Transcriptional origin of *Euglena* chloroplast tRNAs*

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**Abstract**

tRNA-DNA hybridization studies indicate that *Euglena* chloroplast tRNAs are transcriptional products of the chloroplast genome, which contains approximately 26 tRNA cistrons. Hybridization with purified chloroplast tRNA^Phe_ and tRNA^{Amp} shows that the chloroplast genome contains one cistron for each of these two species. No hybridization of chloroplast tRNA with nuclear DNA was observed. tRNAs from *Euglena* cytoplasm, *Escherichia coli*, and *Agmenellum quadruplicatum* do not compete with chloroplast tRNA for hybridization with chloroplast DNA. Evidence is presented that tRNA is at the level of transcription rather than maturation of tRNA precursor molecules.

The chloroplasts of *Euglena gracilis* contain tRNAs and aminoacyl-tRNA synthetases that are (i) induced by light and (ii) exclusively compartmentalized within these organelles (1–3). The synthetases are encoded by nuclear genes and are translated on cytoplasmic ribosomes (2). In the present report we have used tRNA-DNA hybridization to ascertain the intracellular localization of the structural genes for the chloroplast tRNAs.

**Materials and Methods**

*Euglena gracilis* var. *bacillaris* and W2BUL, an ultraviolet-induced mutant lacking detectable chloroplast structure and DNA, were used (4). The blue-green alga, *Agmenellum quadruplicatum* (a gift from Dr. Lonnie O. Ingram), was grown in ASP2 medium (5) and bubbled with 1% CO2 in air.

Preparation of Nucleic Acids. Chloroplasts were isolated as described (2) except that the time of zonal centrifugation was 3 hr. They were stored at −80°C. For the isolation of chloroplast DNA, the frozen chloroplasts were thawed in 0.15 M NaCl, 0.1 M Na2EDTA, and 0.1 M Tris-HCl (pH 8.0), and the DNA was extracted essentially as described (6) by Marmur using boiled pancreatic ribonuclease (100 µg/ml, Sigma, Type IIIA) followed by treatment with predigested Pronase (500 µg/ml, Calbiochem). Nuclear DNA was isolated from W2BUL by the same procedure. After precipitation with isopropanol, the DNA was further purified by preparative CsCl gradient centrifugation (7). The gradients were fractionated so as to retain all DNA species. The amount of nuclear DNA in the chloroplast DNA fraction was determined from the density-gradient profiles.

Chloroplast ribosomes and rRNA were extracted as described (8). rRNA was further purified by Sephadex G-100 chromatography in order to remove any residual tRNA. tRNA was isolated as described, using both DEAE-cellulose and Sephadex G-100 column chromatography (1, 3). Cytoplasmic tRNA was isolated from W2BUL, whereas chloroplast tRNA was extracted from purified chloroplasts (2). The chloroplast tRNA was free of contaminating cytoplasmic tRNA as determined by benzoylated DEAE-cellulose column chromatography of the purified chloroplast tRNA (9). E. coli tRNA was the gift of Dr. G. D. Novelli. Chloroplast tRNA^Phe_ and tRNA^{Amp} were purified by a combination of phenoxacyetylation, benzoylated DEAE-cellulose, and RPC-5 column chromatography (9).

Iodination of tRNA. tRNA labeled with iodine-125 was prepared by a modification of the procedure of Commerford (10, 11). The iodination mixture contained in 0.1 ml of 0.1 M Na acetate-0.44 M acetic acid buffer (pH 4.0): 100 µg of RNA, 4 nmol of I− (consisting of approximately 3.1 mCi of carrier-free NaI (9)) (New England Nuclear, NE 083) and unlabeled KI, and 0.2 µmol of TCl3. The mixture was incubated at 70°C for 20 min and cooled on ice. To eliminate unstable iodine addition products, 25 µl of 0.1 M Na2SO4 and 1 ml of freshly prepared 1 M NH4 acetate-NH4OH buffer (pH 8.0) were added and the mixture was incubated at 70°C for 30 min. The iodinated RNA was dialyzed against 0.001 M potassium phosphate buffer (KPO4, pH 7.0) and then placed onto a 1.4 × 1.8 cm hydroxylapatite C (Clarks ton Chemical Co.) column equilibrated with the same buffer. The column was washed with 0.001 M KPO4 (pH 7.0) until no further radioactivity was eluted (approximately 75 ml), and the RNA was eluted with 0.5 M KPO4 (pH 7.0). The resulting RNA fraction was dialyzed against 2 × SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0); approximately 50% of the RNA was recovered with a specific activity of 1 to 2 × 107 cpm/µg. After alkaline hydrolysis, more than 95% of the radioactivity was no longer precipitable with trichloroacetic acid.

Hybridization Conditions. DNA in 0.1 × SSC was denatured with alkali (0.13 M NaOH) at 35°C, neutralized with HCl, adjusted to 6 × SSC with 20 × SSC, and placed on 25-mm microcellulose filters (Schleicher and Schuell type B6) essentially as described by Gillespie and Spiegelman (12). Filters in groups of four, two DNA-containing filters and two blank filters, were placed in scintillation vials containing the appropriate RNA and 2 ml of 2 × SSC containing 0.1% sodium dodecyl sulfate. The filters were hybridized at 70°C for 20 hr. After hybridization, the filters were removed from the hybridization solution and incubated in 2 × SSC, 0.1% sodium dodecyl sulfate at 70°C for 30 min in order to reduce the background. The filters were washed three times in batches in 2 × SSC, incubated with 2 µg/ml of boiled pancreatic ribonuclease (5 ml per filter) for 1 hr at 25°C. After RNase treatment, the filters were washed three times in 2 × SSC and dried; their radioactivity was determined. The amount of DNA retained on the filter at the end of the hybridization was approximately 55%, as determined by hydrolysis in 1 M HCl (13). All data have been corrected for DNA retention, nuclear DNA contamination, and the adsorption of RNA to blank filters. The blank values were normally less than 0.05% of the input.

**Thermal Stability of the Hybrids.** The *t* <sub>m</sub> (melting temperature) of the tRNA-DNA hybrids was determined in 0.1 × SSC. After the filters were washed in 2 × SSC, they were washed in 0.1 × SSC and placed in a scintillation vial (one filter...
RESULTS AND DISCUSSION

The hybridization of chloroplast tRNA to chloroplast DNA is shown in Fig. 1. These saturation experiments were performed in the presence and absence of unlabeled chloroplast rRNA, because these rRNAs are known to be unstable (8) and it is extremely difficult to obtain from chloroplasts a preparation of unfractionated whole tRNA that is not contaminated with rRNA fragments. Thus, it is possible, by the addition of an excess of unlabeled rRNA, to effectively eliminate by competition any radioactivity that might appear in the hybrid as a result of contaminating rRNA. At saturation in the presence of unlabeled rRNA, chloroplast tRNA hybridizes with 0.74% of the chloroplast genome (Fig. 1). As expected, much higher levels of RNA are required for saturation in the absence of unlabeled tRNA.

Assuming an average molecular weight of 92 x 10^6 for the Euglena chloroplast genome (14, 15) and 26,500 for tRNA, we calculate that the chloroplast genome contains approximately 26 cistrons for the chloroplast tRNAs. Using benzoylated DEAE-cellulose chromatography, we have found (unpublished data) that for most amino acids there is a single species of chloroplast tRNA; however, for methionine and valine, and possibly leucine, tyrosine, and lysine, there appear to be two isoaccepting species. It appears, therefore, that the chloroplast genome contains a sufficient number of cistrons to code for all of the chloroplast tRNAs and that it contains only a single set of these tRNA cistrons.

Similar experiments have been performed using purified chloroplast tRNA^phe and tRNA^asp (Fig. 2), and the amount of DNA complementary to tRNA at infinite tRNA concentration was determined from a double reciprocal plot (16). The results show that approximately 0.023 and 0.036%, respectively, of the DNA exists as hybrid at saturation, indicating that the chloroplast genome contains one cistron for each of these two tRNAs. These results are consistent with the observation that the entire genome contains approximately 26 cistrons.

In order to demonstrate that the hybridization we observe is in fact with chloroplast DNA, we have taken advantage of the fact that the buoyant densities of chloroplast and nuclear DNA are quite different (ρ = 1.685 and 1.707 g/cm^3, respectively) (reviewed in ref. 17). In these experiments (Fig. 3), we have subjected DNA from isolated chloroplasts to CsCl equilibrium centrifugation for hybridization studies. Fig. 3A shows that chloroplast tRNA hybridizes quite efficiently with chloroplast DNA (ρ = 1.685 g/cm^3) and, in the absence of competing unlabeled rRNA, also hybridizes with DNA at a density intermediate between the nuclear (ρ = 1.707 g/cm^3) and chloroplast DNAs. This region of the gradient contains a chloroplast DNA "satellite" which is of higher G+C content than the main band chloroplast DNA, is enriched for chloroplast tRNA cistrons, and is generated by shearing of the circular chloroplast genome during DNA preparation (17-20).

Fig. 3B shows that the presence of an excess of rRNA during hybridization reduces the level of hybridization seen in the satellite region by approximately 80% and that seen in the main band by approximately 45%. The residual hybridization with the satellite DNA suggests that, during the process of DNA preparation, fragments enriched for G+C are produced which carry one or more tRNA cistrons. Both the distribution of hybridization and the preferential competition of rRNA with 125I-labeled RNA hybridizing to the satellite indicate that the tRNA cistrons are not closely linked to the rRNA cistrons. The 50% reduction in hybridization seen in the main-band DNA
results from the fact that not all of the rRNA cistrons are sheared out as fragments (17–20).

Nuclear DNA does not hybridize with chloroplast tRNA (Fig. 3). Thus, under the conditions of our experiments, there is no evidence that the nuclear genome contains sequences complementary to chloroplast tRNAs. These results are in contrast to the observations of Williams et al. (21), who, using bean leaves, found that all seven of the isoaccepting species of tRNA\textsubscript{Leu} hybridized with both chloroplast and nuclear DNA. The isoaccepting species, which increased preferentially during chloroplast development, did not hybridize preferentially with chloroplast DNA.

Purified Euglena chloroplast tRNA\textsubscript{Phe} and tRNA\textsubscript{Amp} have also been used in hybridization experiments (Fig. 4) with CsCl-fractionated DNAs with essentially the same results as obtained with unfractionated tRNA; they hybridize well with main-band chloroplast DNA and to a lesser extent with the satellite band. In the satellite band the tRNA\textsubscript{Amp} hybridizes with DNA of an average buoyant density that is higher than that to which the tRNA\textsubscript{Phe} hybridizes. This suggests that the two tRNA
cistrons are not closely linked. The results with both unfractionated tRNA and the individual tRNAs indicate that, as is the case with mitochondrial DNA (22), the chloroplast tRNA cistrons are probably distributed throughout the chloroplast genome.

Thermal dissociation of the tRNA-DNA hybrids is shown in Fig. 5. In both cases the melting profiles are characteristic of specific hybrids, with \( t_m \) values (in 0.1 X SSC) of 61°C for unfractionated chloroplast tRNA and 63°C for tRNAPhe. The \( t_m \) of polynucleotide complexes is a function of (i) the G+C content (23, 24), (ii) the number (up to approximately 50) of interacting nucleotides (24), (iii) the sequence of the interacting nucleotides (25), and (iv) the number, type, and location of mismatched base pairs (26). The chloroplast tRNAPhe contains 54% G+C (9) and the calculated (23) \( t_m \) in 0.1 X SSC for a DNA-DNA duplex containing 54% G+C is 76°C, whereas the observed value for the chloroplast tRNAPhe-DNA hybrid is 63°C. This lower value is, however, consistent with observed \( t_m \) values in other organisms [Neurospora (27) and E. coli (28)] in which presumably perfectly paired hybrids between tRNA and purified tRNA genes exhibit \( t_m \) values 6-12°C below the \( t_m \) of the corresponding DNA duplex (27, 28). Thus, it seems likely that the lowered \( t_m \) results from the formation of fragments of less than 50 nucleotides as a result of RNase treatment, \(^{125}\)I decay, and thermal scission during hybridization, rather than from mispaired bases.

The specificity of the chloroplast tRNA-DNA hybrids has also been determined by competition experiments (Fig. 6). In the presence of an equal amount of unlabeled chloroplast tRNA, there is a 42% reduction in the amount of chloroplast \(^{125}\)I-labeled tRNA hybridized (Fig. 6A). As the ratio of unlabeled to labeled tRNA increases, the decrease in the amount of \(^{125}\)I-labeled tRNA hybridized is within 10% of the theoretical decrease expected from isotope dilution. Thus it appears that hybridization of the tRNA does not cause nonspecific binding to the DNA-containing filters and that hybridization is only occurring between those portions of the DNA whose base sequence is complementary to chloroplast tRNA.

Cytosolic tRNA isolated from the plastidless mutant, \( \text{W}_{2}\text{BUL} \), does not compete with chloroplast tRNA for hybridization with chloroplast DNA (Fig. 6A). Thus transcription of chloroplast-type tRNA does not occur within the nucleus of \( \text{W}_{2}\text{BUL} \).

Dark-grown Euglena cells are known to contain low levels of the chloroplast tRNAs that rapidly increase upon exposure to light (1, 3). This is reflected in the ability of tRNAs from the light- and dark-grown cells to compete with chloroplast tRNA for hybridization (Fig. 6A). As expected, light-grown whole-cell tRNA is a more effective competitor than dark-grown-whole-cell tRNA. This same relationship holds even when the competing tRNA has not been separated from high molecular weight contaminants by Sephadex G-100 chromatography (data not shown). The concentration-dependence of the competition indicates that approximately 35% of the light-grown whole-cell tRNA is chloroplast tRNA, whereas approximately 5% of the tRNA from dark-grown cells is from this organelle. These results also suggest that the effect of light on tRNA induction is at the level of transcription rather than maturation of precursor molecules, since precursor tRNA would compete in hybridization as effectively as the finished product. These experiments do not, however, rule out the possibility that extremely large precursor molecules are present in dark-grown cells, and may not be in our tRNA preparations.

In order to remove high molecular weight RNA, we subjected the tRNAs used in these experiments to gel filtration on Sephadex G-100 as one of the preparative steps. The chloroplast tRNA of Euglena is quite unstable (8), however, and breaks down to lower molecular weight fragments under the conditions used to isolate chloroplasts and tRNA. Thus, even with the use
of Sephadex G-100, the smaller (approximately 4S) fragments of rRNA contaminate our tRNA preparations. To verify that the observed competition between tRNA and 125I-labeled tRNA is due to these breakdown products, we have studied the concentration-dependence of this competition (Fig. 6B). At ratios of rRNA to 125I-labeled tRNA of 2:1 to 40:1, there is a 50–60% reduction in the amount of 125I-labeled tRNA hybridized (Fig. 6B). A significant amount of tRNA is not competed by tRNA, indicating that tRNA is hybridized to cistrons distinct from the ribosomal cistrons. The 4:1 ratio of rRNA to 125I-labeled tRNA used in the saturation and gradient hybridization experiments is well in excess of the rRNA input required to saturate all of the tRNA cistrons. The saturation values obtained (Fig. 2) and the distribution of tRNA cistrons observed on CsCl density gradients (Fig. 1) are therefore a true reflection of the amount and distribution of the chloroplast tRNA cistrons.

We have also found that the tRNA from either the blue-green alga, A. quadruplicatum, or from E. coli is capable of competing with chloroplast tRNA in hybridization (Fig. 6B). Thus, the hybridization conditions used are highly selective.

Using these stringent hybridization conditions, we have found that the Euglena chloroplast genome contains the structural genes for chloroplast tRNAs. There are approximately 26 tRNA cistrons per genome, or approximately one per tRNA species. These cistrons do not appear to be contiguous with the rRNA cistrons or, in the case of tRNA^Phe and tRNA^Asp, with each other. The mitochondrial genomes of HeLa cells (22, 29) and Xenopus (30) code for only 12 and 15 species of tRNA, respectively. However, the chloroplast genome of Euglena, like the mitochondrial genome of yeast (31) and the chloroplast genome of tobacco (32), contains sufficient information to code for all 20 amino acids involved in protein synthesis. The present experiments also indicate that photoinduction of Euglena chloroplast tRNA occurs at the level of transcription rather than maturation of tRNA precursors.

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