et al., 1979). Within the frame of this model, it is concluded that the PO_4 moieties (and therefore the whole polar head groups as well) reorient at a significantly slower rate in the phase below the subtransition as compared with the $L_{\beta'}$ phase. In addition, the principal axes of the ^{31}P shielding tensor may change their orientation with respect to the effective diffusion axis during the subtransition. This could contribute to the observed shape differences of the ^{31}P powder spectra as well.

In summary, the processes taking place during the subtransition may be visualized as follows: In parallel to changes in the lateral acyl chain packing, the reorientation of the head groups becomes faster. This is possibly accompanied by a conformational change in the head-group region. Finally, some preliminary ^2H NMR results (Füldner, 1980) hint toward a change in the hydration of the head groups at the subtransition.

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Messenger Ribonucleic Acid Transcripts of Pea Chloroplast Deoxyribonucleic Acid†

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ABSTRACT: The total ribonucleic acid (RNA) from pea chloroplasts has been found to hybridize with 50% of the base sequences of pea chloroplast deoxyribonucleic acid (ctDNA). The specificity of hybridization has been analyzed by competition experiments and thermal stability studies of ctDNA–RNA hybrids. The ctDNA-specific RNA does not hybridize with the nuclear DNA. There is about 2–3% reduction in the total hybridization of ctDNA with RNA from the dark grown leaves. About 0.15–0.2% of the RNA from chloroplasts has been found to contain poly(A) tracts. About 20–25% of the ctDNA has been found to hybridize with poly(A+) RNA. Poly(A−) RNA, however, has been found to hybridize with 50% of the ctDNA. The molecular sizes of the mRNA transcripts have been found to range from 0.3×10^6 to 3×10^6.

Chloroplast deoxyribonucleic acids (ctDNAs)[1] from higher plants have been characterized for their structural organization and conformation (Tewari et al., 1977). The molecular sizes of ctDNA from higher plants have been found to range from 85×10^6 for corn ctDNA to 95×10^6 for lettuce ctDNA (Kolodner & Tewari, 1975). The genetic information contained in ctDNA has been studied by carrying out molecular hybridizations between ctDNA and purified stable species of ribonucleic acids (RNAs). Such experiments between pea

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[1] Abbreviations used: ctDNA, chloroplast deoxyribonucleic acid; RNA, ribonucleic acid; rRNA, ribosomal RNA; mRNA, messenger RNA; tRNA, transfer RNA; Tris, tris(hydroxymethyl)aminomethane; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DNase, deoxyribonuclease; RNase, ribonuclease; BSA, bovine serum albumin.

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ctDNA and chloroplast ribosomal RNA (rRNA) have shown that there are two rRNA genes in ctDNA of higher plants (Thomas & Tewari, 1974a,b). Recent studies (Chu et al., 1981) using fine-structure mapping of the rDNA region of the genome, R-loop technique, and DNA–DNA heteroduplex mapping have shown the presence of one rRNA gene in pea ctDNA. However, the presence of two rRNA genes in the ctDNA of corn has been recently confirmed by Bedrook et al. (1977). The hybridization experiments between pea ctDNA and ct-tRNAs have shown that there are ~40 tRNA genes in the organelle DNA. These tRNA genes account for practically all of the 20 aminoacyl-tRNAs (Meeker & Tewari, 1980). The amount of pea ctDNA involved in the coding of rRNA and tRNAs accounts for only ~5% of the genome. Therefore, we have now carried out saturation hybridization experiments with ctDNA and total RNAs from chloroplasts labeled in vivo. The data obtained by such experiments indicate that RNA transcripts present in chloroplasts hybridize to ~50% of the ctDNA, i.e., RNA molecules equivalent to a complete single-stranded chain of ctDNA are transcribed in vivo. The total RNA from chloroplasts has been found to contain poly(A+) sequences. About 20–25% of the pea ctDNA has been found to hybridize with poly(A+) RNA, whereas 50% of the ctDNA hybridizes with poly(A–) RNA.

Experimental Procedures

Isolation of RNA. Pea seedlings, *Pisum sativum*, 7–8 days old, were used for isolating RNA. Total cell RNA was isolated by homogenizing 200 seedlings (~50 g) in a buffer containing 100 mM Tris-HCl (pH 7.5) and 0.1% (v/v) diethyl pyrocarbonate (DEPC). The homogenate was filtered through four layers of cheesecloth and four layers of miracloth. The homogenate was treated with 4% (v/v) Sarkosyl (ICN) and made to a density of 1.60 g/mL with solid CsCl. The solution was centrifuged in a SW27 rotor for 48 h at 20000 rpm. The RNA pellet was dissolved in the extraction buffer containing 50 mM Tris-HCl (pH 7.6), 25 mM KCl, 25 mM $MgCl_2$, and 2% $NaDodSO_4$ and extracted 2 times with water-saturated phenol and 2 times with 98% chloroform–2% isoamyl alcohol (v/v). The RNA was precipitated by the addition of 2 volumes of 95% ethyl alcohol. The precipitated RNA was dissolved in 0.15 M NaCl and 2 mM EDTA (pH 7.5). Total chloroplast RNA was isolated by the above procedure using purified chloroplasts which were obtained by chopping the pea seedlings in a buffer containing 0.3 M sucrose, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0), 5 mM mercaptoethanol, and 0.1% (v/v) DEPC. The homogenate was filtered as before and the chloroplast fraction collected by centrifuging at 1000*g*. The chloroplast fraction was suspended in the homogenizing buffer and purified through discontinuous sucrose gradients as described before (Tewari & Wildman, 1969). The chloroplast fraction collected from 45% sucrose was washed 2 times with the homogenizing medium and collected by centrifuging at 1500*g*. Total polysomal RNA was isolated by homogenizing 100 g of seedlings in a buffer containing 0.25 M sucrose, 25 mM Tris-HCl (pH 8.0), 60 mM KCl, 30 mM $MgCl_2$, and 0.1% DEPC (v/v). The homogenate was filtered through four layers of cheese cloth and four layers of miracloth. The homogenate was treated with 2% Triton X-100 and centrifuged at 3000*g*. The polysomes were pelleted by layering the supernatant fluid on a 4-mL pad of 1.75 M sucrose, 25 mM Tris-HCl (pH 8.0), 20 mM KCl, and 10 mM $MgCl_2$ and centrifuged at 60000 rpm in a 65 rotor for 90 min (Saro et al., 1979). The polysomal pellet was extracted as described before. Chloroplast ribosomal RNA was isolated according to the method of Thomas & Tewari, 1974a,b.

Labeling of Plants. Radioactive labeling of the plants was carried out by cutting the stem of the seedlings and immersing the stem in 100 μL of 0.1 Hoagland solution containing 100 μCi of [^{3}H]uridine and 10 μg/mL gentamycin. The labeling with ^{32}P was carried out in the same manner except that the phosphate was deleted from Hoagland solution. The plants were labeled for 24 h and then kept for another 24 h in Hoagland solution without radioactivity. The plants were exposed alternately to 12 h of light and 12 h of dark periods.

Labeling of DNA in Vitro. Pea ctDNA was isolated as described before (Kolodner & Tewari, 1975). The ctDNA was nick translated essentially by the method of Maniatis et al. (1975) except that the amounts of DNase and DNA polymerase used were lower and the polymerized product was extensively purified. Five micrograms of ctDNA was incubated in 500 μL at 16 °C for 2 h in the presence of 0.5 μg each of dATP, dGTP, dCTP, and [^{32}P]dTTP (50 Ci/mmol), 25 μg of BSA, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 ng of DNase, and 1 unit of DNA polymerase (Boehringer, Mannheim). The reaction was stopped by adding 40 mM EDTA and the solution extracted with phenol. The aqueous phase was passed through a Sephadex G-50 column equilibrated with 0.15 M NaCl and 2 mM EDTA. The DNA-containing fractions were adsorbed to a hydroxylapatite column at 60 °C in 0.12 M phosphate buffer. The temperature of the column was raised to 100 °C, and the single-stranded DNA was eluted from the column in 0.12 M phosphate buffer. The labeled DNA was incubated at 60 °C for 30 min and passed through a hydroxylapatite column at 60 °C. The process was repeated 2 times more to remove hairpin loops (double-standed structures) in the DNA. The DNA solution was then centrifuged at 35000 rpm in an SW 50.1 rotor for 16 h, and the pelleted DNA was dissolved in 0.15 M NaCl and 2 mM EDTA (pH 8.0). The radioactive DNA obtained by this procedure was 99% susceptible to single-stranded nuclease (S1, Boehringer Mannheim). This DNA was found to renature to a level of 96% in the presence of unlabeled ctDNA. The $C_0t_{1/2}$ of the nick-translated DNA was indistinguishable from the $C_0t_{1/2}$ of the unlabeled ctDNA.

DNA–RNA Hybridization. All hybridization experiments were carried out under stringent conditions, T_m (temperature of half-disassociation) −18 °C, to minimize nonspecific hybridization. For hybridization with the RNA labeled in vivo, the ctDNA was immobilized on nitrocellulose filters (Schleicher & Schuell) by the method described before (Thomas & Tewari, 1974a,b). The filters containing DNA were incubated with RNA in a 1-mL solution containing SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.6), 40% (v/v) formamide (MCB), and 0.1% $NaDodSO_4$ at 37 °C for 16 h. In all experiments a control filter containing calf thymus DNA was incubated along with the filter containing ctDNA. After hybridization, the control and ctDNA filters were rinsed twice in 500 mL of SSC at room temperature for 15 min. For removal of nonspecifically bound RNA, the filters were incubated in a 1-mL solution containing SSC, 10 μg of RNase A, and 5 units of RNase T_1 (Sigma) for 30 min at 37 °C. After RNase treatment, the filters were rinsed in 1 L of SSC and dried, and the radioactivity was determined in a Beckman scintillation counter.

The hybridization with the labeled nick-translated DNA and unlabeled RNA was carried out in 0.2 mL of SSC at 65 °C. After the incubation, the solution was diluted to 1 mL, and 10 μg each of single- and double-stranded calf thymus DNA, 300 mM NaCl, 2 mM $ZnSO_4$, and 30 mM sodium acetate (pH 4.6) were added. S1 nuclease was added to this solution,

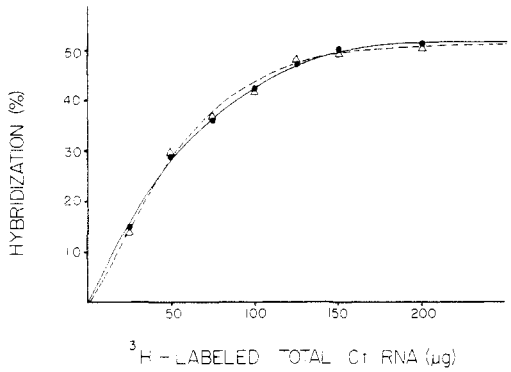

FIGURE 1: Saturation hybridization of ctDNA with ^{3}H-labeled total ctRNA. 0.5 μg of ctDNA bound to nitrocellulose filters was hybridized with labeled RNA as described in the text. The data are averages of six different hybridization experiments. (●) Total ctRNA; (△) ct-poly(A−) RNA.

and the mixture was incubated at 45 °C for 2 h. The same amount of S1 used in these experiments was enough to digest 99% of the single-stranded DNA and was standardized for each batch of the enzyme. At the end of 2 h, 10 μg of RNase A and 5 units of RNase T_1 were added, and the mixture was further incubated at 37 °C for 1 h. The undigested DNA–RNA hybrids were precipitated by the addition of ice-cold Cl_3CCOOH (5% final concentration) in the presence of 25 μg of calf thymus DNA. The control experiments contained calf liver RNA in the same concentrations as the RNA from plant cells.

Isolation of Poly(A+) RNA. The RNA was dissolved in 25% (v/v) formamide, 0.7 M NaCl, 50 mM Tris-HCl (pH 7.5), and 10 mM EDTA and passed through a poly(U)–Sepharose 4B column (Pharmacia) equilibrated in the same buffer (~40 mg of poly(U)–Sepharose/mg of RNA was used). The RNA solution was passed 3 times through the poly(U)–Sepharose column, and the column was extensively washed with the loading buffer until there was no radioactivity in the effluent volume. The bound RNA was then eluted from the column by a buffer containing 90% formamide, 10 mM EDTA, and 10 mM potassium phosphate buffer (pH 7.5). The eluted RNA was diluted 10-fold with the loading buffer and passed through a fresh poly(U)–Sepharose column. This process was repeated 3 times. In the second and third poly(U)–Sepharose columns, >90% of the radioactivity fractionated into poly(A+) RNA was retained.

Results

Hybridization of ctDNA with Total RNA of Chloroplasts. Increasing concentrations of total RNA from chloroplasts were hybridized to 0.5 μg of ctDNA. The data obtained from the saturation hybridization experiments are given in Figure 1. The data show that the hybridization of ctDNA with RNA increases almost linearly up to ~100 μg of RNA and then reaches a plateau at ~150 μg of RNA. Further increases in the RNA concentration to even 500 μg of RNA do not result in an increase in hybridization. At saturation, 50–55% of the ctDNA has hybridized to RNA from chloroplasts. With six different preparations of RNA, the hybridization data obtained by using different concentrations of RNA did not differ by more than 5%. These experiments were carried out with the RNA obtained from purified chloroplasts. In experiments where total cell RNA (i.e., RNA from both chloroplasts and cytoplasm) was used for hybridization, the data were similar to those obtained with the RNA from chloroplasts. At saturation, 50% of the ctDNA was found to be complementary to the total cell RNA (Figure 2). The only difference in the

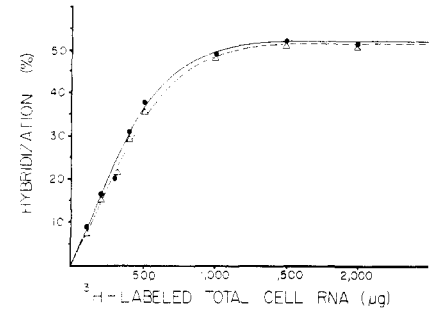

FIGURE 2: Saturation hybridization of ctDNA with total RNA of leaves. 0.5 μg of ctDNA was hybridized with the total RNA of leaves (i.e., RNA containing both cytoplasmic and chloroplastic RNA transcripts). (●) Total RNA from leaves; (△) total poly(A−) RNA.

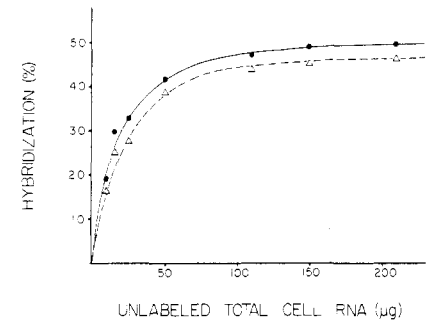

FIGURE 3: Saturation hybridization of nick-translated ctDNA and total RNA of leaves. Pea ctDNA was nick-translated as described in the text. The hybrids were analyzed by their insensitivity to S1 digestion. (●) Total RNA; (△) total RNA from dark grown leaves.

hybridization curves obtained with ctRNA and cell RNA was that it took ~1 mg of total cell RNA to saturate the base sequences in 0.5 μg of ctDNA, compared to ~150 μg of ctRNA needed to saturate the same amount of ctDNA.

The experiments with the saturating amounts of RNA have been possible because the RNA isolated as described under Experimental Procedures is extremely pure and binds very little to the nonspecific DNA. The labeling of plant RNAs under our conditions usually results in specific activities of 3000–10000 cpm/μg. Therefore, the hybridization experiments with 0.5 μg of ctDNA will result in the binding of 1500–5000 cpm to nitrocellulose filters at saturation. In the experiments where 2 mg of RNA of 10000 cpm/μg was used, the control filters were found to bind no more than 100 cpm, which is equivalent to a binding of 0.0005% of the input counts.

If different species of mRNA are labeled to different specific activities because of the different turnover rates of the individual RNA transcripts, the observed counts bound to the filters will not correctly represent the percent ctDNA being hybridized. In other words, the rate of hybridization with the differentially labeled RNAs would not be affected, but the extent of the observed hybridization could be higher or lower than the true hybridization. Therefore, the hybridization experiments were repeated with the nick-translated DNA uniformly labeled in vitro and the unlabeled total cell RNA. The data present in Figure 3 again show that 50% of the ctDNA has base sequences common to the total RNA of the cell. The saturation was almost complete at ~50 μg of RNA compared to ~1 mg of RNA in experiments where DNA immobilized to the nitrocellulose filters was used. However, only ~0.05 μg of nick-translated ctDNA was used in the experiments. The two different methods of hybridization, therefore, show similar levels of saturation of ctDNA.

Hybridization of ctDNA with Total Polysomal RNA. As mentioned above, 50% of the ctDNA sequences are represented in RNA transcripts. In order to find out whether all of these

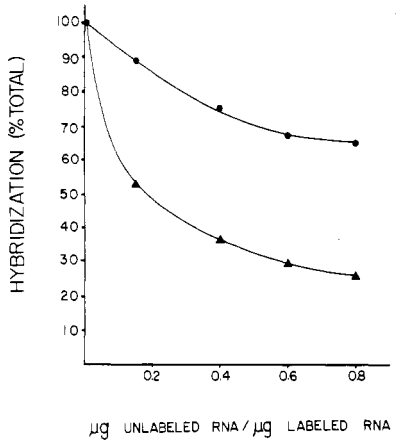

FIGURE 4: Competition hybridization experiments between labeled total cell RNA and unlabeled total RNA from cells and chloroplasts. The hybridization between ctDNA and 1000 μg of labeled total RNA was carried out in the presence of increasing amounts of unlabeled RNA from cells or chloroplasts. (●) Unlabeled total cell RNA; (▲) unlabeled total ctRNA.

transcripts are translated in vivo, we carried out the hybridization experiments with nick-translated ctDNA and RNA isolated from total polysomes. The hybridization data confirmed (data not shown) that at saturation, 45–48% of ctDNA sequences were present on the polysomes. These hybridization data are about 2–5% lower than those obtained with total ctRNA. It is difficult to attach any significance to the lower percent hybridization data, because they represent saturation hybridization to the total genome of the chloroplast. However, these hybridization data show that practically all of the transcribed RNA from the chloroplast genome is probably translated.

Specificity of ctDNA–RNA Hybrids. The amount of hybridization obtained above will be affected if the hybrids formed were not the result of specific base pairing. Therefore, we have carried out competition hybridization experiments where labeled total cell RNA was hybridized with ctDNA in the presence of unlabeled cell RNA and RNA from chloroplasts. As shown in Figure 4, the unlabeled total cell RNA was found to compete with the total labeled cell RNA. When the ratio of unlabeled total RNA to labeled total RNA was 0.2, the reduction in the observed hybridization was found to be ~10%. From purely mathematical calculations, the reduction should have been ~17%. Similarly, at a ratio of 0.4, the reduction was found to be ~22%, as opposed to the mathematically expected value of 29%. The observed reduction in hybridization is, thus, within the limitations of such an experiment. When RNA from chloroplasts was used, the competition was found to be much more than expected by mathematical calculations. For example, the hybridization was observed to be 45% less when the ratio of unlabeled ctRNA to labeled total cell RNA was 0.2. The observed reduction at a ratio of 0.4 was 62% with the unlabeled ctRNA compared to 20% with the unlabeled total cell RNA. These competition hybridization experiments were carried out with 1000 μg of total cell RNA labeled in vivo which was found to saturate 0.5 μg of ctDNA. Similar experiments with purified ctRNA showed that 150–200 μg of ctRNA was needed to saturate the same amount of ctDNA. In other words, 1000 μg of total cell RNA may contain about 150–200 μg of ctRNA. If that is the case, addition of 200 μg of unlabeled ctRNA is equivalent to adding equal amounts of unlabeled total cell RNA to the labeled total cell RNA. In such a case, the observed reduction in hybridization should be ~50%. Our

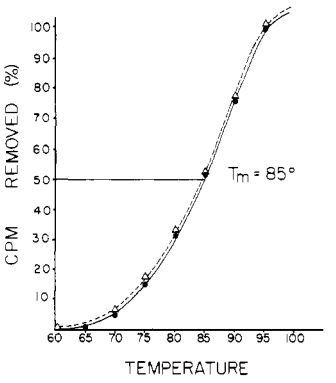

FIGURE 5: Thermal stability of ctDNA–RNA hybrids. (●) Total cell RNA; (△) total cell poly(A+) RNA.

observed reduction of 45% is very close to that expected by theoretical calculations. Similarly, at a ratio of 0.4, unlabeled ctRNA should reduce the hybridization by 67%. The observed reduction was 62%. These results clearly show that ctDNA was hybridized to ctDNA-specific RNAs present in total cell RNA.

The specificity of the hybrids was further tested by measuring the thermal stability of the ctDNA–RNA hybrids according to the procedure of Thomas & Tewari (1974a,b). The melting of the hybrids (Figure 5) was essentially the same whether total cell RNAs or ctRNA was used for hybridizations. The ctDNA–RNA hybrids were stable up to 70 °C, after which they began to melt with a sharp T_m of ~84 ± 1 °C. The T_m of pea ctDNA has been found to be 85 °C. Therefore, the ctDNA–RNA hybrids formed in our experiments represent the transcripts derived from the overall base composition of the ctDNA.

The specificity of the ctDNA–RNA hybrids was also shown by experiments where 25 μg of ctDNA was hybridized with 25 mg of total cell RNA labeled in vivo. The hybrids were washed with 2 × SSC and incubated in 45% (v/v) formamide in SSC to remove unspecific binding. The ribonuclease treatment was omitted from this experiment. The hybrids were incubated in 90% (v/v) formamide for 1 h at 50 °C in SSC to remove the RNA bound to ctDNA. The eluted RNA was diluted to 20% (v/v) formamide and made 0.1 M in sodium acetate (pH unadjusted), and 25 μg of tRNA was added, followed by 3 volumes of 95% ethanol. The precipitated RNA was suspended in SSC and rehybridized with the ctDNA and nuclear DNA. This RNA hybridized with the ctDNA at a $C_0t_{1/2}$ of 2, whereas no hybridization was observed with the nuclear DNA even at a $C_0t_{1/2}$ of 100.

Hybridization of ctDNA with the RNA from Dark-Grown Leaves. Pea seeds were germinated in the dark for ~10 days. The apical leaves were harvested and homogenized in darkness as described under Experimental Procedures. The total cell RNA isolated from the etiolated leaves was hybridized to the nick-translated ctDNA. The saturation curves generated from this RNA were very similar to those observed by the total cell RNA isolated from green leaves (Figure 3). The various experiments, however, showed about 2–3% reduction in the total hybridization of ctDNA with RNA from the etiolated leaves. This level of reduction, even though small, was consistently observed.

Poly(A+) RNA in Chloroplasts. The RNA bound to poly(U)–Sepharose presumably contained poly(A) sequences at the 3′ end. In order to confirm the presence of poly(A), we have analyzed the amounts of poly(A) in our RNA preparations by hybridizing it with radioactive poly(U) (Wilt, 1973). The RNA preparations were incubated with 0.1 μg

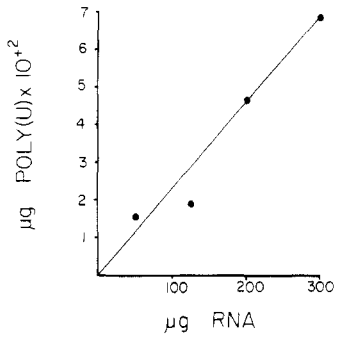

FIGURE 6: Binding of [^{3}H]poly(U) with ctRNA. The details are described in the text.

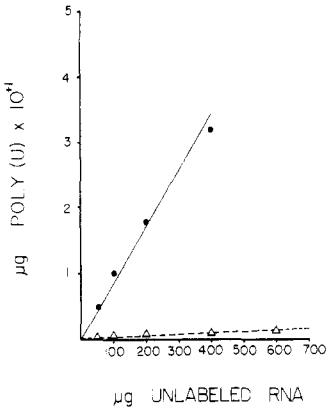

FIGURE 7: Binding of [^{3}H]poly(U) with total cell RNA and total poly(A−) RNA. (●) Total cell RNA; (△) total poly(A−) RNA.

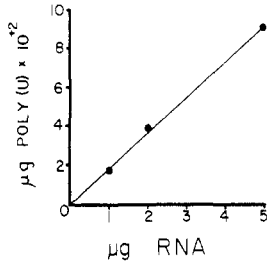

FIGURE 8: Binding of [^{3}H]poly(U) with poly(A+) RNA.

of [^{3}H]poly(U) (New England, specific activity 35 μCi/μmol of P) in 2 × SSC in a volume of 250 μL at 45 °C for 1 h. The solutions were chilled, made to 1.75 mL with 2 × SSC, and treated with 40 μg of RNase and 10 units of RNase T_1 at 4 °C for 30 min. Fifty micrograms of yeast tRNA was added and the hybridized poly(U) precipitated by adding 0.5 mL of 50% Cl_3CCOOH. The hybridization of poly(U) with increasing concentrations of ctRNA is shown in Figure 6. The increasing concentrations of ctRNA were found to bind linearly increasing amounts of poly(U). The poly(A) content of the ctRNA can be determined from the slope of the curve which shows that 2.38 × 10^{-2}% of the ctRNA is poly(A). Similar experiments with total cell RNA are shown in Figure 7. The slope of the curve shows that total cell RNA contains 9.1 × 10^{-2}% of the RNA in poly(A+) form. The specificity of these assays and the ability of the poly(U)–Sepharose column to effectively bind all the poly(A+) RNA is seen in Figure 7 where the [^{3}H]poly(U) binding experiments were performed with the RNA which did not bind to poly(U)–Sepharose. There was practically no hybridization with [^{3}H]poly(U), and the RNA that was not bound to poly(U)–Sepharose (i.e., poly(A−) RNA). Further confirmation of the fractionation procedure is seen in Figure 8 where poly(A+) RNA was used in the hybridization with [^{3}H]-poly(U). The poly(A+) RNA was found to contain 1.81% of RNA in poly(A+) form. The amount of poly(A+) RNA obtained from total cell RNA and ctRNA is shown in Table I. The percent of poly(A+) RNA in total cell RNA was found to range from 0.44 to 0.52%, and, in chloroplasts, it was found to range from 0.15 to 0.21% of the total ctRNA.

Table I: Amount of Poly(A+) RNA Isolated from Pea[a]

source of RNA	total RNA (mg)	poly(A+) RNA (μg)	poly(A+) RNA (% of total)
leaves	35	175	0.50
	42	218	0.52
	38	175	0.46
chloroplasts	6.5	9.8	0.15
	6.2	13.0	0.21
	5.4	9.7	0.18

[a] All RNA preparations were made from 200 seedlings weighing between 40 and 50 g. The poly(A+) RNA is defined as the RNA bound to poly(U)–Sepharose after three cycles of chromatography.

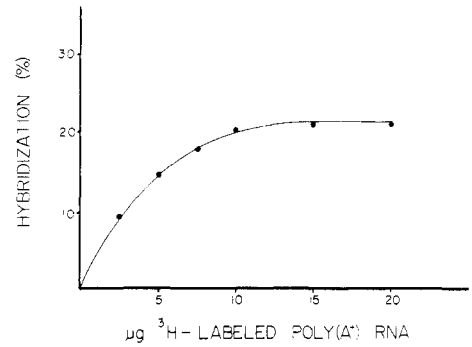

FIGURE 9: Hybridization of ctDNA with in vivo labeled total poly(A+) RNA.

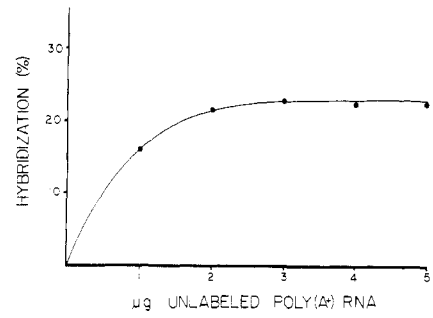

FIGURE 10: Hybridization of nick-translated ctDNA with unlabeled total poly(A+) RNA.

Hybridization of Poly(A+) RNA to ctDNA. We have hybridized ctDNA to the poly(A+) RNA labeled in vivo. At saturation (Figure 9), ~20% of the ctDNA was found to hybridize with poly(A+) RNA. In order to confirm this quantitative hybridization, we also carried out the hybridizations with poly(A+) RNA and nick-translated ctDNA (Figure 10). The data again showed that about 20–25% of the ctDNA has base sequence complementarity with poly(A+) RNA. The hybridization data with the labeled poly(A+) RNA (Figure 9) show that the hybridization is essentially complete at a concentration of ~15 μg. The same experiment with total cell RNA required ~2 mg to saturate a similar amount of ctDNA. The fractionation of RNA in poly(U)–Sepharose has obviously resulted in the tremendous purification of mRNAs from rRNA and tRNAs. The enrichment of the mRNA also is seen in experiments with the nick-translated DNA where 1 μg of poly(A+) RNA effectively saturated all the complementary sequences in the ctDNA.

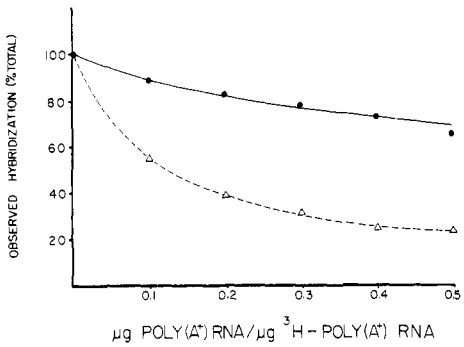

FIGURE 11: Competition hybridization experiments between labeled total cell poly(A+) RNA and unlabeled total poly(A+) RNA from cells (●) and chloroplasts (▲).

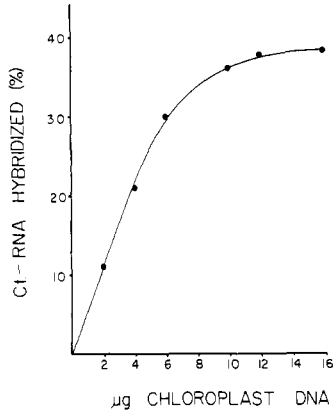

FIGURE 12: Hybridization of ct-poly(A+) RNA with increasing concentrations of ctDNA. 0.1 μg of poly(A+) RNA containing 1000 cpm was hybridized with ctDNA as described in text.

The specificity of the hybrids between ctDNA and cell poly(A+) RNA was tested by competition experiments. In the presence of unlabeled cell poly(A+) RNA, the observed hybridization of ctDNA showed the expected competition (Figure 11). However, the observed hybridization of ctDNA with the labeled total cell poly(A+) RNA was reduced more than 3-fold when carried out in the presence of poly(A+) RNA from chloroplasts. These results again indicate that ctDNA hybridized with ct-poly(A+) RNA. The thermal stability of the ctDNA–poly(A+) RNA (Figure 5) further confirmed the specificity of these hybridizations.

The labeled poly(A+) RNA from chloroplasts was hybridized to increasing concentrations of ctDNA. These experiments were carried out by using 0.1 μg of labeled RNA and hybridizing it with the ctDNA which was sheared to a single-stranded size of ~500 nucleotides. The hybrids were treated with ribonuclease and collected by Cl_3CCOOH precipitation. Figure 12 shows that ~40% of the poly(A+) RNA by weight from chloroplasts was hybridizable to ctDNA. About 10% of the poly(A+) RNA by weight from total leaves was hybridizable to ctDNA.

Hybridization of ctDNA with Poly(A−) RNA. The labeled poly(A−) RNA from cells was hybridized to ctDNA. The level of hybridization obtained with poly(A−) RNA was the same as that obtained with the total RNA; i.e., 50% of the ctDNA was found to hybridize with the poly(A−) RNA. Similar results were obtained by using nick-translated DNA (data not shown) and unlabeled poly(A−) RNA (Figure 1 and 2). The specificity of the hybrids was tested again by carrying out competition hybridization experiments and thermal stability analyses of the hybrids (data not shown). The results are again consistent with the specific sequence hybridization between ctDNA and poly(A−) RNA.

Table II: Analysis of Hybridization Kinetics of Chloroplast RNA[a]

abundance class	obsd $R_0t_{1/2}$ (M s)	% chloroplast genome	calcd[b] $R_0t_{1/2}$ (M s)	%[c] RNA population
1	17.4	20	0.0367	0.2
2	102	19	0.0349	0.03
3	256	10	0.0183	0.007

[a] All hybridizations were performed with 200 μg of total cell RNA and nick-translated ctDNA. [b] Calculated by using $R_0t_{1/2} = 3.3 \times 10^{-3}$ M s for the hybridization of 1.6×10^6 daltons of RNA and 89×10^6 as the complexity of the chloroplast genome. [c] Calculated by using a percent RNA population equal to 100 × calcd $R_0t_{1/2}$/obsd $R_0t_{1/2}$ (Chelm & Hallick, 1976).

Kinetics of ctDNA–RNA Hybridizations. The nick-translated ctDNA was hybridized with 200 μg of total cellular RNA, and the amount of hybrids formed was analyzed by treatment with S_1 nuclease. There were three inflection points (data not shown) at $R_0t_{1/2}$ of 17.5, 102, and 256 M s. The $R_0t_{1/2}$ of ct-rRNA (1.6×10^6 daltons) to homologous DNA has been found to be 3.3×10^{-3} (Chelm & Hallick, 1976). From the molecular weight of 89×10^6 daltons for pea ctDNA and the fraction of ctDNA reacting with each class of RNA, the expected $R_0t_{1/2}$ for each RNA class has been calculated to be 0.0367, 0.349, and 0.183 (Table II). The fraction of the total cellular RNA represented by each class of mRNA can then be calculated to be 0.2%, 0.03%, and 0.007%, respectively, following the method of Chelm & Hallick (1976). These calculations show that the most abundant class of mRNA sequences is present in 30 times the concentration of the least abundant class of mRNA.

Size of mRNA Transcripts of ctDNA. Five micrograms of ctDNA immobilized on a nitrocellulose filter was hybridized with 10 mg of ^{32}P-labeled total cell RNA labeled in vivo. The conditions of the hybridization were as described before. After hybridization, the filter was rinsed 4 times in 500 mL of SSC at room temperature for 1 h. The hybridized RNA was dissociated by incubating the filter at 37 °C for 1 h in a 1-mL solution containing SSC and 90% (v/v) formamide. The ctDNA-specific RNA transcripts were analyzed by electrophoresis as detailed in the legend of Figure 13. The sizes of the ctDNA-specific RNA transcripts were found to range from about 6 S to 30 S (Figure 13). The most important feature of these RNA transcripts is that they fall into 10–12 broad size classes. Even though the data show that RNA transcripts as large as 3×10^6 may be coded by ctDNA, ~75% of the RNA transcripts fall into the 10–18S range. It may be pointed out that the data presented here measure equimolar quantities of all the RNA coded by the ctDNA and do not favor abundant size classes.

Size Distribution of Poly(A) Tracts. The sizes of poly(A) tails in the ctDNA-specific RNA were determined by hybridizing 50 μg of ctDNA with 20 mg of ^{32}P-labeled total cell RNA labeled in vivo. The hybridized RNA was removed as described before and dissolved in 250 μL of 2 × SSC. Fifteen micrograms of RNase and 15 units of RNase T_1 were added, and the solution was incubated at 37 °C for 30 min. The reaction was stopped by adding 250 μL of 1% (v/v) NaDodSO$_4$, 10 mM EDTA, 10 μg of tRNA, and 10 μg of 5S RNA (Boehringer, Mannheim). The mixture was phenol extracted and precipitated in the presence of acetate buffer as described before. The undigested poly(A) was subjected to electrophoresis in 10% acrylamide gels and analyzed according to the method of Stringer et al. (1977). The majority of the poly(A) tracts were found to be about 50–150 nucleotides in

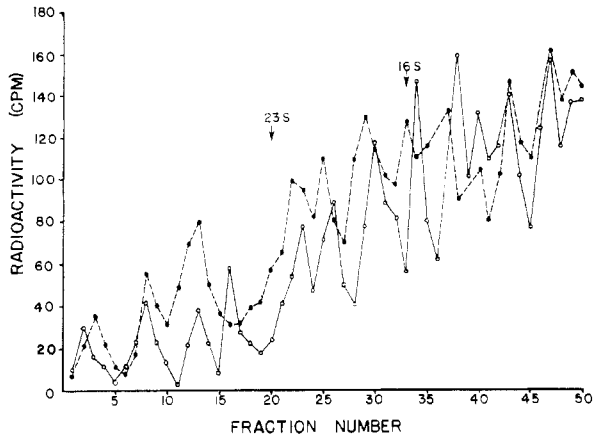

FIGURE 13: Electrophoresis of ctDNA-specific RNA. The RNA dissociated from ctDNA–RNA hybrids was diluted with 4 times the volume of 125 mM sodium acetate (pH unadjusted). The ctDNA-specific RNA was precipitated by adding 25 μg of ct-rRNA and 3 times the volume of 95% ethanol. The resulting RNA was subjected to electrophoresis in a agarose gel containing methylmercury according to the method of Bailey & Davidson (1976). After electrophoresis, the gel was stained with 0.5 μg/mL ethidium bromide and visualized on an ultraviolet transilluminator to determine the position of the marker 16S and 23S rRNA. The gel was cut into 2-mm slices with a mechanical slicer. Each slice was incubated in a 0.4-mL solution of 10% (v/v) perchloric acid at 70 °C for 15 min to dissolve the agarose. 10 mL of Aquasol-2 (NEN) was added and the radioactivity determined.

length (data not shown). However, about one-third of the poly(A) tracts were as large as 250 nucleotides in length.

Discussion

The data reported in the present paper of the transcription of pea ctDNA have shown that 50% of the base sequences of ctDNA are transcribed in vivo. If we assume that only one strand of the duplex DNA is transcribed into RNA, all of the genetic information in pea ctDNA is completely transcribed in vivo. We have shown that ~5% of the ctDNA is complementary to rRNA and tRNAs (Tewari et al., 1977). Thus, 45% of the pea ctDNA or $\sim 40 \times 10^6$ daltons of the molecule is being transcribed in mRNA. Assuming that 1.5 kilobases of DNA are required to code for a polypeptide of ~50 000 daltons, the amount of transcription of pea ctDNA is sufficient to code for ~80 polypeptides. The hybridization between the RNA from etiolated plants and ctDNA has shown that most of genes which are transcribed in light are also transcribed in dark. However, we have consistently observed a 2% difference in the transcription of ctDNA in etiolated and light-grown plants. This difference could conceivably account for three to four genes which are not transcribed in the dark. It is important to note that Bedbrook et al. (1978) have identified a plastid gene which is expressed during photoregulated development of plastids. In their experiments, the nonribosomal RNA fraction of the developing plastids and chloroplasts was found to contain RNA which hybridized to ctDNA from *Bam* fragment 8. This RNA was not found in etioplasts but appeared during the greening of leaves. The RNA which hybridized to *Bam* 8 was found to translate into a 34 500-dalton protein in vitro.

The quantitative analysis of the transcription of the ctDNA has also been carried out in *Euglena* (Chelm & Hallick, 1976). The nick-translated DNA in trace amounts was hybridized to total cell RNA extracted from *Euglena* cells at various times after the onset of chloroplast development. The amount of ctDNA transcribed was found to range from 12 to 23% of the double-stranded molecular weight of the ctDNA. In dark adapted cells, ~17% of the DNA was found to hybridize with the RNA. There was a decrease in the fraction of the genome transcribed at the beginning of light-induced chloroplast development, followed by an increase to 23% of the genome transcribed at the end of 72 h of development. Rawson & Boerma (1977) have also explored the extent of ctDNA transcription in *Euglena* by separating the strands of the ctDNA in alkaline CsCl equilibrium buoyant density gradients, labeling the separated DNA strands with ^{125}I, and hybridizing the DNA with unlabeled total cell RNA. By this procedure, it was observed that 9.5% of the heavy ctDNA and 43% of the light ctDNA strand were complementary to the total cell RNA for a total ~26% of the double-strand molecular weight of the ctDNA.

About 0.18% of the total RNA from pea chloroplasts has been found to contain poly(A) tracts. The fraction of poly(A+) RNA in total RNA of pea chloroplast is practically the same as in the chloroplasts of corn (Haff & Bogorad, 1976). The quantitative hybridization studies with the pea ctDNA and poly(A+) RNA from total chloroplasts or total cell RNA have shown that 20–25% of the base sequences of pea ctDNA are transcribed in poly(A+) RNA chains. The same level of hybridization was obtained whether in vivo labeled RNA or nick-translated ctDNA was used in hybridizations. These data show that only half of the RNA transcripts of ctDNA contain poly(A) tracts. This is in contrast to the results obtained with viral and nuclear transcripts from eukaryotic organisms where practically all mRNAs have been found to contain poly(A) tracts (Davidson et al., 1977). It may be pointed out that recent studies in brain have shown that about half of the total mRNA was poly(A–) mRNA (Van Ness et al., 1978). The studies on *Drosophila* have also shown that ~6000–7000 gene transcripts contain poly(A) tracts, whereas the same number of gene transcripts are also found in nonpoly(A) RNA fraction (Zimmerman et al., 1980).

The experiments with the hybridization of ctDNA with poly(A–) RNA have shown that all of the mRNA species which contain poly(A+) are also found in poly(A–) RNA. The poly(A–) RNA used in the experiments did not contain any poly(A) tracts as assayed by the hybridization with [^3H]poly(U). However, if the RNA was degraded during the isolation and fractionation procedure, the poly(A)-containing tracts will be adsorbed on the poly(U)–Sepharose and the rest of the RNA chain will be present in the poly(A–) RNA. The poly(A–) RNA fraction was analyzed by electrophoresis in 2.5% acrylamide gels and found to contain intact large rRNA chains (data not shown). Therefore, the poly(A–) RNA fraction contained essentially undegraded RNA chains. However, it is possible that the poly(A–) mRNA may contain very short poly(A) chains and, therefore, does not bind to poly(U)–Sepharose.

The mRNA transcripts of ctDNA have been found to range in sizes from 0.3×10^6 to 3×10^6. Most of the mRNA, however, is in the size range of 0.3×10^6 to 1.2×10^6. These RNA chains have been obtained by hybridizing the ctDNA with the in vivo labeled RNA. It is interesting to note that the RNA did not degrade in the hybridization and in the subsequent purification from the DNA–RNA hybrids. It is not known whether some of the mRNAs of chloroplast are transcribed in large sizes and then processed. However, the ability to obtain ctDNA-specific mRNA will help us in understanding and defining all of the mRNA transcripts of ctDNA. We have also analyzed the poly(A) tracts in the mRNA of transcripts by carrying out saturation hybridization of ctDNA with [^{32}P]RNA and then digesting the nonpoly(A)

sequences with ribonucleases. The majority of poly(A) tracts in ct-mRNAs have been found to range from 50 to 150 adenosine residues.

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Isolation and Partial Characterization of Membrane Protein Constituents of Human Neutrophil Receptors for Chemotactic Formylmethionyl Peptides†

Edward J. Goetzl,* Donald W. Foster, and Daniel W. Goldman

ABSTRACT: Plasma membranes of human neutrophils were solubilized in buffer containing a nonionic detergent and applied to a formylmethionylleucylphenylalanine (fMet-Leu-Phe)–Sepharose column that was washed and eluted with the chemotactic peptide fMet-Leu-Phe. Analysis of the eluate by filtration on Bio-Gel P150 in sodium dodecyl sulfate ($NaDodSO_4$) buffer and by $NaDodSO_4$–polyacrylamide gel electrophoresis revealed three predominant membrane proteins of approximate molecular weight 94 000 (MP-1), 68 000 (MP-2), and 40 000 (MP-3), of which MP-2 accounted for 74–93% of the total protein. Purified MP-1 and MP-2 contained an above average content of hydrophobic amino acids, while MP-2 and MP-3 had an above average content of acid and/or amide amino acids and a below average content of basic amino acids. MP-2 and MP-3, but not MP-1, bound [^{3}H]fMet-Leu-Phe in equilibrium dialysis chambers. Both MP-2 and MP-3 exhibited high-affinity sites with a valence of 0.2–0.3 and mean K_A values of 9×10^8 and 2×10^7 M^{-1}, respectively, and low-affinity sites with a valence of 0.3–0.5 and mean K_A values of 3×10^7 and 2×10^6 M^{-1} ($n = 3$). The specificity of the binding of fMet-Leu-Phe was suggested by the failure of MP-2 and MP-3 to bind lipid chemotactic factors and to adhere to a Sepharose column to which had been coupled chemotactic fragments of the fifth component of complement. A series of synthetic formylmethionyl peptides exhibited the same rank order of potency as inhibitors of the binding of [^{3}H]fMet-Leu-Phe by MP-2 and as stimuli of neutrophil chemotaxis. Membrane proteins isolated by fMet-Leu-Phe–Sepharose affinity chromatography may represent constituents of specific human neutrophil receptors for chemotactic peptides.

The chemotactic migration of leukocytes in response to a concentration gradient of a formylmethionyl peptide is initiated by the binding of the peptide stimulus to plasma membrane receptors on rabbit and human polymorphonuclear (PMN)[1] leukocytes and human monocytes (Aswanikumar et al., 1977; Pike et al., 1980; Williams et al., 1977). The relationship of the induction of chemotaxis to the specificity of the leukocyte receptors for formylmethionyl peptides initially was suggested by the close correlation between binding affinity and chemotactic potency for a variety of structurally distinct formylmethionyl peptides (Showell et al., 1976). Chemotactically inactive analogues inhibit the leukocyte chemotactic response to formylmethionyl peptides but not to the chemotactic peptide fragment of the fifth component of complement, and the in-

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[1] Abbreviations used: fMet-Leu-Phe, formylmethionylleucylphenylalanine; MP, membrane protein; PMN, polymorphonuclear; DNase, deoxyribonuclease; PMSF, phenylmethanesulfonyl fluoride; TLCK, *N*-tosyl-L-lysyl chloromethyl ketone; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; NP40, Nonidet P40; $NaDodSO_4$, sodium dodecyl sulfate; 5-HETE, 5-hydroxyeicosatetraenoic acid; LTB, leukotriene B, 5,12-dihydroxyeicosatetraenoic acid; C5fr, chemotactic fragments of the fifth component of complement; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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