Poly(adenylic acid)-Containing RNA from Plastids of Maize†

Lawrence A. Haff* and Lawrence Bogorad

ABSTRACT: Hybridization experiments with chloroplast DNA and ^{125}I-labeled RNA from maize seedlings suggest that chloroplasts and etioplasts contain detectable amounts of RNA that contains poly(adenylic acid) (poly (A)) and was transcribed from chloroplast DNA. About 6% of the total poly(A)-containing RNA isolated from maize seedlings hybridized to chloroplast DNA. Poly(A)-containing RNA could also be isolated directly from purified chloroplasts that were treated with ribonucleases to reduce cytoplasmic contamination. At least 65% of this poly(A)-containing RNA hybridized to chloroplast DNA. Chloroplast poly(A) tracts average about 45 nucleotides in length, one-half the average size of poly(A) tracts from whole cells. The poly(A) tracts themselves are probably added to plastid RNAs following their transcription, because maize chloroplast DNA was found not to contain poly(dT).

A high proportion of messenger RNA (mRNA) from eukaryotic organisms contains polyadenylic acid (poly(A))[1] tracts. Recently, some mRNA from bacteria (Nakazato et al., 1975) and RNA from mitochondria (Hirsch and Penman, 1974; Attardi et al., 1974) also have been shown to contain poly(A). The existence of poly(A) tracts in prokaryotes and in mitochondria is not surprising, since corresponding poly(A) polymerases have been isolated from *Escherichia coli* (Ohasa and Tsugita, 1972) and mitochondria of animal cells (Rose et al., 1975). Chloroplasts of at least one higher plant, wheat, also contain poly(A) polymerase (Burkard and Keller, 1974), so it might be expected that some chloroplast RNA also contains poly(A). We report here that poly(A)-containing RNA can be isolated from maize chloroplasts, and that this RNA hybridized to chloroplast DNA.

Experimental Procedures

The following materials were obtained from indicated sources: maize seeds (*Zea mays* FR9^{c}MS × FR37), Illinois Foundation Seeds (Champaign, Ill.); corn germ, Lauhoff Grain Co. (Danville, Ill.); deoxyribonuclease, all ribonucleases, nuclease-free Pronase, and yeast tRNA, Sigma Chemical Co. (St. Louis, Mo.); Sarkosyl (sodium *N*-lauroylsarcosinate), K & K Laboratories (Plainview, N.Y.); membrane filters, type B-6, Schleicher & Schuell (Keene, N.H.); carrier-free ^{125}I and Aquasol, New England Nuclear (Boston, Mass.); optical grade cesium chloride and synthetic polynucleotides, Calbiochem (LaJolla, Calif.); radioactive synthetic polynucleotides, Schwarz/Mann (Orangeburg, N.Y.); cyanogen bromide activated Sepharose 4B, Pharmacia (Piscataway, N.J.); Bio-Glass G-75 and G-175, Bio-Rad Laboratories (Richmond, Calif.).

Procedures for RNA Extraction. Maize seedlings were grown and harvested as previously described (Bottomley et al., 1971). Whole cell RNA was isolated from 50 g of maize leaves homogenized in 100 ml of 0.1 M Tris-HCl (pH 9) and an equal volume of 90% phenol–10% cresol–1% Sarkosyl–0.1% 8-hydroxyquinoline. Following initial extraction, the crude RNA was extracted three more times with the phenol solution and precipitated with ethanol.

Plastid Isolation and Ribonuclease Treatment of Chloroplasts. Chloroplasts and etioplasts were isolated from maize leaves as previously described (Bottomley et al., 1971). Some chloroplasts were then treated with ribonuclease. The chloroplasts isolated from up to 1 kg of maize seedlings were suspended in 50 ml of homogenization buffer containing 50 μg/ml of DNase, 50 units/ml of RNase T_2, 6000 units/ml of RNase T_1, and 0.05 μCi of [^{3}H]poly(A). The sample was incubated at 4 °C for 90 min and then centrifuged at 5000 rpm for 15 min in the Sorvall SS-34 rotor. The pelleted chloroplasts were suspended in homogenization buffer and banded in sucrose gradients as previously described (Bottomley et al., 1971). The centrifuge tubes were punctured and fractions containing the ribonuclease-free chloroplasts were collected.

Plastid RNA Isolation. Plastids were lysed in 20 ml of 50 mM Tris-HCl (pH 9)–1% Sarkosyl–150 μg/ml of Pronase at 4 °C for 10 min. Diethyl pyrocarbonate (0.6%) and an equal volume of 90% phenol–10% cresol–0.1% 8-hydroxyquinoline were added and the RNA was isolated by repeated extractions with the phenol solution. Plastid rRNA was isolated from plastid ribosomes prepared as described by Vasconcelos and Bogorad (1971).

Purification and Radioiodination of RNA Preparations. All RNA preparations were purified by cesium chloride gradient centrifugation and labeled with ^{125}I by the procedures of Scherberg and Refetoff (1974). Labile ^{125}I was hydrolyzed from RNA by incubation of the sample in 0.5 M Tris-HCl (pH 8) at 60 °C for 10 min. Unbound ^{125}I was then removed by a second cesium chloride gradient centrifugation.

Isolation of Chloroplast DNA and Whole DNA from Maize. Maize chloroplast DNA was isolated by the method of Kolodner and Tewari (1972) from deoxyribonuclease-treated chloroplasts, and its purity was confirmed by analytical cesium chloride gradient centrifugation as shown in Figure 1. Chloroplast DNA had a hyperchromicity of 38% at 100 °C and a T_m of 85 °C in SSC. Ninety percent of the hyperchromicity of denatured chloroplast DNA disappeared when the DNA was incubated to C_0t = 1 in SSC solution at 60 °C. "Whole"

† From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138. *Received February 5, 1976.* Supported in part by National Institute of General Medical Sciences Research Grant GM 20470 and by the Maria Moors Cabot Foundation of Harvard University.

[1] Abbreviations used are: Poly(A)$^{+}$ RNA, RNA which contains poly(adenylic acid); poly(A)$^{-}$ RNA, RNA lacking poly(adenylic acid); SSC, 0.15 M NaCl–0.015 M trisodium citrate; EDTA, (ethylenedinitrito)tetracetic acid; C_0t and R_0t were defined as mol of DNA or RNA nucleotides per liter times seconds, respectively; T_m was defined as the temperature at which 50% of a hybrid nucleic acid melted. Enzymes: RNase T_1 (EC 2.7.7.26); RNase T_2 (EC 2.7.7.17); RNase A (EC 2.7.7.16); DNase (EC 3.1.4.5).

maize DNA was isolated from maize chromatin. Green maize seedlings were homogenized as described by Bottomley et al. (1971), passed through two layers of Miracloth, and centrifuged at 1500*g* in a Sorvall GSA rotor. The pellet was resuspended and subjected to sucrose gradient centrifugation as described by Bottomley et al. (1971). DNA isolated from the pellet is referred to in this paper as "whole" DNA. The method of preparation and cesium chloride centrifugation both suggested that whole maize DNA consists largely of nuclear DNA with some organelle DNA (see Figure 1).

DNA–RNA Hybridization Conditions. All DNA preparations were immobilized on membrane filters. Hybridizations were carried out by incubating the immobilized DNA with RNA at 37 °C in 1 ml of solution containing fourfold concentrated SSC and 40% formamide. Hybrids were then purified as described by Gillespie (1968).

Determination of Radioactivity. Samples containing ^{125}I were counted in a Picker Liquimat Model 110 γ-ray counter. Samples containing ^{3}H were counted in a Packard Model 3375 scintillation counter with Aquasol as the scintillation fluid and corrected for quenching using internal standards.

Poly(U)-Sepharose Chromatography. Poly(U)-Sepharose was prepared from cyanogen bromide activated Sepharose 4B as described by the manufacturer. Up to 1 mg of RNA was applied to 1-ml poly(U)-Sepharose columns at 20 °C in a buffer containing 0.7 M NaCl–50 mM Tris-HCl (pH 7.5)–10 mM EDTA–25% formamide, and the columns were washed with this buffer. Bound RNA was eluted from the column with a buffer containing 90% formamide–10 mM EDTA–10 mM potassium phosphate (pH 7.5)–0.2% Sarkosyl. A buffer (2.6 ml) containing 0.069 M Tris-HCl (pH 7.5)–0.97 M NaCl–10 mM EDTA was added to each ml of RNA eluted from the column, and the chromatography was repeated twice.

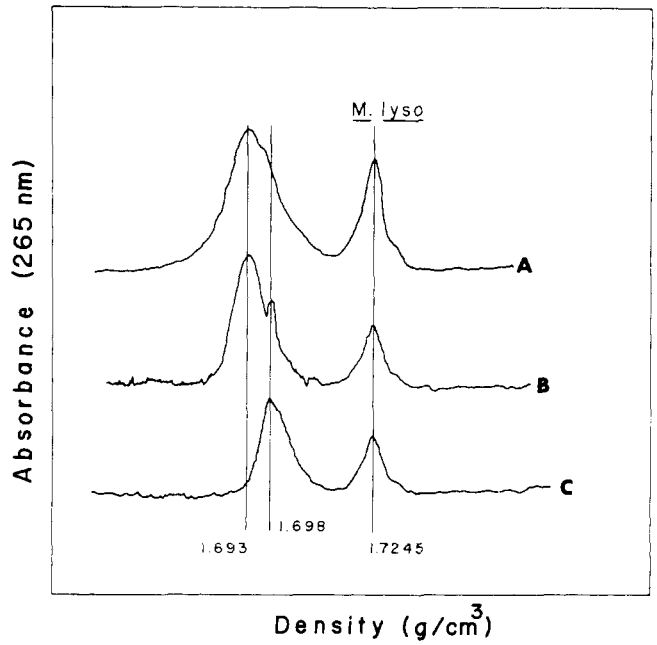

FIGURE 1: Photoelectric scans of several DNA preparations following isopycnic-cesium chloride gradient centrifugation. Samples were centrifuged for 24 h at 25 °C and 44 700 rpm in a Spinco Model E analytical ultracentrifuge. The CsCl solutions were adjusted to an initial buoyant density of 1.700 g/cm^3. *Micrococcus lysodeikticus* DNA of density 1.7245 was used as a marker in each run. Buoyant densities were determined by the method of Schildkraut et al. (1962). (A) Upper scan: whole maize DNA, prepared as described under Experimental Procedures. (B) Middle scan: chloroplast DNA, prepared as described under Experimental Procedures, but without DNase treatment of the chloroplast. (C) Bottom scan: chloroplast DNA, prepared as described under Experimental Procedures, including DNase treatment of the chloroplasts.

Results

Preparation and Purity of Poly(A)-Containing RNA. Poly(A)-containing RNA was isolated from ^{125}I-labeled total RNA preparations by poly(U)-Sepharose chromatography. Three cycles of chromatography were always performed, although a single cycle yielded a product free of non-poly(A)-containing RNA. During the second cycle of chromatography of a typical preparation of poly(A)$^+$ RNA[1] (as well as additional cycles), only about 6% of the poly(A)$^+$ RNA failed to bind to the column. The failure of a small percentage of poly(A)$^+$ RNA to bind appeared to be due to slow breakdown of the RNA. It was minimized by limiting the number of manipulations of poly(A)$^+$ RNA preparations and by storing them in liquid nitrogen.

Detection of Chloroplast Poly(A)$^+$ RNA. Poly(A)$^+$ RNA was isolated from purified chloroplasts that were incubated in a solution containing RNases T_1 and T_2 to diminish possible contamination of the plastids with cytoplasmic poly(A)$^+$ RNA (see Experimental Procedures). [^{3}H]Poly(A) was added to the mixture during the incubation to monitor the course of digestion. Following the incubation, the mixture was free of radioactive acid-insoluble poly(A), indicating that enough nuclease was added to degrade unprotected poly(A) tracts. Radioactive heteropolymers were also used to monitor the course of the digestion, but all were hydrolyzed much faster than poly(A). Etioplasts could not be purified by this procedure, since they lysed during the incubation yielding only low amounts of RNA.

Chloroplasts were freed of added RNases by banding them in sucrose gradients as described under Experimental Procedures, and labeled poly(A)$^+$ RNA was isolated from them as previously described. As shown in Table I, poly(A)$^+$ RNA comprised about 0.5% of the total RNA from RNase-treated chloroplasts. This labeled poly(A)$^+$ RNA was incubated simultaneously with chloroplast DNA and whole maize DNA bound to separate filters, as shown in Figure 2A. Sixty-five percent of the labeled poly(A)$^+$ RNA hybridized directly to chloroplast DNA, at relatively low C_0t values, while only about 10% of the poly(A)$^+$ RNA hybridized to whole maize DNA, even at C_0t values up to 150. Most of the remaining poly(A)$^+$ RNA was bound to the filter-immobilized chloroplast DNA but was released from the hybrid during the RNase A treatment employed to purify DNA–RNA hybrids. This RNA could be of either plastid or cytoplasmic origin.

Poly(A)$^+$ RNA was also isolated from purified maize plastids that were not treated with RNases. As shown in Figure 2B, about 24% of the poly(A)$^+$ RNA isolated from either chloroplasts or etioplasts hybridized to chloroplast DNA. These data suggest that the ribonuclease treatment was necessary to remove contaminating cytoplasmic poly(A)$^+$ RNA. We examined the thermal stability of the hybrids formed between plastid poly(A)$^+$ RNA and chloroplast DNA. As shown in Figure 3, these hybrids melted with a T_m of about 87 °C in SSC, while plastid rRNA–chloroplast DNA hybrids melted with a T_m of about 90.5 °C.

As shown in Figure 2C, when labeled poly(A)$^+$ RNA was isolated from whole leaf tissue of etiolated or green maize seedlings, a maximum of only 6% of the labeled poly(A)$^+$ RNA hybridized to chloroplast DNA. The total yields of poly(A)$^+$ RNA from various sources are shown in Table I.

Poly(A) Content of RNase-Treated Chloroplasts. Because the radioiodination procedure (Scherberg and Refetoff, 1974)

TABLE I: Amounts of Poly(A)$^+$ RNA Isolated from Maize.[a]

	Type of Seedlings	Source of RNA: Leaves	Plastids	Ribonuclease-Treated Plastids
Yield of RNA (mg of RNA/kg of leaf tissue)	Green	250	2.5	0.5
	Etiolated	250	2.5	
% poly(A)$^+$ RNA	Green	0.53	0.12	0.47
	Etiolated	0.94	0.18	
% poly(A)$^+$ RNA hybridizable to chloroplast DNA	Green	6.0	24.0	70
	Etiolated	6.4	24.0	
% total RNA containing poly(A) and hybridizable to chloroplast DNA	Green	0.032	0.028	0.33
	Etiolated	0.060	0.044	

[a] All RNA preparations were labeled with ^{125}I as described under Experimental Procedures. The "% poly(A)$^+$ RNA" was defined as the fraction of total RNA bound to poly(U)-Sepharose following three cycles of chromatography. Values for "% poly(A)$^+$ RNA hybridizable to chloroplast DNA" are the maximal hybridization values taken from Figure 2A,B,C. The "% total RNA containing poly(A) and hybridizable to chloroplast DNA" is equal to the corresponding values in line B × line C/100.

does not label adenosine residues, it was impossible to directly assay the amounts of poly(A) in our preparations. However, poly(A) can be assayed by hybridization with radioactive poly(U) (Wilt, 1973). Model hybridizations with purified poly(A) established that, under the hybridization conditions employed, an average of 1.06 (±0.05) mol of poly(A) nucleotides hybridized per mole of [^{3}H]poly(U) nucleotides, and that these hybrids were resistant to digestion with RNases T_1, T_2, and RNase A. As shown in Figure 4, [^{3}H]poly(U) hybridized to the total nonradioactive RNA isolated from either whole maize leaf tissue or from RNase-treated chloroplasts, but did not hybridize to purified plastid rRNA. The relative poly(A) content of each preparation is approximately equal to the slope of the corresponding line in Figure 4, i.e., 5.8×10^{-2}% of whole leaf RNA and 2.3×10^{-2}% of the RNA from RNase-treated chloroplasts consisted of poly(A). Since poly(A)$^+$ RNA comprised about 0.5% of the total RNA from either preparation, it can be calculated that 11% of the poly(A)$^+$ RNA from leaf tissue, and 5.0% of the poly(A)$^+$ RNA from RNase-treated chloroplasts, is poly(A) itself.

Size Distribution of Maize Chloroplast Poly(A) Tracts. The assay just described was slightly modified to estimate the size of poly(A) tracts. If radioactive poly(U) is used in great excess over the quantity of poly(A) in an RNA mixture, and if the poly(U) tracts are substantially larger than the poly(A) tested, then the hybrids formed after treatment with RNase A and T_1 must consist of a poly(A) tract and a poly(U) fragment the same length as the poly(A). Once dissociated, the size of the radioactive poly(U) fragment can be determined by molecular sieving in a denaturing solvent, 90% formamide.

Radioactive poly(U) was "sized" by this treatment following hybridization to RNA isolated from either whole leaf tissue or from RNase-treated chloroplasts. These poly(U) fragments were completely excluded from a Bio-Glass G-75 column, which excludes poly(U) tracts longer than about 29 nucleotides. However, as shown in Figure 5, neither preparation of poly(U) fragments was excluded by Bio-Glass G-175, which excludes poly(U) fragments over about 200 nucleotides in length. The [^{3}H]poly(U) fragments "sized" from the RNA of whole leaf tissue eluted from the column slightly before the yeast tRNA marker. On the other hand, the [^{3}H]poly(U) fragments "sized" from RNA obtained from ribonuclease-treated chloroplasts eluted slightly after the yeast tRNA marker. Undegraded [^{3}H]poly(U) was completely excluded from the Bio-Glass G-175 column.

Increasing the ribonuclease concentrations fivefold during the "sizing" treatment failed to alter the elution pattern of "sized" [^{3}H]poly(U), indicating that the original poly(A)·[^{3}H]poly(U) hybrids were resistant to "nicking" by the ribonucleases. The data from Figure 5 indicated that chloroplast poly(A) tracts average 30–60 nucleotides in length, while poly(A) tracts from whole leaf tissue averages 80–100 nucleotides in length.

Size Distribution of Maize Chloroplast Poly(A)$^+$ RNA. RNA was isolated from RNase-treated chloroplasts, labeled with ^{125}I, and separated into poly(A)$^+$ and poly(A)$^-$ fractions by poly(U)-Sepharose chromatography. These fractions were layered onto a sucrose gradient and centrifuged together with standards of 16S, 18S, 23S, and 28S maize rRNA, and 4S yeast tRNA. As shown in Figure 6, the bulk of the poly(A)$^+$ RNA sedimented between 6 and 13 S, although a minor fraction sedimented as rapidly as 22 S. Poly(A)$^-$ RNA, on the other hand, sedimented predominantly in the regions of the rRNA standards. The sedimentation coefficients of the poly(A)$^+$ RNA suggest that most poly(A)$^+$ RNAs fall in the molecular weight range from 100 000 to 350 000.

Lack of Poly(dT) Sequences in Maize Chloroplast DNA. The chloroplast poly(A) tracts detected in the previous experiments could be added after transcription by a poly(A) polymerase, or added during synthesis of the RNA by transcription of poly(dT) in chloroplast DNA. We tested the existence of poly(dT) in maize chloroplast DNA by hybridization of [^{3}H]poly(A) to chloroplast DNA immobilized on membrane filters. Up to 0.67 μg of [^{3}H]poly(A) was incubated with 24 μg of chloroplast DNA. As shown in Table II, a small amount of [^{3}H]poly(A) was bound to the filters, but this complex was degraded by treatment with RNase T_2. If chloroplast DNA contained only one sequence of $(dT)_{20}$, the smallest oligonucleotide likely to form a stable duplex with poly(A) (Walker, 1969), about 800 cpm of RNase T_2-resistant hybrid should have been detected. Since it was not, maize chloroplast DNA contains less than 1 oligo(dT) sequence, 20 residues or less in length, per molecule of chloroplast DNA.

Discussion

A major interest in the study of chloroplasts has been to determine if messenger RNAs coding for particular chloroplast proteins are transcribed from chloroplast DNA. A number of studies have indicated that mRNA exists within plastids, yet no evidence has been presented against the hypothesis that

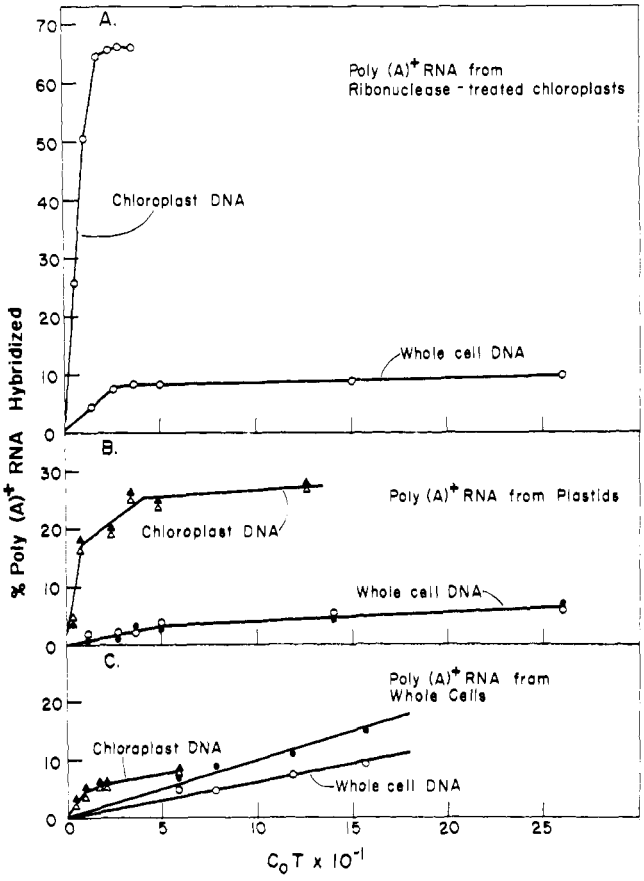

FIGURE 2: (A) Hybridization of poly(A)$^+$ RNA isolated from ribonuclease-treated chloroplasts to maize DNAs. In each experiment, about 0.05 μg of ^{125}I-labeled poly(A)$^+$ RNA (sp act. 2.8 $\times$ 10^4 cpm/μg) was incubated with membrane filters containing up to 50 μg of chloroplast DNA or up to 100 μg of whole maize DNA. The hybridizations were carried out as described under Experimental Procedures with an incubation time of 44 h. (B) Hybridizations of poly(A)$^+$ RNA isolated from plastid preparations to maize DNAs. In each experiment, about 0.1 μg of ^{125}I-labeled poly(A)$^+$ RNA (sp act. 2.5 $\times$ 10^4 cpm/μg) was incubated with a membrane filter containing up to 150 μg of chloroplast DNA or up to 500 μg of whole maize DNA. The hybridizations were carried out as described under Experimental Procedures with an incubation time of 42 h. (C) Hybridization of poly(A)$^+$ RNA isolated from maize leaves to maize DNAs. In each experiment, about 0.1 μg of ^{125}I-labeled poly(A)$^+$ RNA (sp act. 2.5 $\times$ 10^4 cpm/μg) was incubated with membrane filters containing up to 150 μg of chloroplast DNA or up to 400 μg of whole maize DNA. The hybridizations were carried out as described under Experimental Procedures with an incubation time of 31 h. (○) Poly(A)$^+$ RNA from green maize leaves, incubated with whole maize DNA; (●) poly(A)$^+$ RNA from etiolated maize leaves, incubated with whole maize DNA; (△) poly(A)$^+$ RNA from green maize leaves, incubated with chloroplast DNA; (▲) poly(A)$^+$ RNA from etiolated maize leaves, incubated with chloroplast DNA.

these are transcripts of nuclear DNA. However, at least one polypeptide (the large subunit of ribulose-1,5-diphosphate carboxylase) is synthesized within chloroplasts (for reviews see Woodcock and Bogorad, 1971; Ellis, 1975).

We reported here that a small proportion of maize poly(A)$^+$ RNA appears to be specific to the chloroplast. The absolute amount of isolatable chloroplast poly(A)$^+$ RNA averaged about 1 μg/kg of maize leaves. The total yields from repeated experiments can vary by a factor of 2. This plastid poly(A)-containing RNA may serve a mRNA function, since up to now only mRNAs (or their precursors) have been found to contain poly(A) tracts of substantial length.

Most of the poly(A)$^+$ RNA isolated from RNase-treated chloroplasts forms RNase-stable hybrids with chloroplast

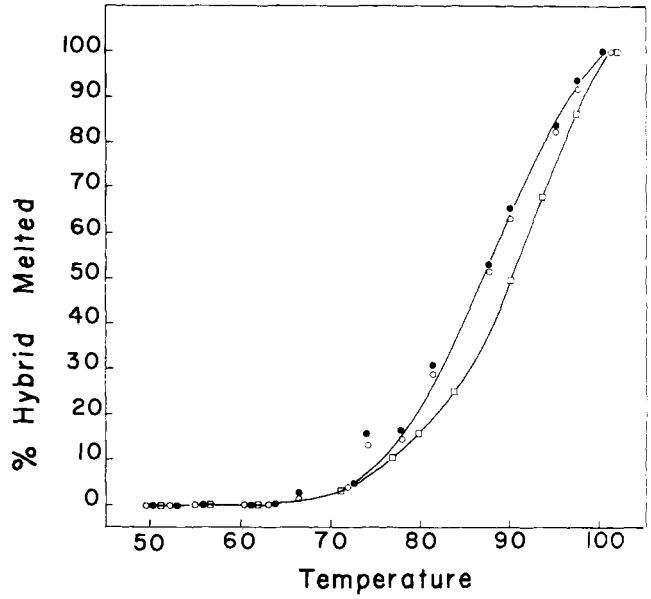

FIGURE 3: Melting curves of hybrids formed between ^{125}I-labeled poly(A)$^+$ RNA from chloroplasts or etioplasts, and chloroplast DNA. Membrane filters, bearing hybrids formed between chloroplast DNA and ^{125}I-labeled poly(A)$^+$ RNA isolated from either etioplasts or chloroplasts, taken from the experiment shown in Figure 2A were combined and rinsed with SSC. The filters were equilibrated with 50 °C SSC for 5 min and the supernatant solution, containing melted poly(A)$^+$ RNA, was withdrawn with a Pasteur pipet. Fresh SSC solution, 2–5 °C warmer than the first solution, was added, and the procedure was repeated up to a temperature of 100 °C. The procedure was also repeated with a hybrid of ^{125}I-labeled plastid rRNA and chloroplast DNA (0.2 μg of rRNA of sp act. 5.5 $\times$ 10^4 cpm/μg and 5 μg of chloroplast DNA). (○) Poly(A)$^+$ RNA from chloroplasts, hybridized with chloroplast DNA; (●) poly(A)$^+$ RNA from etioplasts, hybridized with chloroplast DNA; (□) plastid rRNA, hybridized with chloroplast DNA.

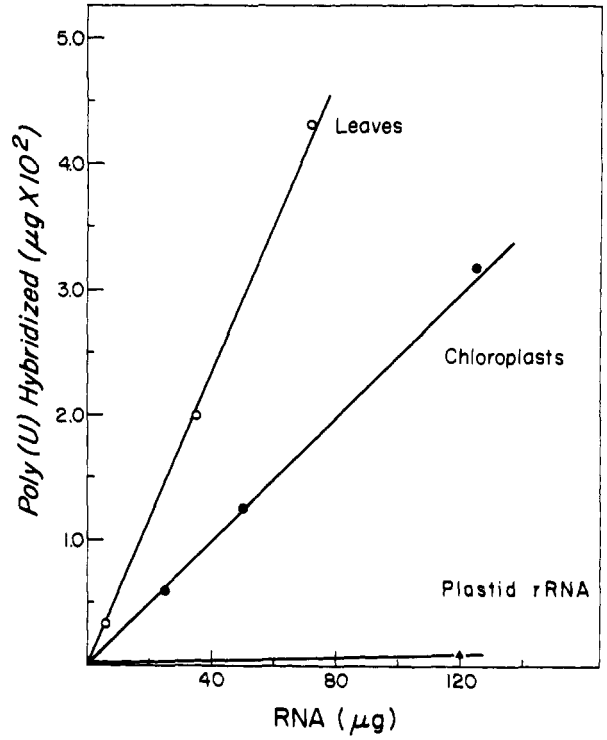

FIGURE 4: Detection of poly(A) in various RNA preparations by hybridization to [^{3}H]poly(U). The indicated amounts of RNA from each RNA preparation were hybridized to [^{3}H]poly(U) and treated with ribonucleases A and T$_1$ using the assay described by Wilt (1973). (○) Total RNA from green maize leaves; (●) total RNA from ribonuclease-treated chloroplasts (RNased chloroplasts); (▲) plastid rRNA.

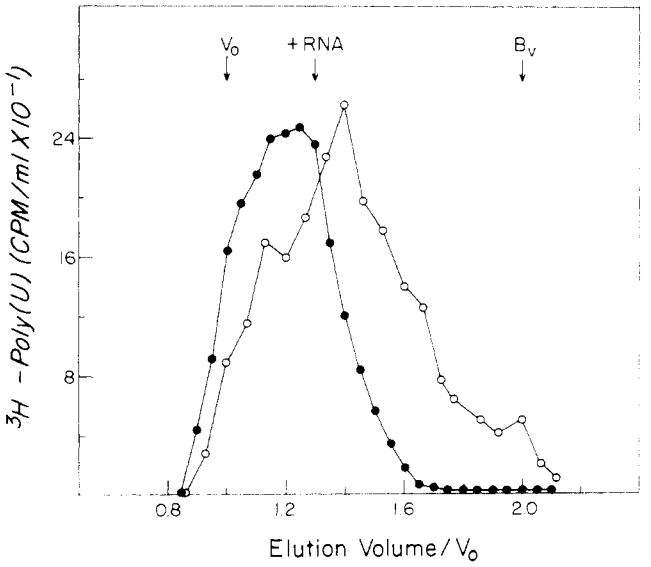

FIGURE 5: Exclusion chromatography of [^{3}H]poly(U) fragments. About 750 μg of RNA from either ribonuclease-treated chloroplasts, or from whole green maize leaves, was hybridized to 1 μCi of [^{3}H]poly(U) (32 μg) in 2.5 ml of twofold concentrated SSC at 45 °C for 45 min. Three volumes of chilled 2 × SSC containing 40 μg/ml of RNase A was added to the sample, which was then incubated at 4 °C for 30 min. The sample was extracted one time with phenol–10% cresol (pH 9). Poly(A) (100 μg) and yeast RNA (200 μg) were added to the sample, which was then precipitated with ethanol. After dissolving the sample in 90% formamide (pH 7) and heating briefly to 100 °C, the sample was chromatographed on a 0.8 × 59 cm column of Bio-Glass G-175, equilibrated with 90% formamide. The void volume (V_0) of the column was determined by the elution position of blue dextran, and the bed volume (B_v) determined from the column dimensions. Exclusion limits were given by the manufacturer. (○) [^{3}H]Poly(U) fragments sized from RNA isolated from ribonuclease-treated chloroplasts; (●) [^{3}H]poly(U) fragments sized from whole green maize leaf RNA.

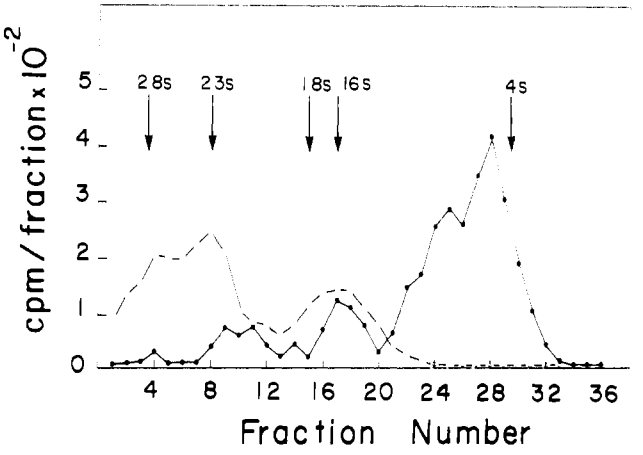

FIGURE 6: Sucrose gradient centrifugation of RNAs isolated from ribonuclease-treated chloroplasts. ^{125}I-labeled poly(A)$^+$ or poly(A)$^-$ RNA was centrifuged in linear 5–20% (w/v) gradients of sucrose in 0.15 M NaCl–25 mM Tricine-KOH (pH 7)–5 mM $MgCl_2$. Internal standards of maize cytoplasmic and chloroplastic rRNAs and yeast tRNA were centrifuged with the samples for 17 h at 24 000 rpm in a Spinco SW40 rotor at 4 °C. Fractions were collected from punctured tubes and acid-insoluble radioactivity of the fractions was determined. Cytoplasmic rRNA was prepared from corn germ ribosomes. (●) Radioactivity in poly(A)$^+$ RNA from ribonuclease-treated chloroplasts; (○) Radioactivity in poly(A)$^-$ RNA from ribonuclease-treated chloroplasts.

TABLE II: Hybridization of [^{3}H]Poly(A) to Chloroplast DNA.[a]

DNA Tested	[^{3}H]Poly(A) (μg)	RNase Treatment	cpm
24 μg of chloroplast DNA	0.168		595
	0.335		828
	0.500		598
	0.670		449
	0.168	+	109
	0.335	+	78
	0.500	+	−127
	0.670	+	67
0.1 μg of poly(dT)	0.670	+	20 700

[a] The indicated amounts of filter-bound DNA were incubated with up to 0.67 μg of [^{3}H]poly(A) (2.9 × 10^5 cpm) in 1 ml of 4 × SSC containing 35% formamide, at 37 °C for 17 h. The filters were washed with 2 × SSC, and half of the filters were incubated in 2 × SSC containing 10 μg/ml of RNase T_2 at 4 °C for 1 h. The filters were then washed with 2 × SSC and counted. Background values averaged about 150 cpm at the highest input level of [^{3}H]poly(A).

DNA. In addition, we have found that most of the remaining RNA forms a "ribonuclease-sensitive" complex with chloroplast DNA. This complex contains unlabeled poly(A) that could be detected by the poly(U) hybridization assay (data not shown) and labeled RNA that may have hybridized with poor fidelity to chloroplast DNA.

The reactivity in all our poly(A)$^+$ RNA preparations could be completely degraded to an acid-soluble state with RNase A, T_1, or T_2 (indicating, as expected, that the poly(A) tracts themselves were not labeled with ^{125}I). The fact that this labeled RNA was prepared from chloroplasts incubated in RNases T_1 and T_2 is a strong indication that the poly(A)$^+$ RNA was originally protected from RNase digestion by a physical barrier, probably the outer membrane of the chloroplast. Hartley and Ellis (1973) used ribonuclease A to remove RNA external to the outer membrane of spinach chloroplasts. We also attempted to prepare chloroplast RNA from maize chloroplasts treated with ribonuclease A or ribonuclease A immobilized onto Sepharose using the cyanogen bromide technique (Porath, 1974). The sucrose gradient determined sedimentation coefficients of these RNA preparations declined upon a few days storage at −20 °C, which was not true of RNA prepared from plastids treated with ribonucleases T_1 and T_2. For reasons that remain unclear, it appears that ribonuclease A is difficult to remove from plastids treated with this nuclease.

The evidence suggests that poly(A) tracts are added to plastid RNAs following their transcription, since chloroplasts contain a poly(A) polymerase capable of this function (Burkard and Keller, 1974) and since maize chloroplast DNA appeared to lack poly(dT) sequences. The average size of chloroplast poly(A) sequences was relatively short (about 45 nucleotides), half the size of whole cell poly(A) sequences. Generally, poly(A) tracts from prokaryotes (Nakazato et al., 1975) and from mitochondria (Perlman et al., 1973) have also been found to be shorter than poly(A) tract added to nuclear-encoded mRNAs. This size difference could be related to the observation that nuclei of some eukaryotes contain two poly(A) polymerases. The function of one of these polymerases may be to elongate relatively short poly(A) tracts (Haff and Keller, 1973a,b, 1975).

As shown in Figure 3, plastid poly(A)$^+$ RNA chloroplast DNA hybrids melted with a T_m of about 87 °C in SSC. Since the T_m of double-stranded chloroplast DNA itself is 85 °C, plastid poly(A)$^+$ RNA may have a (guanosine + cytosine) base content about the same as chloroplast DNA. This melting

curve also indicated that little of the hybridization of the poly(A)$^+$ RNA occurred through the poly(A) tracts themselves, since poly(A)·poly(dT) hybrids melt with a T_m of about 70 °C in SSC.

The sedimentation rate distribution of plastid poly(A)$^+$ RNA (Figure 6) was similar to the sedimentation behavior of mitochondrial poly(A)$^+$ RNA (cf. Ojala and Attardi, 1974). Although we report relatively low sedimentation values for plastid poly(A)$^+$ RNA, most plastid poly (A)$^+$ RNAs would appear to be large enough to code for small proteins. Both the isolation and radioiodination procedures could have caused some degradation of poly(A)$^+$ RNA. However, gross degradation is ruled out, since labeled poly(A)$^-$ RNA sedimented similarly to intact plastid rRNA. Since any degradation of poly(A)$^+$ RNA would result in lowered yields, the yields of poly(A)$^+$ RNA presented in Table I must be treated as minimums.

Although we found that at least 0.5% of total chloroplast RNA contains poly(A), our data do not reveal how many plastid mRNAs do not contain poly(A). Indeed, Sagher et al. (1976) and Wheeler and Hartley (1975) have detected mRNA activity for the large subunit of ribulose-1,5-diphosphate carboxylase, and this RNA does not appear to contain poly(A). Since only about 3% of *E. coli* mRNAs contain poly(A) (Nakazato et al., 1975), a low degree of polyadenylation may be a general characteristic of mRNAs from both prokaryotes and organelles of eukaryotes.

Acknowledgment

We thank George Kidd for performing the analytical ultracentrifugation.

References

Attardi, G., Constantino, P., and Ojala, D. (1974), *Biog. Mitochondria, Proc. Int. Conf.*, 9.

Bottomley, W., Smith, H. J., and Bogorad, L. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2412.

Burkard, G., and Keller, E. B. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 389.

Davidson, E. H., and Britten, R. J. (1973), *Q. Rev. Biol. 48,* 565.

Ellis, R. J. (1974), in Membrane Biogenesis, Tzagoloff, A., Ed., New York, N.Y., Plenum Press, p. 247.

Gillespie, D. (1968), *Methods Enzymol. 12B,* 641.

Haff, L. A., and Bogorad, L. (1976), *Biochemistry 15* (preceding paper in this issue).

Haff, L. A., and Keller, E. B. (1973a), *Biochem. Biophys. Res. Commun 54,* 1611.

Haff, L. A., and Keller, E. B. (1973b), *Biochem. Biophys. Res. Commun 51,* 704.

Haff, L. A., and Keller, E. B. (1975), *J. Biol. Chem. 250,* 1838.

Hartley, M. R., and Ellis, R. J. (1973), *Biochem. J. 134,* 249.

Hirsch, M., and Penman, S. (1974), *Cell 3,* 335.

Kolodner, R., and Tewari, K. K. (1972), *J. Biol. Chem. 247,* 6355.

Nakazato, H., Venkatesan, S., and Edmonds, M. (1975), *Nature (London) 256,* 144.

Ohasa, S., and Tsugita, A. (1972), *Nature (London), New Biol. 240,* 39.

Ojala, D., and Attardi, G. (1974), *J. Mol. Biol. 88,* 205.

Perlman, S., Abelson, H. T., and Penman, S. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 350.

Porath, J. (1974), *Methods Enzymol. 34,* 13.

Rose, K., Morris, H., and Jacob, S. T. (1975), *Biochemistry 14,* 1025.

Sagher, D., Edelman, M., and Jacob, K. (1976), *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Scherberg, N. H., and Refetoff, S. (1974), *J. Biol. Chem. 249,* 2143.

Schildkraut, C. L., Marmur, J., and Doty, P. (1962), *J. Mol. Biol. 4,* 430.

Tewari, K. K., and Wildman, S. G. (1970), *Symp. Soc. Exp. Biol. 24,* 147.

Vasconcelos, A. C. L., and Bogorad, L. (1971), *Biochim. Biophys. Acta 228,* 492.

Walker, P. M. B. (1969), *Prog. Nucleic Acid Res. Mol. Biol. 9,* 301.

Wheeler, A. M., and Hartley, M. R. (1975), *Nature (London) 257,* 66.

Wilt, F. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2345.

Winters, M. A., and Edmonds, M. (1973), *J. Biol. Chem. 248,* 4756.

Woodcock, C. L. F., and Bogorad, L. (1971), *Struct. Funct. Chloroplasts,* 89.