Synthesis of Poly(A)-Containing RNA by Isolated Spinach Chloroplasts†

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ABSTRACT: Chloroplasts were isolated from spinach leaves and the intact chloroplasts separated by centrifugation on gradients of silica sol. Chloroplasts prepared in this way were almost completely free of cytoplasmic rRNA. The purified chloroplasts were incubated with $^{32}PO_4$ in the light. The nucleic acids were then extracted and the RNA was fractionated into poly(A)-lacking RNA and poly(A)-containing RNA (poly(A)-RNA) via oligo(dT)-cellulose chromatography. The poly(A)-RNA had a mean size of approximately 18–20 S as determined by polyacrylamide gel electrophoresis. The poly(A)-RNA was digested with RNase A and RNase T_1, and the resulting poly(A) segments were subjected to electrophoresis on a 10% w/v polyacrylamide gel (98% v/v formamide). Radioactivity was incorporated into both poly(A)-RNA and poly(A)-lacking RNA and into the poly(A) segments themselves. The poly(A) segments were between 10 and 45 residues long and alkaline hydrolysis of poly(A) segments followed by descending paper chromatography showed that they were composed primarily of adenine residues. There was no $^{32}PO_4$ incorporation into acid-insoluble material in the dark. We conclude that isolated chloroplasts are capable of synthesizing poly(A)-RNA.

In the course of investigating the synthesis of macromolecules by isolated chloroplasts, we became interested in their capacity to form polyadenylated RNAs. Chloroplasts have been clearly shown to make discrete species of RNA. Bohnert et al. (1976) and Hartley et al. (1977), for example, demonstrated that isolated chloroplasts synthesize precursors of ribosomal RNAs and Serfling & Adler (1976) reported the formation of an RNA species that migrates in polyacrylamide gels coincident with tRNAs. Although newly synthesized mRNAs of eukaryotes are commonly found to contain 150–200 adenine residues covalently linked to their 3′ ends, the RNAs of chloroplasts displaying messenger activities are almost exclusively nonpoly(A) species (Wheeler & Hartley, 1975; Edelman et al., 1977; Howell et al., 1977). Specifically the mRNA for the large subunit of ribulose bisphosphate carboxylase, which is known from several lines of evidence to be coded on chloroplast DNA, is not polyadenylated (Howell et al., 1977; Edelman et al., 1977). Haff & Bogorad (1976) isolated poly(A)-RNA from maize chloroplasts. The poly(A) segments were about 40 nucleotides long, which corresponds closely to the lengths reported to be synthesized by isolated mitochondria (Aujame & Freeman, 1976; Ross & Jacob, 1976). They also observed that 65% of it hybridized to chloroplast DNA. Their data make it very likely that polyadenylation of a portion of the chloroplast-coded RNA occurs within the chloroplast, but, until now, the synthesis of poly(A)-RNA by isolated chloroplasts has not been demonstrated directly. A preliminary account of this work has been presented previously (Bartolf & Price, 1977).

Methods

Chloroplast Preparation. Intact spinach (*Spinacia oleracea* L., var. Bloomsdale Long Standing) chloroplasts were isolated, as described by Morgenthaler et al. (1974, 1975), from either 60 g or 120 g of whole leaves grown in the greenhouse or from commercial spinach purchased locally. The isopycnically banded intact chloroplasts were washed twice and resuspended for labeling in sorbitol/Tricine (*N*-tris(hydroxymethyl)glycine) medium (0.33 M sorbitol, 50 mM Tricine/KOH (pH 8.4)) (Morgenthaler & Mendiola-Morgenthaler, 1976).

Nucleic Acid Labeling. Chloroplasts were labeled with carrier-free $^{32}PO_4$ (New England Nuclear Corp.) by mixing the chloroplast suspension with approximately 1 mCi of $^{32}PO_4$/60 g of starting material. Prior to mixing with the chloroplast suspension, the $^{32}PO_4$ was mixed with an equal volume of double-strength sorbitol/Tricine medium. The total incubation mixture contained approximately 1 mg of chlorophyll in 4 mL. Incubation was for 40 min at an illumination of approximately 20 000 lx passed through a red filter (filter no. 14, Kliegl Brothers). From the time the leaves were homogenized until the labeled RNA was extracted, all operations were carried out by using the aseptic technique wherever possible in order to minimize bacterial contamination. The radioactivity was measured without scintillation fluid via Cerenkov counting as described by Mardh (1975), except that 7-mL polypropylene vials and glass adapters were used. In the studies on the time course of incorporation, 10-μL aliquots of the chloroplast suspension were spotted on 1-cm squares of filter paper and processed as described by Bollum (1966). Other variations in counting technique with different assay requirements are described below.

RNA Extraction. At the end of the labeling period, the chloroplasts were washed twice in sorbitol/Tricine medium. The pellet from the second wash was suspended in ice-cold extraction buffer (0.05 M Tris, 0.5% sodium dodecyl sulfate ($NaDodSO_4$), 5 mM $MgCl_2$ (pH 7.6)) containing 3% (v/v) diethyl pyrocarbonate in the proportions of 9 mL of buffer/60 g of starting material. The mixture was incubated at 37 °C for 5 min with occasional mixing. This was followed by centrifugation at 8000*g* for 15 min at room temperature. The supernatant fluid was made 1.7 M with respect to NaCl and then incubated at 37 °C for 5 min with occasional mixing.

The mixture was centrifuged at 10000*g* for 20 min at 4 °C. Two volumes of cold absolute ethanol was added to the supernatant fluid and it was then let stand at least 2 h at −10 °C. The nucleic acid precipitate was collected by centrifugation at 8000*g* for 15 min at 4 °C and, after drying in a vacuum desiccator for 15 min, resuspended in a minimal volume of deionized H_2O (Solymosy et al., 1968).

Oligo(dT)-Cellulose Fractionation. The RNA was fractionated into poly(A)-RNA and poly(A)-lacking RNA on a column consisting of a Pasteur pipet containing 0.2 g of ol-

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igo(dT)-cellulose (type T-2, Collaborative Research Inc.). The procedure was that of Aviv & Leder (1972) with the following modifications. The sample was applied to the column and the column washed at 4 °C in order to bind poly(A) tails less than about 30 nucleotides long (Nadel et al., 1976); since a significant number of counts remained on the column after elution at room temperature, the poly(A) was eluted at 45–50 °C; the application buffer was 0.5 M NaCl, 0.001 M EDTA, 0.01 M Tris-HCl (pH 7.5), and the elution buffer was 0.001 M EDTA, 0.01 M Tris-HCl (pH 7.5); in one experiment, dimethyl sulfoxide was used to dissociate poly(A)-lacking RNA aggregates from the poly(A)-containing RNA, prior to rechromatography on the oligo(dT)-cellulose column (Bantle et al., 1976); and the column was prerun with unlabeled spinach nucleic acid in order to block the nonspecific binding sites described by Bantle et al.

Other Techniques. The nuclease digestion was at 37 °C for 1 h with approximately 800 μg/mL RNase A (5× crystallized, Sigma), 8 μg/mL RNase T_1 (grade III, Sigma) and 150 μg/mL DNase I (chromatographically pure, Sigma) in 10 mM Tris, 500 mM KCl, 0.2 mM $MgCl_2$ (pH 7.6) (Kitos et al., 1972). The reaction was stopped by the addition of $NaDodSO_4$ to a final concentration of 0.5% (w/v) and diethyl pyrocarbonate to a final concentration of 0.5% (v/v). After the addition of *E. coli* tRNA (type XXI, Sigma) as carrier, the mixture was made 0.3 M in NaCl and precipitated with 2 volumes of absolute ethanol.

Alkaline hydrolysis was for 18 h at 37 °C in 0.3 M KOH (Edelman et al., 1970). The method developed by Lane (1963) was used for one-dimensional paper chromatography of the alkaline hydrolysate. Electrophoresis in formamide–polyacrylamide gels (98% formamide) was as described by Duesberg & Vogt (1973). Electrophoresis in agarose–acrylamide (0.5% agarose) was as described by Peacock & Dingman (1967) and Dingman & Peacock (1971). Chlorophyll was estimated by the method of Arnon (1949).

Results

Purity of Chloroplasts. Before we attempted to detect polyadenylation of RNA by isolated spinach chloroplasts, we examined our chloroplast preparations for cytoplasmic RNA contamination. Our criterion for cytoplasmic contamination was the presence of rRNA in the chloroplast preparations. Since the 23S component of the rRNA of spinach chloroplasts can degrade to an 18S component, indistinguishable by polyacrylamide gel electrophoresis from the 18S component of cytoplasmic rRNA (Leaver & Ingle, 1971), we looked at the 25S cytoplasmic component.

The analysis of rRNA in several stages of chloroplast purification is shown in Figure 1. We see that crude chloroplasts obtained by differential centrifugation contain about 4% 25S rRNA (profile b). We note that studies with chloroplasts have been usually carried out with preparations at this level of purity. When these crude chloroplasts are sedimented into silica sol gradients, two green bands are obtained corresponding to stripped thylakoid membranes and intact chloroplasts (Morgenthaler et al., 1975). The RNAs of these preparations are shown in profiles c and d of Figure 1. We see that the 25S RNA is recovered exclusively with the stripped thylakoid membranes. No 25S RNA is detectable in the zone of intact chloroplasts even when the gel is greatly overloaded (not shown). We conclude that contamination of gradient-purified chloroplasts by cytoplasmic RNA is insignificant.

Light-Dependent $^{32}PO_4$ Incorporation by Isolated Spinach Chloroplasts. Our gradient-purified spinach chloroplasts have been shown previously to incorporate [^{14}C]leucine into proteins, with light as the sole energy source (Morgenthaler & Mendiola-Morgenthaler, 1976). Figure 2 shows the time course of incorporation of $^{32}PO_4$ into acid-insoluble material in the light. There is no significant incorporation of $^{32}PO_4$ in the dark. Incorporation proceeds for 30 min, while for the next 35 min the total amount of incorporated material decreases slightly. Incubation in the presence of RNase A does not reduce the extent of incorporation, an indication that cytoplasmic contaminants are not responsible for the incorporation.

Synthesis of Poly(A)-Containing RNA. Total RNA was extracted from labeled chloroplasts and fractionated into poly(A)-RNA and poly(A)-lacking RNA via oligo(dT)-cellulose chromatography. The poly(A)-RNA was treated with dimethyl sulfoxide, to disaggregate any poly(A)-lacking RNA bound to it, and then rechromatographed (Bantle et al., 1976). The twice-chromatographed poly(A)-RNA was disaggregated

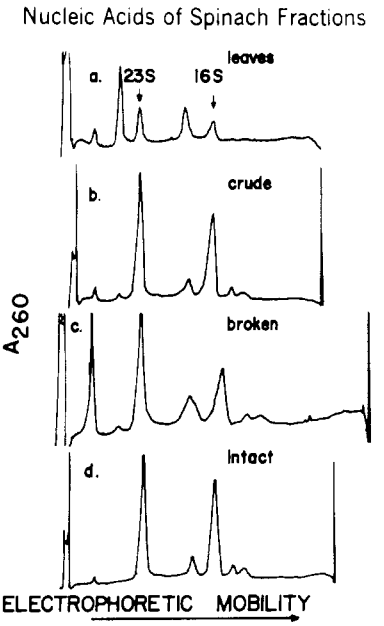

FIGURE 1: Gel electrophoretic profiles of total spinach nucleic acid and nucleic acid from various chloroplast fractions. The samples were subjected to electrophoresis on agarose–acrylamide (3% acrylamide) composite gels for 8.5 h at 4 mA/gel and the gels scanned at 260 nm: (a) total spinach nucleic acid; (b) nucleic acid from crude chloroplasts; (c) nucleic acid from broken chloroplasts; and (d) nucleic acid from intact chloroplasts.

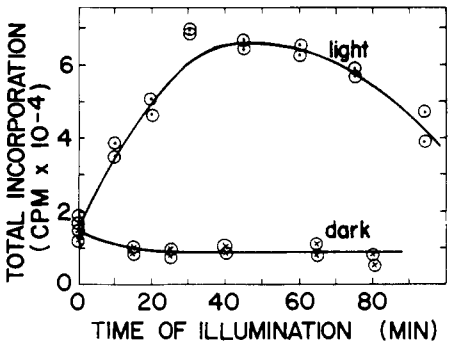

FIGURE 2: Time course of incorporation of $^{32}PO_4$ into acid-insoluble material by isolated spinach chloroplasts. The $^{32}PO_4$ labeling was as described under Methods, except for a reduction in scale, i.e., 0.3 mL of intact chloroplasts and 0.15 mCi of $^{32}PO_4$ were used. The conditions for the light (●) and dark (X) incubated chloroplasts were identical, except that the test tube in the dark was covered with aluminum foil. Ten-microliter aliquots were removed from the incubation mixtures at the times indicated and processed as described under Methods.

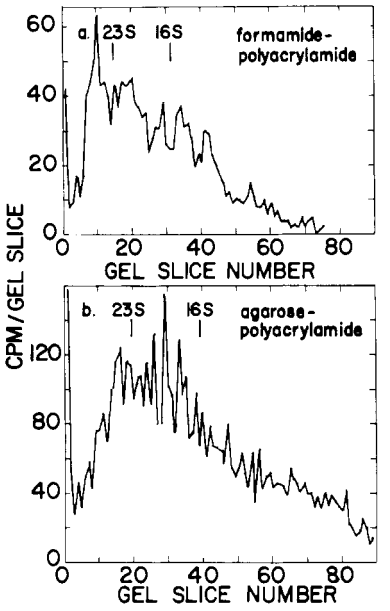

FIGURE 3: Polyacrylamide gel electrophoresis of poly(A)-RNA. (a) Formamide–polyacrylamide gel (4% acrylamide) run for 10 h at 75 V (4 gels) on 12-cm gels and (b) agarose–acrylamide composite gel (3% acrylamide) run for 7 h at 2.5 mA/gel on 10-cm gels. The standards were run simultaneously on separate gels and are from *E. coli*. The gels were frozen in dry ice, sliced into 1-mm sections, placed on filter paper disks, and counted via Cerenkov counting. The samples were counted twice for 10 min; a background of 33 cpm was subtracted from each data point.

by heating at 65 °C for 5 min followed by rapid chilling and then subjected to polyacrylamide gel electrophoresis. As seen in Figure 3, poly(A)-RNA is heterogeneous in size ranging from larger than 23 S to smaller than 16 S. In Figure 3a the sample was electrophoresed on a formamide–polyacrylamide gel (4% acrylamide), whereas in Figure 3b the sample was run on an agarose–polyacrylamide gel (3% acrylamide). Although the samples in each of the two gels were from separate experiments with different batches of spinach, the two gel systems yield similar results. While in Figure 3a there is some indication of discrete RNA species, it is obvious from a comparison of the results of the two gel systems that the poly(A)-RNA is heterogeneous in size. The completely denaturing formamide system was a priori the system of choice, but in practice these gels often proved too fragile to analyze.

The small amount of radioactivity depicted in Figure 3 may seem inconsistent with the large amount of incorporation shown in Figure 2. The chloroplasts of Figure 2 were obtained from young greenhouse spinach, while those of Figure 3 were isolated from mature commercial spinach. We consistently observed that the former incorporate $^{32}PO_4$ into acid-precipitable radioactivity at a much greater rate than the latter. While the greenhouse spinach is preferred and used when available, technical difficulties in growing it often necessitated the use of commercial spinach.

Size of Poly(A) Segments. The poly(A)-RNA was digested with the nucleases DNase I, RNase T_1, and RNase A under conditions which leave stretches of adenylic acid intact (see Methods). The hydrolysate was then analyzed on a formamide–polyacrylamide gel (10% acrylamide) (Figure 4). Calibration of the gel was by 5S and 4S *E. coli* standards and an A10 standard (Collaborative Research), as indicated in the figure. The poly(A) segments range from approximately 10 to 45 nucleotides in length with the greatest number of segments at the lower end of this range. We do not know the identity of the radioactive material near the top of the gel (left)

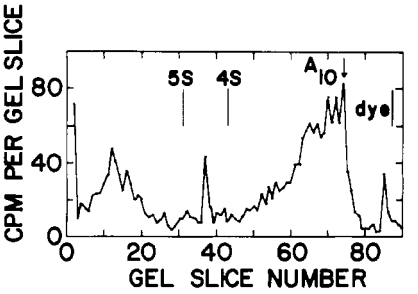

FIGURE 4: Formamide–polyacrylamide (10% acrylamide) gel electrophoresis of nuclease digested poly(A)-RNA. Electrophoresis in 10-cm gels at 60 V for 9 h. The gel was processed as in Figure 3. The 5S and 4S *E. coli* standards were run simultaneously on a separate gel. The A_{10} standard was run, together with 5S and 4S standards, on a separate occasion.

but assume it represents undigested poly(A)-RNA; although some material is always observed in this region of the gel, the amount varies and in some instances is barely detectable. We did not consistently observe the blip of radioactivity between the 5S and 4S markers.

Composition of Poly(A) Segments. Poly(A) segments were obtained as described for Figure 4 and eluted from gel slices in the appropriate region of the gel. They were then subjected to alkaline hydrolysis and paper chromatography; and the paper chromatogram was fractionated and counted via Cerenkov radiation. This resulted in 93.5% AMP, 3.1% CMP, 2.0% GMP, and 1.3% UMP. Nuclease digestion under the present conditions should yield an AMP value upon alkaline hydrolysis of 100% for 3′-poly(A)-terminated molecules. Among the possible reasons for obtaining less than 100% AMP are contamination by small nonpoly(A) molecules, incomplete nuclease digestion, or poly(A) segments which are not 3′ terminally located.

A similar analysis of poly(A)-RNA showed that all four nucleotides are labeled; that is, we are observing synthesis of the entire RNA molecule in the isolated chloroplasts rather than merely polyadenylation of preexisting RNA molecules.

Turnover of Poly(A)-RNA. Several experiments were performed in order to examine the possible turnover of poly(A) segments in isolated spinach chloroplasts. Chloroplasts were labeled for up to 10 min and chased for up to 25 min. The chase was accomplished either by adding cold phosphate to a final concentration of 0.25 mM or by sedimenting the chloroplasts and resuspending them in fresh sorbitol/Tricine medium. The gel profiles of the poly(A) segments obtained with labeling times as short as 5 min are essentially the same as in Figure 4; i.e., the poly(A) segments range from 10 to 45 nucleotides in length with the smallest segments predominating. With increasing times of chase following the initial pulse of $^{32}PO_4$, the size range of the poly(A) segments remains the same, but the larger segments predominate.

The results of a typical pulse-chase experiment are shown in Figure 5 in which the time of labeling is 10 min (Figure 5a) followed by chases of either 2 min (Figure 5b) or 5 min (Figure 5c). In other experiments (not shown) in which the time of chase was increased, the larger segments became even more predominant. Unlike the case in eukaryotic systems, chloroplast poly(A) segments do not appear to become shorter within the relatively short times (less than 30 min) of our experiments.

Discussion

The incorporation of highly purified chloroplasts of $^{32}PO_4$ into species of RNA that bind to oligo(dT)-cellulose and the

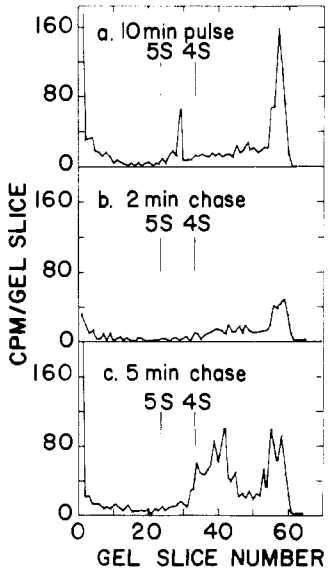

FIGURE 5: Formamide–polyacrylamide (6% acrylamide) gel electrophoresis of nuclease-digested poly(A)-RNA from a pulse-chase experiment. Electrophoresis on 10-cm gels for 5.25 h at 60 V. The chase was by addition of cold phosphate to a final concentration of 0.25 mM. The gels were processed as in Figure 3.

subsequent excision of ^{32}P-labeled segments of poly(A) provide the most direct kind of evidence that chloroplasts are capable of polyadenylation of RNA. These findings complement the compelling but indirect evidence of Haff & Bogorad (1975), who found that poly(A)-RNA from maize chloroplasts hybridizes to the extent of 65% with maize chloroplast DNA and that the chloroplast DNA lacks poly(dT). The detection by Burkard & Keller (1974), moreover, of a poly(A) polymerase in wheat chloroplasts provides a likely mechanism for polyadenylation.

We find that spinach chloroplasts form poly(A) segments 10–45 nucleotides long. This is shorter than the 30–60 unit segments reported by Haff & Bogorad. The difference may be due to species difference or to different analytical techniques, gel electrophoresis vs. exclusion chromatography, or relatively short time labeling by the isolated chloroplasts vs. a steady-state distribution.

The results of the pulse-chase experiments are puzzling. In eukaryotic systems, the poly(A) tails tend to become degraded with time, whereas our data are not consistent with a decrease in the length of poly(A) segments with time.

The function of poly(A)-RNA in chloroplasts remains obscure. From the relatively limited observations cited in the introduction, the bulk of the mRNA activity of chloroplasts appears to be poly(A) negative. Whether poly(A)-containing species represent a separate class of messengers, messengers in a different state, or serve some still unsuspected function remains for future determination.

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