Transfer Ribonucleic Acid Genes in the Chloroplast Deoxyribonucleic Acid of Pea Leaves†

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ABSTRACT: The saturation hybridization between pea ctDNA and ^{125}I-labeled pea ct-tRNAs has shown that 1.2% of the peak ctDNA codes for tRNA genes. The observed level of hybridization has been found to result from specific base pairings between ctDNA and ct-RNA as shown by competition hybridization experiments and thermal stability studies on DNA–tRNA hybrids. The level of hybridization obtained in this study amounts to the presence of ~40 tRNA genes in pea ctDNA. The tRNAs from the cytoplasm of the pea leaves, *Escherichia coli*, yeast, and calf thymus did not compete with the pea ct-tRNAs for the common base sequences in pea ctDNA. The presence of 17 aminoacyl-tRNA synthetases and their corresponding tRNAs was demonstrated in chloroplast. The acylation of ct-tRNAs proceeds with the same rate whether the partially purified tRNA synthetases from chloroplasts of *E. coli* are used. The aminoacylation of the three amino acids glutamic acid, glutamine, and cysteine proceeded very slowly in chloroplasts. The individually labeled aminoacyl-tRNAs hybridized with pea ctDNA. The hybridization follows true saturation rates, and the melting profiles of aminoacyl-tRNA–ctDNA indicate the formation of specific base pairs between the ctDNA and tRNA. Seventeen aminoacyl-tRNA genes have been identified in the pea ctDNA.

Chloroplast DNA from higher plants has been shown to exist in circular conformation with molecular weights ranging from 85×10^6 for corn ctDNA to 95×10^6 for lettuce ctDNA (Kolodner & Tewari, 1975a). The excellent agreement between the molecular sizes of these circular DNA molecules and the molecular weights of the unique sequences of these ctDNAs determined by renaturation kinetics suggests that the sequence of a circular ctDNA molecule represents the entire information content of the ctDNA (Tewari & Wildman, 1970; Tewari, 1971; Kolodner & Tewari, 1972). This suggestion was confirmed by the denaturation mapping studies with pea ctDNA (Kolodner & Tewari, 1975b). The denaturation maps of pea ctDNA, at all levels of denaturation, were demonstrated to be consistent with each other. All of the pea ctDNA molecules were found to be of similar base sequences.

The genetic information contained in ctDNA can be studied by molecular hybridization experiments involving ctDNA and RNAs of chloroplasts. By use of this approach, pea ctDNA was proven to contain ribosomal (r) RNA genes (Thomas & Tewari, 1974a). The data also showed that the genes for the 23S and 16S rRNA were nonoverlapping in pea ctDNA. These studies were extended to ctDNAs from bean, lettuce, spinach, corn, and oats (Thomas & Tewari, 1974b). The amount of hybridization between labeled pea ct-rRNA and ctDNAs from each higher plant showed that there were two ct-rRNA gene equivalents in these DNAs. The competition hybridization and thermal stability experiments involving homologous and heterologous ct-rRNA–ctDNA systems proved that the base sequences of rRNAs are very similar in these higher plants. These data have recently been confirmed by Bedbrook et al. (1977) for corn ctDNA and for spinach ctDNA by Herrmann et al. (1976). Using DNA fragments produced by restriction endonucleases, they have shown that corn and spinach ctDNAs contain two rRNA genes.

We now report the results of hybridization of pea ctDNA with pea ct-tRNAs (Tewari et al., 1977). The data show that there are 40 ± 10 tRNA genes in ctDNA. Competition hybridization experiments have demonstrated that ct-tRNAs do not have any common base sequences with tRNAs from cytoplasm. Also, ct-tRNAs do not share base sequences with the bacterial, yeast, or animal tRNAs. The ct-tRNAs have been acylated with individually labeled amino acids in the presence of aminoacyl-tRNA synthetases from chloroplasts and/or *Escherichia coli*, and the isolated genes for at least 17 aminoacyl-tRNAs can easily be identified in pea ctDNA. The amino acids glutamine, glutamic acid, and cysteine have been found to charge very poorly to the tRNAs. The hybridization data obtained with these amino acids suggest that their tRNAs might also be coded by pea ctDNA but the data are not conclusive. The hybridization data with aminoacyl-tRNAs have been confirmed by the thermal denaturation of the ctDNA–aminoacyl-tRNA hybrids.

Experimental Procedures

Chloroplast tRNA. In a typical experiment, 10–14-day-old pea leaves were homogenized in 3 L of cold (4 °C) STM buffer [0.3 M sucrose, 50 mM Tris-OH (pH 7.6), 5 mM $MgCl_2$, 0.1 mM mercaptoethanol, and ~0.05% diethyl pyrocarbonate] in two 500-g batches by using two 5-s bursts in a gallon-size Waring blender. All steps were carried out at 4 °C unless otherwise stated. The homogenate was filtered through four layers of cheesecloth and four layers of miracloth (Calbiochem) and centrifuged at 1500*g* for 15 min in a Sorvall centrifuge. The chloroplast pellet (containing nuclei and some mitochondria) was gently suspended in 225 mL of STM and recentrifuged at 1478*g* for 15 min. The procedure was repeated 1 more time. The washed chloroplast pellet was suspended with 125 mL of TM [50 mM Tris (pH 7.6) and 5 mM $MgCl_2$] and made to a final concentration of 0.2% Lubrol (Lubrol WX, ICI). After incubation for 15 min on ice, the lysed chloroplasts were centrifuged for 20 min at 35000*g*. The pellet (containing nuclei and chloroplast membranes) was discarded, and the supernatant was further centrifuged for 5 h at 80000*g* in a Beckman L3-50 ultracentrifuge. The top two-thirds of the 80000*g* supernatant then was carefully pipetted off by using

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a sterile glass syringe. To this was added a half-volume of extraction buffer [50 mM Tris (pH 7.6), 25 mM KCl, 25 mM $MgCl_2$, and 2% $NaDodSO_4$]. Phenol extraction was done per Thomas & Tewari (1974b). Two volumes of 95% ethanol was added to the aqueous phase and RNA precipitated overnight at −20 °C. The RNA precipitate was collected by centrifugation at 12000*g* for 10 min. The pellet was dissolved in 10 mL of TM [25 mM Tris (pH 7.6) and 5 mM $MgCl_2$], and 2.5-mL aliquots (~60–80 A_{260nm} units) were layered on 5–20% linear sucrose gradients and centrifuged at 80000*g* for ~40 h. The RNA banding at 4 S was collected by using a Beckman flow cell (1-cm path length) and a recording spectrophotometer. The pooled 4S RNA peaks then were precipitated by the addition of 2 volumes of 95% ethanol and stored overnight at −20 °C. The RNA was collected by centrifugation at 12000*g* for 10 min and dissolved in 2.5 mL of 1 × SSC.

In another variation of the above method, ct-tRNAs were obtained by using 2% Triton for the lysing of chloroplasts, and the RNA was loaded on a DEAE-cellulose column equilibrated with 0.05 M KCl. The column was washed with 0.2 M KCl and the tRNA was eluted with 1 M KCl. The cell tRNAs (i.e., tRNAs from chloroplasts and cytoplasm) were prepared by homogenizing the freshly harvested leaves in extraction buffer containing 0.05% diethyl pyrocarbonate. All the other steps were essentially the same as described above. The cytoplasmic tRNA was obtained from the roots of 6–8-day-old pea seedlings grown in sterile vermiculite.

Chloroplast DNA. The pea ctDNA was obtained by the method of Kolodner & Tewari (1975a). The absolute purity of all ctDNA preparations was tested by analyzing them in analytical CsCl density gradients following the denaturation and renaturation at a C_0t of 2.0 (Thomas & Tewari, 1974a). The quantitative amounts of DNA in our preparations were extremely important in order to arrive at an accurate number of genes. For this purpose, the DNA preparations have been analyzed by their hyperchromicity, and the DNA content has been calculated by taking the hyperchromicity of pure ctDNA preparations to be 36%.

Radioiodination of tRNAs. There are a number of radioiodination procedures described in the literature. The goal of all these methods is to remove the unstable radioactive intermediates formed during iodination, to minimize the chain breakage of RNA due to iodination, and to purify the radioiodinated RNA to yield acceptable levels of radioactivity in control experiments. We have intensively studied iodinated tRNA for all these criterion utilizing a number of different iodination methods (Tereba & McCarthy, 1973; Orosz & Wetmur, 1974; Meeker et al., 1976). The most suitable procedure is described here. The iodination was carried out at 60 °C for 30 min in 100 μL of a solution containing 1 mg/mL RNA, 7×10^{-5} M KI, 2×10^{-5} M $TlCl_3$, 50 mM sodium acetate (pH 4.8), and 1 m Ci/mL $Na^{125}I$ (4.7×10^{-7} M). The reaction was terminated by chilling the reactants to 4 °C. The reaction was brought to pH 9.0 by adding 1 M ammonium acetate buffer (pH 9.0) to a final concentration of 0.25 M. A freshly made solution of 0.1 M Na_2SO_3 was added to a final concentration of 0.05 M, and the iodination solution was reheated at 60 °C for 60 min. The iodinated RNA was precipitated by adding 2 volumes of alcohol. The precipitated RNA was loaded on a hydroxylapatite (HAP) column equilibrated with 0.01 M phosphate buffer (pH 6.8). The HAP-bound [^{125}I]RNA was exhaustively washed with the loading buffer until no radioactivity or A_{260nm} absorbing material was eluted. The [^{125}I]tRNA was then eluted from the HAP column with 0.25 M phosphate buffer (pH 6.8). Fractions containing 90% of the A_{260} material were pooled and further purified on a Sephadex G-25 column.

Aminoacylation of tRNAs. Aminoacylation reactions were carried out by using a number of aminoacyl-tRNA synthetase enzyme preparations. In the first procedure, 200 g of 8–10-day-old pea seedlings was chopped in 250 mL of STM without diethyl pyrocarbonate at 4 °C, filtered through four layers of cheese- and miracloth, and then centrifuged at 1478*g* for 15 min. The supernatant was decanted, and the chloroplasts were gently resuspended with 100 mL of STM and repelleted as above. The washed chloroplasts were then resuspended with 20 mL of TM [50 mM Tris, 5 mM $MgCl_2$ (pH 7.4) and 0.1 mM SHEtOH], and 5-mL aliquots were layered on discontinuous TM-buffered sucrose gradients containing 10 mL of 60% sucrose, 10 mL of 45% sucrose, and 10 mL of 30% sucrose in SW27 nitrocellulose centrifuge tubes. The gradients were centrifuged at 80000*g* for 1 h at 4 °C, and the chloroplasts banding at the interface of the 60–45% sucrose layer were collected by pipetting with a sterile glass syringe. The pooled chloroplasts were diluted with an equal volume of TM buffer and centrifuged at 1478*g* for 15 min. The pellet was resuspended to a final volume of 20 mL with TM buffer, disbursed into 1-mL aliquots, and kept frozen at −20 °C. Frozen chloroplasts were thawed at room temperature, lysed with Lubrol at a final concentration of 0.25%, and assayed for aminoacyl-tRNA synthetase activity. Alternatively, 50 g of 10–12-day-old pea seedlings was chopped in 150 mL of STM at 4 °C, filtered through cheese- and miracloth, and centrifuged at 1478*g* for 15 min. The chloroplast pellet was resuspended with 50 mL of STM and repelleted at 1478*g* for 15 min. The washed chloroplasts were suspended in 1 mL of TM to which $^1/_{10}$ volume of 10% Triton–TM was added. After lysis, the preparation was either assayed directly for aminoacyl-tRNA synthetase enzyme activity or was further purified by using DEAE-cellulose (Whatman DE-52) chromatography and/or Sephadex G-100 gel filtration. When the enzymes were purified by DEAE-cellulose, the lysed chloroplasts were adjusted to 0.2 M potassium phosphate (pH 6.8) by adding $^1/_5$ volume of 1 M potassium phosphate buffer (pH 6.8, 0.5 M KH_2PO_4 and 0.5 M K_2HPO_4), and 1–2-mL aliquots were loaded on a Pasteur pipet column (0.5 × 7 cm) containing DEAE-cellulose equilibrated with 0.2 M potassium phosphate (pH 6.8) and 0.1 mM SHEtOH. The synthetases eluting in the second and third void volumes of the column were pooled and either dialyzed or run through Sephadex G-100. Sephadex G-100 gel filtration was performed by using a 0.9 × 25 cm column equilibrated with TM. The synthetases were found to elute in the void volume of the column. The *E. coli* aminoacyl-tRNA synthetases were obtained from Miles Laboratories. The tRNAs were dissolved in 2 M Tris buffer (pH 8.2) and incubated for 90 min at 37 °C to ensure that all tRNA molecules were completely deacylated. Enzymatic assays for aminoacyl-tRNA synthetase activity were carried out at 37 °C in 200-μL volumes containg 50 mM Tris-OH (pH 7.4), 1 mM ATP, 2–5 μg of pea ct-tRNA, 5–10 μCi/mL ^{3}H-labeled amino acid, 5 mM 2-mercaptoethanol, 2–40 mM $MgCl_2$, and 10 μL of enzyme. Large-scale acylations of ct-tRNAs were carried out at 37 °C in 2-mL volumes containing the same concentrations of reagents as in the activity assay except that the ^{3}H-labeled amino acid concentrations were increased to 10–40 μCi/mL and the ct-tRNA was increased to 20–50 μg of RNA/mL. After the proper time interval, the acylation reaction was stopped by adding $^1/_{10}$ volume of 1 M sodium acetate (pH 5.0) and $^1/_{10}$ volume of 10% $NaDodSO_4$. The solution was phenol extracted by adding an equal volume

of water-saturated phenol–0.05% 8-hydroxyquinoline. The aqueous phase was phenol extracted a second time, followed by three extractions with ether. The ether-extracted aqueous phase was then purged of ether with N_2 gas and precipitated by adding 1 volume of 95% ethanol and storing overnight at −20 °C. The ethanol precipitate was centrifuged at 8714*g* for 10 min, and the pellet was air-dried for 10 min and then resuspended with 2 mL of 2 × SSC (pH 4.8). The A_{260nm} of the [^{3}H]aminoacyl-ct-tRNA solution was determined, and appropriate aliquots were taken for the determination of specific activity.

Hybridization. DNA bound to nitrocellulose filters were prepared as per Thomas & Tewari (1974b). Closed vials containing both a pea ctDNA filter and a control with an equivalent amount of calf thymus DNA were incubated for the various time intervals at 37 °C in a volume of 2 mL containing 1 × SSC (pH 7.6), 30% formamide, and 0.1% $NaDodSO_4$. After the incubation, the filters were rinsed with 5 mL of 2 × SSC at room temperature and incubated at 65 °C for 30 min in a fresh 5 mL of 2 × SSC. The filters were placed in another 5 mL of 2 × SSC and incubated with 50 μg of RNase A/mL for 30 min at 37 °C. Finally, each of the filters was rinsed with 30 mL of 2 × SSC at room temperature on a Millipore filter apparatus and dried and the radioactivity determined. When hybridizations were done with aminoacylated-ct-tRNAs, the incubation was carried out in 2 mL containing 2 × SSC (pH 4.8), 40% formamide, and 0.1% $NaDodSO_4$. After the hybridization, the filters were rinsed with 1 L of 4 × SSC (pH 6.0) at room temperature for 10 min and reincubated for 1 h in 2 × SSC (pH 4.8) and 40% formamide at 37 °C. After reincubation, the filters were again rinsed with 1 L of 2 × SSC (pH 6.0) at room temperature for 10 min. Finally, the filters were washed with 4 × SSC (pH 6.0) as described above.

Thermal Denaturation. The thermal stability of the ^{125}I-labeled pea ct-tRNA–pea ctDNA hybrid was determined by hybridizing the ^{125}I-labeled pea ct-tRNA at 1 μg of RNA/mL in 1 × SSC, 30% formamide, and 0.1% $NaDodSO_4$ at 37 °C for 20 h by using two sets of ctDNA filters. After hybridization, the filters were rinsed with 3 10-mL volumes of 2 × SSC (pH 7.6) at room temperature. The experimental filters then were placed in separate vials containg 2 mL of 40% formamide in 1 × SSC, preequilibrated at 37 °C. The filters were incubated for 10 min and then transferred to vials containing 50% formamide in 1 × SSC at 37 °C and incubated for 10 min. This procedure then was followed using 60, 70, 75, and 80% formamide. The eluted radioactivity for each step was determined by adding 50 μg of calf thymus DNA and 45 mL of ice-cold 5% Cl_3AcOH and placing the solutions on ice for 20 min. The Cl_3AcOH precipitate was collected on GF/A glass filters, washed with 5% Cl_3AcOH and 70% ethanol, dried, and counted.

The thermal stabilities of the aminoacyl-tRNA–ctDNA hybrids were determined in the following manner. The pea ct-tRNAs were aminoacylated with *E. coli* aminoacyl-tRNA synthetase and purified as described above. Aminoacylated ct-tRNA was hybridized to filters containing 50 μg of ctDNA for 4 h at 37 °C in 2 × SSC (pH 5.0), 30% formamide, and 0.1% $NaDodSO_4$. After hybridization, the filters were processed as described above.

Results

Chloroplast tRNAs. The isolation of RNA as described under Experimental Procedures does not result in the degradation of the 23S and 16S RNA chains of rRNA because we routinely obtain 2:1 ratios for the areas of the 23S and 16S rRNA peaks. The centrifugation of tRNAs in sucrose gradients for 40 h completely separates the 4S RNA from 16S and 23S RNA. The tRNA peak collected from sucrose gradients was further analyzed by electrophoresis in 10% acrylamide gels. The RNA preparation essentially showed only one peak which ran well ahead of the 5S RNA marker. These analyses clearly indicated that 4S RNA utilized for hybridization is completely free of 5S and degraded chains of rRNA.

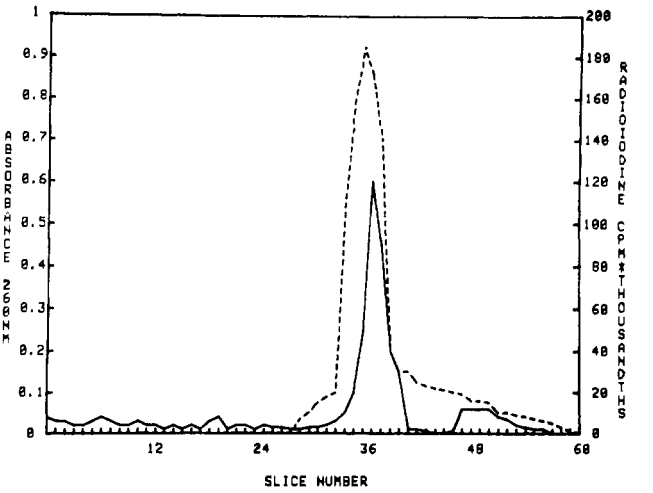

FIGURE 1: Polyacrylamide gel electrophoresis of ^{125}I-labeled pea ct-tRNA using 10% acrylamide–$^1/_3$% bis(acrylamide). Migration is from left to right. 0.5 μg of ^{125}I-labeled pea ct-tRNA (sp act. = 1.91 × 10^6 cpm/μg of RNA) purified through HAP and Sephadex G-25 from the iodination procedure (Experimental Procedures) and 25 μg of unlabeled pea ct-tRNA were layered onto the gel and electrophoresed at 4 mA/gel for 5 h at 23 °C.

Purity of Iodinated tRNAs. The advantage of the iodinated RNA in hybridization studies lies in the ability to label RNA to a very high specific activity. However, the iodinated RNA has been found to give very high blanks in the hybridization using DNA–nitrocellulose filters. The iodination procedure described in this communication gave background radioactivity of only 0.01–0.03% of the input radioactivity which is a greater than 10-fold improvement in the background over the other iodination procedures used. Further, the number of counts bound to the ctDNA filters routinely exceeded the background counts by a factor of 10–20, and the level of hybridization obtained in different experiments was highly reproducible. The [^{125}I]tRNA prepared by this method was also found to contain stable [^{125}I]cytosine when RNA was exposed to different concentrations of formamide. The iodinated RNA was electrophoresed in 10% acrylamide gels and found to exhibit no degradation as seen in Figure 1.

Number of tRNA Genes in ctDNA. The purity of ctDNA and tRNAs is of utmost importance in order to arrive at the quantitative number of tRNA genes in ctDNA. The purity of tRNAs and ctDNA has already been described. The data obtained on the time course of hybridization using ctDNA and [^{125}I]ct-tRNAs are presented in Figure 2. The data show that the rate of hybridization increases with the time of incubation and is essentially complete after 20 h of incubation. Further increase in the time of hybridization did not result in any significant change in the level of hybridization. The amount of hybridization of ctDNA with ct-tRNAs was found to be ~1.2 ± 0.3%. This level of hybridization has been obtained in a number of experiments carried out over a period of 2 years with the specific activities of tRNAs (counts per minute per microgram) ranging from about 200 000 to 3 × 10^6 (data not shown). The presence of degraded rRNA in tRNAs can significantly affect the quantitation of tRNA genes in ctDNA.

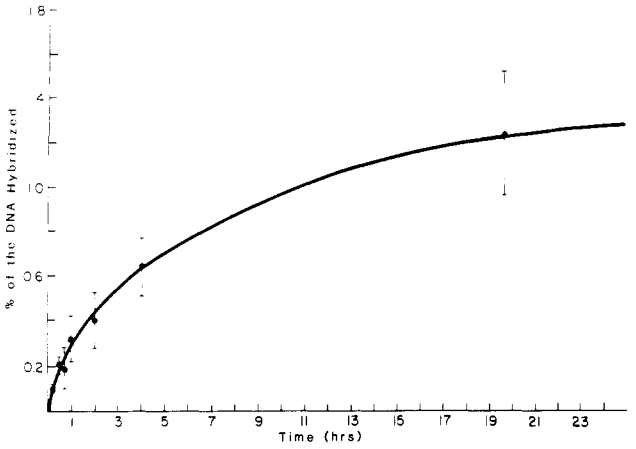

FIGURE 2: Time course of saturation hybridization of total ^{125}I-labeled pea tRNA with 0.5 μg of pea ctDNA. Hybridizations were done in a 2-mL volume containing 1 μg of [^{125}I]tRNA/mL, 1 × SSC (pH 7.6), 40% formamide, and 0.1% $NaDodSO_4$ and incubated at 37 °C for the incubated time periods.

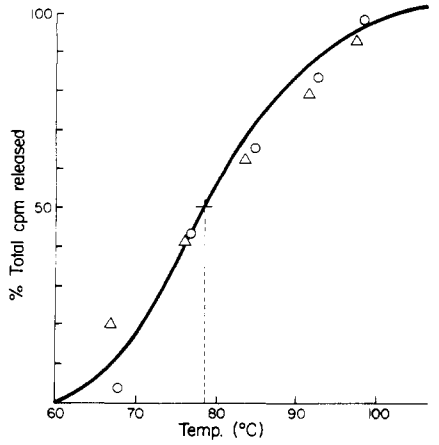

FIGURE 3: Thermal denaturation of the ^{125}I-labeled pea ct-tRNA–pea ctDNA hybrid. The experimental conditions were those described under Experimental Procedures. Data points are the averages of four separate determinations.

Even though our tRNA preparations did not contain any measurable amounts of degraded rRNA, we carried out the hybridization between ctDNA and ^{125}I-labeled tRNAs in the presence of 10 times the concentration of unlabeled ct-rRNA over labeled ct-tRNAs. The amount of hybridization with ct-tRNAs was not affected in these experiments. The data of Figure 2 were analyzed by a double-reciprocal plot and these data gave a straight line, indicating a single-component kinetics of hybridization. The extrapolation of the hybridization at infinite time yielded a value of ~1%, which was very close to the value obtained in experiments with saturating concentrations of tRNAs. As expected, the amount of observed hybridization was found to be proportional to the concentrations of ctDNA.

The base-pairing specificity of ctDNA–tRNA hybrids was analyzed by denaturation of the hybrids using increasing concentrations of formamide from 40 to 80% in 1 × SSC. The addition of 1% formamide to salt concentration of 1 × SSC reduces the temperature required to denature nucleic acids by 0.7 °C (Kolodner & Tewari, 1975b). Therefore, the concentrations of formamide used in denaturation experiments are equivalent to the temperature range of 37–93 °C. The results of four separate denaturation experiments are plotted in Figure 3. The ctDNA–tRNA hybrids were relatively stable until 70 °C, after which they began to melt. At 90 °C, ~80% of the hybrids were found to dissociate. At 93 °C, the melting of the hybrids was complete. The T_m (temperature of half-dissociation) of the ctDNA–tRNA hybrids was found to be 80 °C.

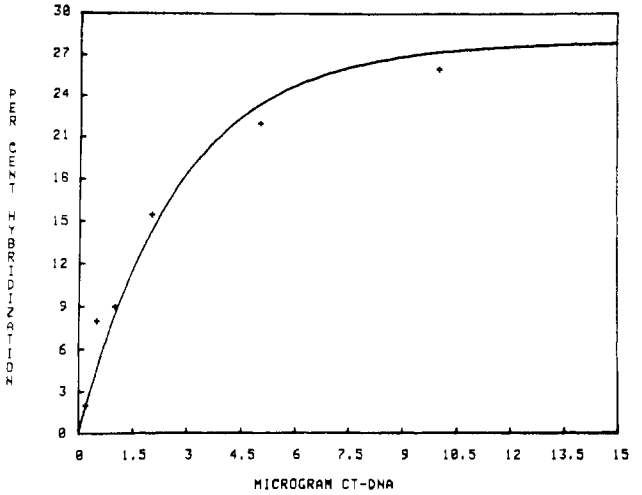

FIGURE 4: DNA excess hybridization of ^{125}I-labeled pea ct-tRNA against varying amounts of pea ctDNA. Experimental conditions were 1 × SSC, 40% formamide, 0.1% $NaDodSO_4$ 0.0052 μg/mL ct-tRNA and incubation for 25 h at 37 °C. (^{125}I-Labeled pea ct-tRNA sp act. = 6.73 × 10^5 cpm/μg of RNA.)

Chloroplast tRNAs Are Unique. The base-pairing specificity between [^{125}I]ct-tRNAs and ctDNA was further tested by competition with cytoplasmic tRNAs. The cytoplasmic tRNAs were obtained from young roots of the pea plants because of the inevitable contamination of cytoplasmic (cyt) tRNAs with ct-tRNAs in tRNAs isolated from leaves. This is due to the disruption of the mobile phase of the chloroplasts during the preparation of plastids. The pea cyt-tRNAs did not compete with the ^{125}I-labeled pea ct-tRNAs for the complementary base sequences in ctDNA. This lack of competition was seen even at a C/H ratio of 10:1. The cyt-tRNAs was also found not to hybridize with ctDNA. In experiments where ctDNA was hybridized with the total cell [^{125}I]tRNAs (i.e., tRNAs containing both the cytoplasmic and chloroplastic species), the amount of hybridization was found to be ~1%, a value close to that obtained by hybridizing ctDNA with ct-tRNAs. The ctDNA-specific tRNAs have not been found to hybridize with nuclear (n) DNA. This conclusion was derived from the following experiment. Five to ten micrograms of ctDNA was saturated with [^{125}I]ct-tRNAs, and the hybridized tRNAs were eluted by incubating the hybrid in 90% formamide at 50 °C. The ctDNA specific tRNAs were then hybridized to 50–100 μg of nDNA and 0.5–1.0 μg of ctDNA. The nDNA was not found to hybridize with the tRNAs but the [^{125}I]ct-tRNAs did hybridize with ctDNA. The specificity of base pairing between ctDNA and ct-tRNAs was further studied by using tRNAs from *E. coli* (prokaryote), yeast (a lower eukaryote), and calf liver (a higher eukaryote). None of these tRNAs were found to compete with ct-tRNAs for the complementary base sequences in ctDNA.

Amount of ctDNA-Specific tRNAs in Pea Leaves. In order to find out the amount of ctDNA specific tRNAs in chloroplasts and total tRNAs of cells, we carried out DNA excess hybridizations. Highly labeled [^{125}I]tRNAs in concentrations of 0.0046–0.052 μg of RNA/mL were incubated with increasing concentrations of ctDNA on the nitrocellulose filters. The amount of tRNAs hybridized at saturation amounts to ~30% (Figure 4). The same amount of hybridization was obtained whether ct-tRNAs or total cell tRNAs were used in the experiments.

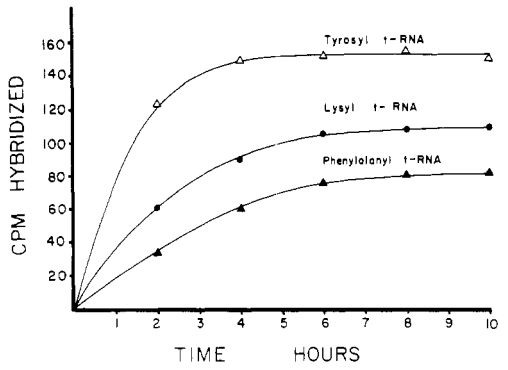

FIGURE 5: Time course of hybridization of tyrosyl- (△), lysyl- (●), and phenylalanyl-tRNA (▲) with the pea ctDNA. The hybridization was carried out as described under Experimental Procedures.

Aminoacyl-tRNA Synthetases in Chloroplasts. The above experiments have shown that ~1% of the pea ctDNA is complementary to tRNAs. In order to find out whether all of the 20 aminoacyl-tRNAs are coded by ctDNA, it was decided to acylate ct-tRNAs with radioactive amino acids and hybridize them with ctDNA. In order to carry out these experiments, it was necessary to demonstrate the presence of aminoacyl-tRNA synthetases in chloroplasts. The presence of only a few aminoacyl-tRNA synthetases in pea chloroplasts has been shown before (Aliev et al., 1967; Aliev & Filippovich, 1968). Therefore, it was decided to study the acylation of each amino acid with tRNAs and synthetases isolated from chloroplasts.

The presence of many aminoacyl-tRNA synthetases was shown in lysed chloroplasts without separating the tRNAs and synthetases. The observed acylating activity for certain amino acids in these preparations was enhanced by partial purification of the synthetases. The synthetases from chloroplasts were fractionated on either a DEAE-cellulose column or Sephadex G-100 for the acylation reactions. The tRNAs for these experiments were obtained by sucrose gradient centrifugation or DEAE-cellulose chromatography. Using a combination of different tRNAs and synthetase preparations, the acylation of all the 20 amino acids is shown in Table I. All of the amino acids except glutamic acid, glutamine, and cysteine actively charged the ct-tRNAs by using synthetases from chloroplasts. The amount of observed acylation with different amino acids was found to range from 2 to 50 pmol/mg of ct-tRNAs. For example, the acylation by alanine, tryptophan, proline, serine, isoleucine, methionine, glycine, threonine, aspartic acid, and tyrosine ranged from about 2 to 10 pmol/mg of ct-tRNA. The amino acids arginine and phenylalanine, however, were found to charge from about 20 to 50 pmol/mg of ct-tRNA. The ct-tRNAs were also charged with each individual amino acid by using synthetases from *E. coli*. As seen from the data of Table I, there was no difference in acylation whether the synthetases from chloroplasts or from *E. coli* were used. The ct-tRNAs were also acylated by using the total synthetases for the cell. The amount of observed acylation in these experiments was significantly higher than that obtained by using synthetases from chloroplasts or *E. coli*. This observation is consistent with the presence of cyt-tRNAs in chloroplasts which are acylated only by cytoplasmic synthetases.

In order to affect the maximum acylation of tRNAs by each amino acid, we determined the Mg^{2+} requirement for the acylation of each amino acid by keeping the concentration of ATP (1 mM) constant. The optimal Mg^{2+} requirement for different amino acids was found to vary from 2 to 10 mM. After determination of the optimum Mg^{2+} concentration for the acylation reaction, the optimum time for acylation was

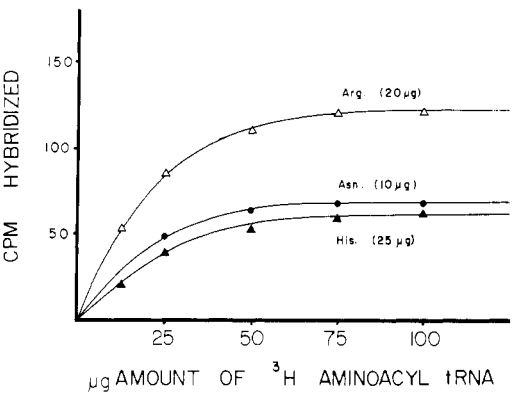

FIGURE 6: Saturation hybridization of arginyl- (△), asparaginyl- (●), and histidyl-tRNA (▲) with the pea ctDNA.

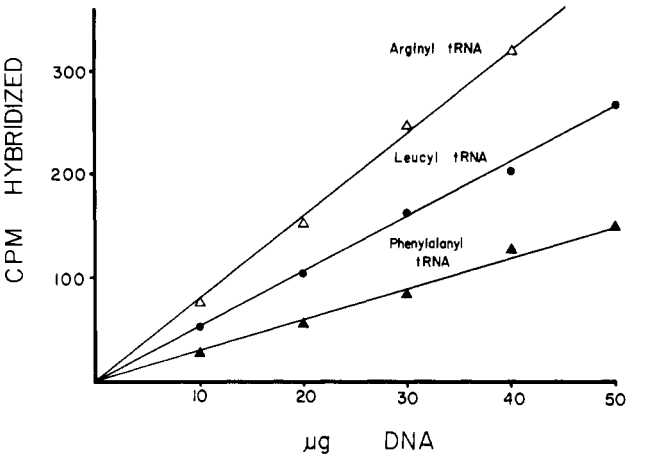

FIGURE 7: Hybridization of arginyl- (△), leucyl- (●), and phenylalanyl-tRNA (▲) with the increasing concentrations of pea ctDNA.

determined for each amino acid which was found to range from 10 to 30 min for different amino acids.

Hybridization of Aminoacylated ct-tRNAs with ctDNA. Typical examples of the saturation hybridization experiments between individually labeled aminoacyl-tRNA and ctDNA are shown in Figure 5. In the case of each of the three aminoacyl-tRNAs presented in the graph (tyrosyl, lysyl, and phenylalanyl-tRNAs), the amount of the hybridization increased with time. The hybridization was essentially complete in ~6 h. Further incubation for 10 h did not increase the amount of radioactivity bound to the ctDNA. It may be pointed out that although the observed levels of counts bound to the ctDNA are seemingly small, the hybridized radioactivity represents meaningful data. In the first place, the number of counts bound to the calf thymus DNA filters under similar experimental conditions never amounted to more than 10% of the counts bound to the ctDNA filters. In addition, the hybridization followed a time course of saturation. In the case of certain other amino acids (Arg, Asn, and His), the saturation hybridization experiments were carried out with the increasing amounts of individually labeled ct-tRNAs (Figure 6). The data again show typical saturation curves. For further confirmation of the validity of these experiments, labeled aminoacyl-tRNA (Arg, Leu, and Phe) was hybridized with increasing amounts of ctDNA. The radioactivity bound to the filter increased linearly with increasing concentrations of ctDNA (Figure 7). These data again confirm the reliability of the observed hybridizations. All of the above experiments were carried out by using *E. coli* synthetases to obtain labeled aminoacyl-tRNAs. The experiments also were carried out by using synthetases from chloroplasts. For example, ct-tRNAs

Table I: Aminoacylation of tRNAs from Chloroplasts

amino acid[c]	source of tRNAs[a]	source of aminoacyl-tRNA synthetases[b]	sp act. (pmol of radioactive amino acid incorpd per mg of tRNA)	amino acid	source of tRNAs	aminoacyl-tRNA synthetases	sp act. (pmol of radioactive amino acid incorpd per mg of tRNA)
Ala	a	1	2.5	Leu	a	1	2.3
	b	1	3.0		b	2	7.5
	c	2	2.5		c	2	8.0
	c	3	4.2		c	3	6.9
	c	4	3.5		c	4	6.7
Arg	a	1	4.8	Lys	b	2	2.6
	b	1	34.2		c	3	2.8
	c	2	28.8		c	4	2.2
	c	3	36.7	Met	a	1	1.7
	c	4	22.8		b	2	2.8
Asn	a	1	8.6		c	2	5.8
	c	2	15.8		c	3	9.0
	c	3	16.4		c	4	9.1
	c	4	14.9	Phe	a	1	10.4
Asp	a	1	14.2		b	1	20.6
	c	2	28.0		c	2	28.0
	c	3	25.3		c	3	62.0
	c	4	29.2		c	4	66.6
Cys	a	1	0.1	Pro	a	1	0.8
	c	2	0.2		c	2	4.6
	c	3	0.1		c	4	7.3
	c	4	0.2	Ser	a	1	3.1
Glu	b	2	0.2		c	2	4.5
	c	3	0.2		c	4	5.3
	c	4	0.2	Thr	a	1	6.6
Gln	b	2	0.1		c	2	49.5
	c	3	0.3		c	4	16.6
	c	4	0.2	Trp	a	1	8.6
Gly	a	1	1.7		c	2	9.3
	c	2	2.0		c	4	7.7
	c	4	2.7	Tyr	a	1	0.5
His	b	2	2.8		c	2	3.7
	c	3	3.7		c	4	3.9
	c	4	3.8				
Ile	a	1	0.3				
	b	1	0.4				
	c	2	3.9				
	c	4	3.5				

[a] a, Lysed chloroplasts, endogenous tRNAs; b, tRNAs from chloroplasts purified on sucrose density gradients; c, tRNAs from chloroplasts purified through the DEAE-cellulose column. [b] 1, Lysed chloroplasts, endogenous synthetases; 2, synthetases from chloroplasts purified through DEAE-cellulose; 3, synthetases from chloroplasts purified through Sephadex G-100; 4, *E. coli* synthetases. [c] The specific activities of amino acids in Ci/mol were as follows: Ala, 13; Arg, 23; Asn, 48; Asp, 15.8; Cys, 10.8; Glu, 49; Gln, 24; Gly, 11.4; His, 28; Ile, 104; Leu, 60: Lys, 26; Met, 0.2; Phe, 16; Pro, 35; Ser, 3; Thr, 2; Trp, 5.4; Tyr, 48.

were charged with [^{3}H]phenylalanine by using synthetases from chloroplasts. The isolated tRNAs was found to have a specific activity of ~320 cpm/μg of tRNAs. This RNA was hybridized to 10 μg of ctDNA. After the hybridization, ~30 cpm was bound to the filter, accounting for about one-tenth of the observed specific activity. When [^{3}H]phenylalanine was used in acylating ct-tRNA with *E. coli* aminoacyl-tRNA synthetases, the specific activity of the isolated tRNA was found to be 1030 cpm/μg of tRNAs, and the observed level of hybridization was 86 cpm by using 10 μg of ctDNA. This level of hybridization accounted for about one-twelfth of the specific activity of the tRNAs. Thus, the level of hybridization of ctDNA with [^{3}H]Phe-tRNAs was about the same whether the ct-tRNAs were charged with the synthetases from chloroplasts or *E. coli*. Similarly, the tRNAs charged with the [^{3}H]methionine by using the synthetases from chloroplasts were found to have a specific activity of 340 cpm/μg of tRNAs and hybridized with 10 μg of ctDNA to give 23 cpm. The tRNAs charged with [^{35}S]methionine by using the *E. coli* synthetases were found to have a specific activity of 890 cpm/μg of tRNAs and hybridized with 10 μg of pea ctDNA to give 70 cpm. Thus, the amount of hybridization obtained with aminoacyl-tRNAs and ctDNA was essentially the same whether they were charged by using synthetases from chloroplasts or *E. coli*.

The saturation hybridizations described above have been carried out with all of the 20 aminoacyl-tRNAs. The data obtained by using tRNAs from two different preparations are given in Table II. The numbers of cpm bound to the nitrocellulose filter are the averages of three to five determinations. The amount of ctDNA used for different aminoacyl-tRNA was always enough to yield at least more than 25 cpm above the control filters. Whenever the number of counts bound to ctDNA was lower than 25, the experiment was carried out with higher concentrations of ctDNA on nitrocellulose filters. In every case, the number of counts bound to ctDNA increased with the increase in the concentration of ctDNA. The ctDNA in these experiments ranged from 10 to 100 μg. As is evident from the Table II, all of the 20 amino acids, except glutamic acid, glutamine, and cysteine, unambiguously hybridized with ctDNA.

The specificity of the hybrids formed by aminoacyl-tRNA–ctRNA was determined by carrying out the thermal stability of the hybrids. Typical data obtained are shown in Figure 8 for [^{3}H]lysyl and [^{3}H]isoleucyl-tRNA. The ctDNA–[^{3}H]isoleucyl-tRNA was found to melt sharply as a

Table II: Hybridization of Aminoacyl tRNAs with the Pea ctDNA[a]

amino acid	source of tRNA[b]	source of tRNA synthetases[c]	sp act. of amino-acyl tRNAs (cpm/μg of total tRNA)	amt of ctDNA bound to nitrocellulose filter (μg)	cpm bound to ctDNA at saturation	presence of aminoacyl-tRNA gene in ctDNA
Ala	b	3	102	50	65	+
			178	20	36	
Arg	b	1	800	10	78	+
			760	20	127	
Asn	b	2	1595	10	66	+
			1720	20	142	
Asp	c	3	1462	10	160	+
Cys	b	2	25	100	16	?
	a	3	33	100	21	
Glu	c	3	30	100	14	?
	a	2	15	100	12	
	b	2	14	100	15	
Gln	a	2	43	100	12	?
	b	2	61	100	45	
Gly	c	3	480	20	58	+
			710	20	84	
His	b	3	132	50	105	+
			171	25	63	
Ile	c	3	1185	10	59	+
			1400	10	75	
Leu	a	3	420	10	52	+
			510	10	69	
Lys	a	2	503	10	44	+
			670	10	72	
Met	b	1	2964	10	109	+
			1950	10	83	
Phe	c	1	323	10	30	
			401	25	98	
Pro	c	1	182	25	35	+
			210	25	42	
Ser	a	3	592	10	23	+
			641	25	107	
Thr	c	3	297	20	24	+
			321	50	62	
Trp	c	3	120	25	27	+
			162	50	61	
Tyr	a	3	1225	10	68	+
			1416	10	72	
Val	c	3	444	10	31	+
			460	25	77	

[a] All data are an average of three to five determinations. [b] a, ct-tRNAs prepared by sucrose gradient; b, ct-tRNAs prepared by DEAE-cellulose column; c, total tRNAs from cell. [c] 1, tRNA synthetase from chloroplasts purified through DEAE-cellulose or Sephadex G-100; 2, cell synthetases purified through DEAE-cellulose; 3, *E. coli* synthetases.

single component with a T_m of ~84 °C, which is close to the T_m of ctDNA. Similarly, the [^{3}H]lysyl-tRNA–ctDNA hybrid was found to melt at a T_m of ~91 °C. These studies have indicated that aminoacyl-tRNA–ctDNA hybrids observed in our studies result from specific base pairing of DNA and tRNAs.

Discussion

The various methods (Tereba & McCarthy, 1973; Orosz & Wetmur, 1974; Meeker et al., 1976) tested for the radioiodination of tRNA were all capable of labeling tRNA to very high specific activities [(1–10) × 10^6 cpm/μg]. However, these methods did not yield iodinated tRNA which could be used in quantitative hybridization studies. The iodinated RNA which was found to be the most suitable in these studies was obtained as described under Experimental Procedures. The fractionation of RNA on HAP column reduced the background counts to less than 0.01% of the input counts in the hybridization experiments. In a typical experiment where 2 μg of RNA of a specific activity of ~3 × 10^6 was used, the control filters were found to bind 600–1200 counts whereas the experimental filters were found to bind 15 000–20 000 counts. The iodinated tRNA was undegraded, as shown by acrylamide gel electrophoresis, and the radioactivity in tRNA was totally stable to formamide concentrations of as high as 90%.

The technique of hybridization to determine base complementarity of RNA chains to ctDNA has been shown to give reliable estimates on the number of rRNA genes in ctDNA (Thomas & Tewari, 1974a,b). The hybridization data obtained here by using iodinated tRNA indicate that ~1.2 ± 0.2% of ctDNA contain sequences complementary to ctDNA. Since the molecular weight of pea ctDNA has been found to be ~90 × 10^6 and those of tRNAs average ~25 000, it can be calculated that pea ctDNA contains 43 ± 10 tRNA genes. These hybridization data are a pretty reliable estimate of the number of tRNA genes in ctDNA because tRNAs used in these experiments are not contaminated with rRNAs. The use of in vitro iodination of tRNAs has also circumvented the possible contamination of tRNAs by RNAs that are rapidly labeled in vivo. The hybridization between ctDNA and tRNAs has been found to result from specific base pairing between the two components as reflected by the T_m of 81 °C between DNA–tRNA hybrids. The T_m of ctDNA has been found to be 85 °C. Thus, the T_m of DNA–RNA hybrids is only 4 °C lower than the T_m of DNA–DNA hybrids, an observation which has been reported for many such systems. The data in this paper also have shown that the base sequences of ct-

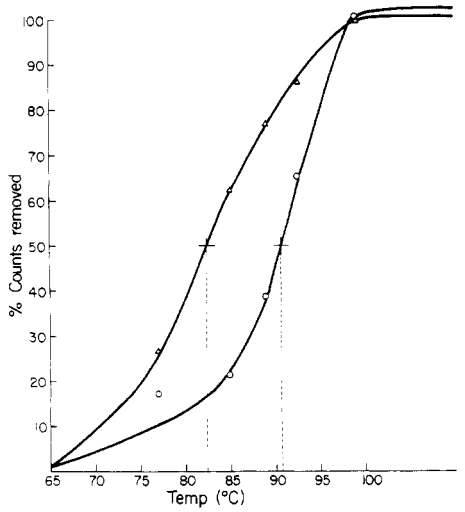

FIGURE 8: Thermal denaturation of [^{3}H]lysyl- and [^{3}H]isoleucyl-ct-tRNAs hybridized to 20 μg of pea ctDNA. Hybridizations of [^{3}H]lysyl- and [^{3}H]isoleucyl-ct-tRNAs were for 4 h as described under Experimental Procedures. Data points are averages of duplicate sets of filters. The specific activity of [^{3}H]lysyl-ct-tRNA was 503 cpm/μg of RNA, and the pea ctDNA filters bound an average of 440 cpm above the background value of 10 cpm. The specific activity of [^{3}H]isoleucyl-ct-tRNA was 1320 cpm/μg of RNA, and the pea ctDNA filters bound an average of 600 cpm above background. (○) [^{3}H]lysyl-ct-tRNA; (△) [^{3}H]isoleucyl-ct-tRNA.

tRNAs are unique since the tRNAs from cytoplasm of pea roots, *E. coli*, yeast, and calf liver were found not to compete for the complementary ct-tRNA base sequences in ctDNA. In contrast, when the competition was carried out by using unlabeled ct-tRNAs, expected levels of competition were observed. The uniqueness of the base sequences of ct-tRNA is also evident from the data where ctDNA-specific tRNA was not found to hybridize with the pea nuclear DNA.

The hybridization of ct-tRNAs with an excess of ctDNA has shown that only ~30% of the tRNAs present in chloroplasts are coded by ctDNA. These data demonstrate that cyt-tRNAs are present in chloroplasts. The consistent presence of cyt-tRNAs in chloroplasts has also been observed by Weil et al. (1976). It is not quite clear whether the presence of cyt-tRNAs in chloroplasts represents the actual in vivo state of the cell or is an artifact of the isolation procedures. However, the importation of cyt-tRNAs into organelles has been reported (Chiu et al., 1975). The DNA excess hybridizations with total cell tRNAs have shown that ~30% of the total cell tRNAs are specific to ctDNA. These data, therefore, have not shown any significant enrichment of ctDNA-specific tRNAs in plastids. The possibility of export of tRNAs from organelles has not been eliminated. The observation that 30% of the cell tRNAs are coded by ctDNA is significant since ctDNA has a molecular size of only 90×10^6 compared to a molecular size of 3×10^{12} for the nuclear DNA and accounts for only 5% of the total cell DNA.

The nature of the tRNAs and their aminoacyl-tRNA synthetases has been extensively studied in bean leaves by Weil et al. (1977). They have been able to demonstrate the presence of 18 aminoacyl-tRNA synthetase activities in both the chloroplastic and cytoplasmic enzymes. The ctRNAs were seen to be better acylated by the synthetases from chloroplasts and the cytoplasmic tRNAs were found to prefer cytoplasmic enzymes. The synthetases from *E. coli* were found to acylate ct-tRNAs with the same efficiency as the enzyme from chloroplasts. The present studies have identified the presence of at least 17 aminoacyl-tRNA synthetases and their tRNAs in pea chloroplasts. The activity of these enzymes could be demonstrated in lysed chloroplasts (Table I) without adding exogenous tRNAs or synthetases. However, the activities in general increased 2–7-fold by using purified ct-tRNAs and partially purified synthetases. The amount of aminoacylation was observed to vary widely for each amino acid. The synthetase activities, expressed as picomoles of amino acid attached per milligram of tRNA, vary from a low of 2 to a high of ~50. The observed levels of acylation are dependent upon the specific activity of a specific synthetase as well as on the concentration of the corresponding tRNA in the total tRNA preparation. Since total ct-tRNA has been used in the present studies, it is difficult to calculate the acylation of a specific aminoacyl-tRNA. The data only indicate that all of the aminoacyl-tRNA synthetases and their corresponding tRNAs are present in chloroplasts. It is interesting to note that all of the amino acids were charged to tRNAs to the same extent whether the tRNA synthetases from chloroplasts were utilized or the tRNA synthetases from *E. coli* were used. Guillemaut & Weil (1975) have also shown that ct-tRNAs can be as efficiently charged by synthetases from chloroplasts as well as from *E. coli*. The three amino acids glutamic acid, glutamine, and cysteine have been found to charge very poorly in the acylation reaction in the present study. The reason for the poor acylation shown by these amino acids is not understood.

The individually labeled aminoacyl-tRNA hybridized with ctDNA. The saturation hybridization curves have been generated for the labeled aminoacyl-tRNAs for each amino acid (some examples are shown in Figures 5–7). These hybridizations were seen to result from specific base pairing because the T_m of ctDNA–aminoacyl-tRNA hybrids was found to be very close to the T_m of ctDNA. In addition, the amount of hybridization obtained was directly proportional to the amount of ctDNA. From the hybridization of labeled aminoacyl-tRNA to ctDNA, it is not possible to calculate the number of genes for each of the tRNAs because the amounts of specific aminoacyl-tRNA in total ct-tRNAs are not known and it is not possible to know whether all of the specific aminoacyl-tRNAs have been acylated. The data only show that if a particular [^{3}H]aminoacyl-tRNA hybridizes with the ctDNA, the ctDNA must contain at least one gene for that tRNA. Such hybridizations using individually labled aminoacyl-tRNA (Table II) indicate that ~17 aminoacyl-tRNA genes are present in ctDNA. All of these aminoacyl-tRNAs hybridize to a significant extent to the ctDNA as shown in Table II. The amount of hybridization reflects the minimal amount because of the nature of these experiments. The percent of ctDNA specific to aminoacyl-tRNA in these experiments range from a low of ~0.005 to a high of 0.015. It is interesting to note that if we assume that a value of 0.005 represents the presence of 1 gene, then these data would indicate the ctDNA to have ~36 tRNA genes. This number of genes is again very close to that observed with the total tRNAs. We recognize that these calculations merely show that the data are meaningful and do not give the absolute number of each of the individual tRNA genes because of the limitations described above.

Haff & Bogorad (1976) have also shown the presence of 17 aminoacyl-tRNA genes in corn ctDNA. They were not able to detect tRNAs for the amino acids cysteine, methionine, and glutamine. In experiments with pea leaves, the tRNAs of cysteine and glutamine have been found to hybridize very poorly with ctDNA so that their presence in ctDNA cannot be detected with certainty. However, the methionyl-tRNA gene has been identified in pea ctDNA. On the other hand, glutamyl-tRNA has not been detected in pea ctDNA which

has been found to be present in corn ctDNA. The inability to localize the above three tRNA genes in ctDNA of corn and pea probably is a result of inadequate charging of the tRNAs rather than the lack of their genes in ctDNA.

A number of isoaccepting species of tRNAs have been reported in chloroplasts (Guillemaut & Weil, 1975). In experiments using three isoaccepting species of leucyl-tRNAs and two isoaccepting species of phenylalanyl-tRNA, the hybridization data obtained by Weil et al. (1977) have shown that the isoaccepting species of leucyl-tRNA are coded by the same gene(s). The same was found to be true for the isoaccepting species of phenylalanyl-tRNA. The multiplicity of tRNA genes in ctDNA must be tightly restricted because we have found that there are only ~40 tRNA genes in ctDNA. In addition, the data of Table II do not support the gross multiplicity of any particular aminoacyl-tRNA.

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Conformational Changes in Deoxyribonucleic Acid during Transcription†

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ABSTRACT: Circular dichroism (CD) was used to examine changes in secondary structure of calf thymus DNA during in vitro transcription. Formation of a binary complex between DNA and RNA polymerase (nucleoside triphosphate:nucleotidyltransferase, EC 2.7.7.6) did not alter the CD spectrum of the DNA. Alterations in ellipticity in the spectral region between 245 and 300 nm occurred during synthesis of RNA. This change was consistent with a B- to A-like form transition in polynucleotide conformation. The increment of ellipticity consisted of two separate components; component I was insensitive to treatment with pancreatic ribonuclease whereas component II was a ribonuclease labile fraction. Cleavage by restriction endonucleases did not produce or significantly alter the ellipticity of transcription. In contrast, between 50% and 60% of the component I ellipticity was sensitive to pancreatic DNase I. The data indicate that component I is a property of DNA and suggest that the alteration in secondary conformation which affects this component extends cooperatively beyond the DNase I insensitive DNA–RNA polymerase complexes.

On the basis of X-ray diffraction data for DNA, RNA, and DNA/RNA hybrids, it has been suggested that the ability of DNA to be transcribed may be regulated by its secondary structure. DNA can be identified in a number of conformations (for review, cf. Arnott, 1970). These are dependent upon the degree of hydration and the type and concentration of counterions present. In contrast, RNA or DNA/RNA hybrids have more limited conformational flexibility and have been found to exist only in an A-like conformational form (Arnott et al., 1968, 1973). From such data it has been suggested that a B- to A-form transition in the DNA would favor its transcription (Arnott et al., 1968).

There has been little direct experimental work to support or contradict a major role for the structure of DNA in regulating transcription. Indirect biochemical evidence obtained by Travers' group (Travers et al., 1973; Travers, 1974) and by Nankanishi et al. (1974, 1975) indicates that variations in temperature and ionic strength, as well as the presence of

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