclusion that the chloroplast ATP and GTP polymerization reactions are due to two distinct enzymes, a poly-(A) polymerase and a separate poly(G) polymerase, is based largely on the fact that upon elution from DEAE-cellulose the two peaks of activity did not correspond (Fig. 1 and 2). The differences in primer requirement supports this conclusion. Further purification will be necessary to see if the two activities can be completely separated.

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