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**Chloroplast DNA codes for transfer RNA**

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**Received 1 June 1976.**

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**ABSTRACT**

Transfer RNA's were isolated from Euglena gracilis. Chloroplast cistrons for tRNA were quantitated by hybridizing tRNA to ct DNA. Species of tRNA hybridizing to ct DNA were partially purified by hybridization-chromatography. The tRNA's hybridizing to ct DNA and nuclear DNA appear to be different. Total cellular tRNA was hybridized to ct DNA to an equivalent of approximately 25 cistrons. The total cellular tRNA was also separated into 2 fractions by chromatography on dihydroxyboryl substituted amino ethyl cellulose. Fraction I hybridized to both nuclear and ct DNA. Hybridizations to ct DNA indicated approximately 18 cistrons. Fraction II-tRNA hybridized only to ct DNA, saturating at a level of approximately 7 cistrons. The tRNA from isolated chloroplasts hybridized to both chloroplast and nuclear DNA. The level of hybridization to ct DNA indicated approximately 18 cistrons. Fraction II-type tRNA could not be detected in the isolated chloroplasts.

**INTRODUCTION**

An important question in molecular biology remaining to be answered is the extent that tRNA regulates cellular processes. In organelles, the number of aminoacyl-acceptor species of tRNA coded by the organelle genome is a key to ascertaining the autonomy of that organelle. Mitochondria from a number of organisms contain specific tRNA's which are coded by the mitochondrial DNA. Casey et al.,<sup>1,2,3</sup> Halbreich and Rabinowitz,<sup>4</sup> Cohen et al.,<sup>5</sup> Cohen and Rabinowitz<sup>6</sup> and Carnevallis et al.;<sup>7</sup> have shown that the mitochondrial DNA from yeast codes for at least 14 different aminoacyl-acceptor species of tRNA.

The presence of tRNA in chloroplasts has been established by Schwartz et al.,<sup>8</sup> Burkard et al.,<sup>9,10</sup> and Guillemaut et al.,<sup>11,12,13</sup> Barnett has shown that light induces the formation of several species of tRNA in Euglena.<sup>14</sup> The location of the cistrons for tRNA in these studies was not identified. Williams et al.,<sup>15</sup> reported hybridization attempts with tRNA's specific for leucine from bean chloroplasts, but their results were inconclusive.

The cistrons on the chloroplast DNA (ct DNA) which code for tRNA can be identified conclusively in Euglena gracilis because the ct DNA can be isolated as intact molecules without contamination by other species of DNA. Analysis by hybridization is possible because sufficient quantities of the ct DNA can be prepared.

The procedure for isolation of tRNA described by Roe<sup>16</sup> allows preparation of large quantities of RNA enriched for tRNA. The recent development of DBAE (acetylated dihydroxyboryl substituted amino ethyl) cellulose by McCutchen, Gilham and Söll<sup>17</sup> allows separation of the tRNA from fragments of RNA with a high molecular weight. The DBAE-cellulose also allows isolation of tRNA's which apparently contain the modified nucleoside Q, [7-(4,5-cis-dihydroxy-1-cyclopenten-3-yl-amino methyl)-7-deaza guanosine].

The tRNA from isolated chloroplasts and from whole cells was hybridized to ct DNA. Results of hybridization with purified tRNA indicated the presence of tRNA-cistrons on the chloroplast genome. Exclusive hybridization to ct DNA was observed in tRNA in Fraction II from DBAE cellulose.

### MATERIALS AND METHODS

#### Growth of cells

Cultures of Euglena gracilis strain Z were grown mixotrophically in a chemically defined medium.<sup>18</sup> Cells were harvested by centrifugation of cultures in the mid-exponential phase of growth.

#### Buffers and reagents

The radioactive <sup>14</sup>C-algal hydrolysate used to characterize the aminoacylation was purchased from Schwartz Mann. Acetylated dihydroxyboryl substituted aminoethyl (DBAE) cellulose was purchased from Collaborative Research.

Na<sup>125</sup>I for in vitro labeling of RNA was purchased from Amersham Searle. Pronase (nuclease-free) from Calbiochem was used in isolating DNA. Buffers used in the procedures described are as follows:

SSC	standard saline citrate	0.15 M NaCl, 0.015 M sodium citrate pH 7.0
SV	saline versene	0.1 M EDTA (ethylene diamine tetraacetate) 0.15 M NaCl pH 8.0

Iodination buffer	buffer used by Orosz and Wetmur <sup>23</sup>	0.4 M NaCl, 15 mM NaH <sub>2</sub> PO <sub>4</sub> 0.2 mM EDTA, pH 6.0
Buffer T	buffer used by Roe <sup>16</sup> for extraction of tRNA	0.14 M sodium acetate pH 4.5
Buffer C	used to desalt aminoacyl synthetase - similar to buffer described by Roe <sup>16</sup>	0.2 M Tris HCl pH 7.6 0.01 M MgCl <sub>2</sub> , 0.01 M β-mercaptoethanol, 20% glycerol
Buffer A	used by McCutchen <i>et al.</i> <sup>17</sup> to remove high molecular weight RNA fragments	0.2 M NaCl, 0.01 M MgCl <sub>2</sub> 0.05 M morpholine, 20% ethanol pH 8.7
Buffer I	used by McCutchen <i>et al.</i> <sup>17</sup> to isolate tRNA's containing the modified base Q	1.0 M NaCl, 0.1 M MgCl <sub>2</sub> 0.05 M morpholine, pH 8.7

Isolation of chloroplasts

Chloroplasts were isolated by flotation on 2.0 M sucrose (Eisenstadt and Brawerman<sup>19</sup>) as modified by Kissil and Buetow.<sup>20</sup> The procedure was further modified by the replacement of MgCl<sub>2</sub> with 100 mM ethylene diamine tetraacetate in all buffers when DNA was to be isolated from the chloroplasts.

Isolation of DNA

Chloroplast DNA was prepared from isolated chloroplasts. The chloroplasts were lysed in SV with 2% sodium dodecyl sulfate and pronase at a concentration of 5.0 mg/ml. The lysate was incubated for 4 hours at 37° C and centrifuged in a preparative CsCl equilibrium density gradient.<sup>21</sup>

Nuclear DNA was prepared in a similar manner from a bleached mutant, W<sub>1</sub>ZHL. The mutant does not contain detectable amounts of chloroplast DNA when assayed to a sensitivity that would detect a half molecule per cell (Mielenz and Hershberger, manuscript in preparation). Whole cells were suspended in SSC, pronase was added to a concentration of 5.0 mg/ml, sodium dodecyl sulfate was added to a final concentration of 2%, and the incubation was continued for 4 hours. The DNA was isolated by preparative centrifugation to equilibrium in CsCl-gradients. The DNA was removed from CsCl by pelleting the DNA in a Spinco SW 50.1 rotor at 50,000 rpm at 4° C for 8 hours. The DNA was dissolved in the desired buffer and concentrations of DNA were

determined by measuring ultraviolet-absorption at 260 nm ( $A_{260}$ ). A value of 50  $\mu\text{g/ml}$  per  $A_{260}$  unit was assumed.

### Preparation of transfer RNA

Transfer RNA was prepared from isolated chloroplasts or from whole cells by a modification of the procedure described by Roe.<sup>16</sup> The cells or chloroplasts were suspended in Buffer T with 2% sodium dodecyl sulfate and extracted twice with phenol-cresol.<sup>22</sup> Chromatography on DEAE-cellulose was used to isolate tRNA as described by Roe.<sup>16</sup> This tRNA was then chromatographed on DBAE-cellulose in Buffer A to purify further the tRNA by removing fragments of other species of RNA.<sup>17</sup> The tRNA was further separated into 2 fractions, one of which (Fraction II) bound to DBAE cellulose in Buffer I. Precipitation with ethanol was used to concentrate the tRNA's. The quantity of RNA was determined by ultraviolet-absorption at a wavelength of 260 nm. A value of 40  $\mu\text{g/ml}$  per  $A_{260}$  unit was assumed.

### Labeling of transfer RNA

The tRNA was labeled in vitro with  $^{125}\text{I}$  using a modification of the procedure of Orosz and Wetmur.<sup>23</sup> The reaction mixture contained 0.1 M sodium acetate,  $6 \times 10^{-4}$  M  $\text{TlCl}_3$ , and  $1 \times 10^{-4}$  M non-radioactive KI, about 400  $\mu\text{Ci/ml}$   $\text{Na } ^{125}\text{I}$  (for specific activities of approximately  $1 \times 10^6$  cpm/ $\mu\text{g}$  tRNA) and 200  $\mu\text{g/ml}$  RNA. The reagents were reacted at 60° C for one hour before terminating the reaction by quenching on ice and adding an equal volume of Iodination buffer. Unreacted iodine was removed by passing the reaction mixture through Sephadex G-25 equilibrated with Iodination buffer. The RNA was then incubated at 60° C for five hours to remove unstable intermediates. After the five-hour incubation, the RNA was further purified by chromatography on Whatman CF 11-cellulose.<sup>24</sup> The radioactive RNA was used within three days. A Nuclear Chicago gamma radiation counter was used to quantitate radioactivity.

### Measuring the integrity of the radioactive RNA

The RNA was tested for integrity by measuring precipitability with trichloroacetic acid and sensitivity to ribonuclease. Precipitability with trichloroacetic acid was measured by suspending the tRNA in 1 x SSC, adding an equal volume of 10% trichloroacetic acid, adding 200  $\mu\text{g}$  of nonradioactive yeast RNA as carrier RNA, and incubating at 0 to 4° C for 15 minutes. This mixture was filtered, under suction, onto glass fiber filters (Reeve Angel 934 AH) which were then washed extensively with 10% trichloroacetic acid and counted in the gamma counter. Sensitivity to ribonuclease was measured by incubating the tRNA in 1 x SSC with 0.02 mg/ml ribonuclease A and 10 units/

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ml ribonuclease T<sub>1</sub> for 30 minutes at 30° C and determining the radioactivity that was not precipitable with trichloroacetic acid.

The tRNA was analyzed by electrophoresis on 10% polyacrylamide cross-linked with 1.5% bis acrylamide according to Peacock and Dingman.<sup>25</sup> Yeast tRNA (Sigma) was used as a marker for measuring the relative mobility of the radioactive samples. The gels were fixed in 1.0 M acetic acid for four hours, stained for RNA in a solution containing 0.2% methylene blue, 0.4 M acetic acid, 0.4 M sodium acetate for four hours, and destained by diffusion in water. The position of the stained band was marked and the gel was fractionated into 1.0 mm slices. Radioactivity of each slice was measured in the gamma counter.

#### Hybridization procedure

Hybridizations were performed as described by Gillespie and Spiegelman.<sup>26</sup> The DNA was denatured at pH 13.0 in 3 x SSC, neutralized with 6.6 x SSC at pH 7.0, and bound to nitrocellulose filters (Schleicher and Schuell BA85). Each filter containing DNA was placed in a small vial along with a blank filter and RNA in 270 µl of 1 x SSC. Duplicate hybridization mixtures were incubated at 57° C for 18 hours and quenched on ice. The filters were washed three times in 1 x SSC then placed in vials containing 270 µl of a mixture of ribonuclease A at 0.02 mg/ml and ribonuclease T<sub>1</sub> at 10 units/ml. Solutions of ribonuclease were boiled for ten minutes as a precaution against contamination by deoxyribonucleases. The filters were incubated with the ribonuclease for 30 minutes at 30° C and washed exhaustively with 100 ml of 1 x SSC on each side of the individual filters. Binding to heterologous controls was determined in parallel reactions with *E. coli* DNA. The hybridizations were corrected by subtracting the binding to the heterologous controls and results with duplicate samples were averaged.

#### Chloroplast DNA column

The ct DNA was bound to Sepharose 4B by the procedure of Poonian *et al.*<sup>27</sup> Investigations to determine the optimum conditions for the reaction (to be published elsewhere) dictated the following conditions: 250 µg DNA/ml Sepharose 4B, 200 mg of CNBr/ml of Sepharose 4B. The activated Sepharose 4B was incubated with denatured ct DNA at 4° C for 22 hours. The residual activity of the activated Sepharose was eliminated by washing the column with 100 mM ethanolamine. The DNA bound to the Sepharose with an efficiency of 90%. The tRNA to be hybridized was loaded on the column in 1 x SSC-50% formamide and incubated at 22° C for 18 hours. The column was washed with cold 1 x SSC-50% formamide until the effluent was free of RNA that did not hybridize.

dize. The bound tRNA was eluted with 1 x SSC-90% formamide and precipitated with ethanol.

### Preparation of aminoacyl tRNA synthetase

Total cellular aminoacyl synthetase was prepared from Euglena by the procedure of Barnett et al.<sup>14</sup> and stored as the ammonium sulfate precipitate at -20° C. Before use of the aminoacyl synthetase, the enzyme was exchanged into Buffer C on Sephadex G-50.

### Aminoacylation reaction

The tRNA's were aminoacylated with saturating amounts of enzyme using the reaction conditions of Roe<sup>16</sup> at 25° C. Analytical reactions were performed in volumes of 50  $\mu$ l and assayed by measuring the radioactivity that bound to DEAE-filter discs as described by Santi and Anderson.<sup>28</sup> Radioactivity was counted as previously described.<sup>29</sup>

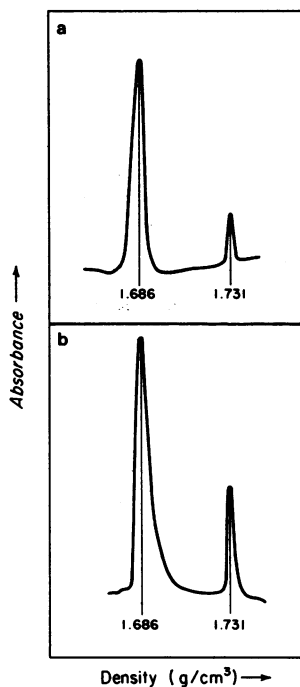
## RESULTS

### DNA samples

The ct DNA used in all hybridizations was prepared from isolated chloroplasts. This DNA was isolated as intact, circular molecules with a molecular weight of 90 to 100 x 10<sup>6</sup> daltons (Mielenz and Hershberger, manuscript in preparation). The DNA was examined by analytical ultracentrifugation to determine if the samples were contaminated by nuclear and mitochondrial DNA. A single, homogeneous band was seen in neutral CsCl equilibrium density gradients (Figure 1A). The DNA exhibited the buoyant density of ct DNA, 1.685 g/cm<sup>3</sup>; but bands for nuclear DNA with a buoyant density of 1.708 g/cm<sup>3</sup>, and mitochondrial DNA with a buoyant density of 1.689 g/cm<sup>3</sup> were not present. Denaturation followed by renaturation provides a more sensitive assay for detecting mitochondrial DNA because mitochondrial DNA does not fully renature to its native density.<sup>30</sup> The density of the renatured mitochondrial DNA is 0.011 g/cm<sup>3</sup> greater than the density of renatured chloroplast DNA. A single, homogenous band with the density of renatured ct DNA was observed when the samples were analyzed by this more sensitive method (Figure 1B). The ct DNA, therefore, was free of contamination by other species of DNA.

### Radioactive RNA

The in vitro labeling of RNA with <sup>125</sup>I has proven very successful for preparing samples with specific activities of 10<sup>4</sup> to 10<sup>7</sup> cpm/ $\mu$ g of RNA. The reaction conditions were selected for optimum incorporation of <sup>125</sup>I and desired specific activities were obtained by controlling the amount of <sup>125</sup>I



**Figure 1.** Densitometer tracing of ultraviolet absorption photographs. DNA was centrifuged to equilibrium in a CsCl density gradient at 44,000 rpm. Chloroplast DNA (1.686 g/cm<sup>3</sup>) and *Micrococcus luteus* DNA (1.731 g/cm<sup>3</sup>) are shown. A. Native ct DNA. B. Denatured-renatured ct DNA.

added to the reaction mixture (Mielenz, McCrea, Milner and Hershberger to be published elsewhere). The radioactive RNA was purified through chromatography on CF 11-cellulose and assayed for precipitability with trichloroacetic acid and sensitivity to hydrolysis by ribonuclease. More than 98% of the radioactivity was precipitable with trichloroacetic acid; greater than 96% of the radioactivity was converted to acid soluble products by treatment with ribonuclease.

The RNA was analyzed by electrophoresis on polyacrylamide disc gels to ascertain its integrity. Samples were isolated by chromatography on DEAE-cellulose and were iodinated and electrophoresed with yeast tRNA as a marker for measuring relative mobilities. Migration of the major radioactive band was coincident with the yeast tRNA. A minor radioactive component exhibited the mobility expected for 5S RNA (Figure 2A). Samples purified by chromatography on DBAE-cellulose were iodinated and analyzed by electrophoresis as described above. All radioactive RNA migrated to the same position as the yeast tRNA marker (Figure 2B), demonstrating that the iodinated RNA behaved

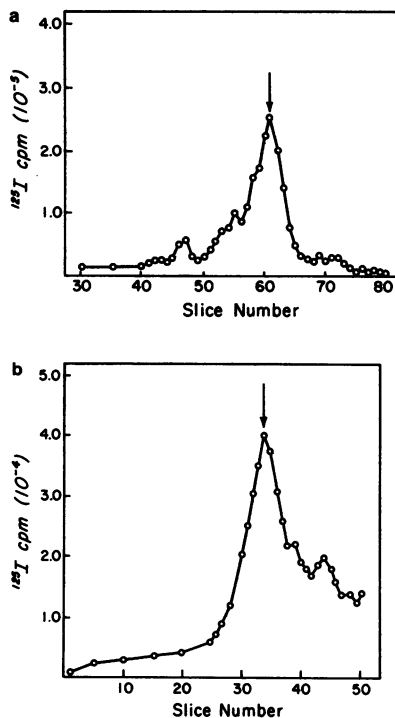


Figure 2. Electrophoresis of  $^{125}\text{I}$ -tRNA on 10% polyacrylamide disc gels. The top of the gels is to the left, the arrow marks the migration of the yeast tRNA stainable marker. A. tRNA purified by chromatography on DEAE cellulose. B. tRNA purified further by chromatography on DBAE cellulose.

as 4S RNA. Similar results were obtained when iodinated tRNA was electrophoresed in the presence of formamide (Milner, unpublished observation).

#### Aminoacylation of tRNA

The tRNA was assayed by measuring aminoacylation with a mixture of  $^{14}\text{C}$ -amino acids from an algal hydrolysate. The mixture of whole cell tRNA prepared by chromatography on DEAE-cellulose was aminoacylated to 0.02 pmoles of amino acid per  $\mu\text{g}$  of tRNA. After purification by chromatography on DBAE-cellulose, the total cellular tRNA was aminoacylated to 13 pmoles of amino acid per  $\mu\text{g}$  of tRNA. These values do not represent absolute measurements of tRNA but they do indicate that the reputed fractions of tRNA can be aminoacylated.

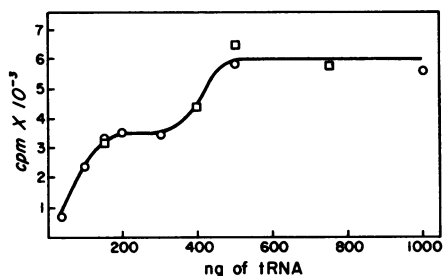
#### Hybridization with chloroplast tRNA

Chloroplast tRNA was prepared from isolated chloroplasts by chromatography on DEAE-cellulose. The RNA was iodinated and used for hybridization experiments with chloroplast and nuclear DNA. Non-radioactive DNA was used



for all of the hybridization reactions described below. Control experiments with  $^3\text{H}$  labeled DNA were performed and showed that virtually 100% of the DNA was retained on the filters under the reaction conditions (J. Milner, unpublished results).

Results of the hybridization reactions (Figure 3) indicated that 0.48%



**Figure 3.** Hybridization of  $^{125}\text{I}$ -tRNA from isolated chloroplasts (specific activity of  $1.24 \times 10^6$  cpm/ $\mu\text{g}$  of tRNA) to ct DNA ( $1 \mu\text{g}$ ). The plateau represents hybridization to approximately 0.48% of the ct DNA. Corrections have been made for heterologous binding. The  $\circ$  and  $\square$  represent data obtained in two separate experiments.

of the chloroplast DNA hybridized at saturation. Hybridization to nuclear DNA, however, was also significant. Binding to *E. coli* DNA as a heterologous control was less than 10% the level of reaction with chloroplast or nuclear DNA. This indicated that the chloroplast and nuclear hybridization results represented true hybridization rather than a non-specific reaction. The specificity of the hybridization was tested further by measuring the level of hybridization at temperatures in the range of  $53^\circ\text{C}$  to  $66^\circ\text{C}$  ( $16^\circ$  to  $29^\circ$  below  $T_m$  under our conditions). The level of hybridization from  $53^\circ\text{C}$  to  $63^\circ\text{C}$  was independent of temperature (Table 1), indicating that the binding was indeed specific hybridization.<sup>31</sup>

Hybridization of tRNA to ct DNA at different temperatures	
TEMPERATURE	CPM HYBRIDIZED
$53^\circ\text{C}$	8642
$57^\circ\text{C}$	7371
$60^\circ\text{C}$	7169
$63^\circ\text{C}$	8590
$66^\circ\text{C}$	6329

Transfer RNA from isolated chloroplasts (100 ng, specific activity of  $5 \times 10^6$  cpm/ $\mu\text{g}$  of tRNA) was hybridized to ct DNA ( $1 \mu\text{g}$ ).

Hybridization of tRNA from isolated chloroplasts to nuclear DNA raised the question, whether or not the same species of tRNA hybridize to both chloroplast and nuclear DNA? To answer this question, the tRNA hybridizing to ct DNA was isolated by hybridization chromatography on ct DNA bound to Sepharose 4B. A portion of the RNA employed for the experiments in Figure 3 was fractionated. Ten ng of the RNA before hybridization chromatography and 10 ng of the RNA isolated by hybridization chromatography were used in identical reactions (Table II). This amount of tRNA is less than the amount of tRNA required to saturate the ct DNA. Approximately 10% of the RNA in the reaction mixture hybridized to DNA. Seventy percent of the RNA hybridizing to nuclear DNA was removed by the chromatography, and 81% of the hybridizing RNA hybridized to ct DNA after the chromatographic separation. The tRNA that hybridizes to ct DNA, therefore, is not the same tRNA that hybridizes to nuclear DNA at less than saturating levels of tRNA.

**TABLE II**

Hybridization with tRNA from isolated chloroplasts to nuclear and chloroplast DNA before and after chromatography on ct DNA-Sepharose 4B

<u>DNA</u>	<u>CPM HYBRIDIZED</u>	
	Before Column	After Column
Nuclear	2674	790
Chloroplast	2830	3397

One  $\mu\text{g}$  of DNA was on each filter disc and 10 ng of  $^{125}\text{I}$  tRNA with a specific activity of  $5 \times 10^6$  cpm/ $\mu\text{g}$  of tRNA was in each reaction.

The tRNA from isolated chloroplasts was further fractionated in Buffer I on DBAE-cellulose as previously described<sup>17</sup> to separate Fraction I and Fraction II tRNA. The recovery of RNA in Fraction II was very low and aminoacylation could not be detected. Similar results were obtained when cultures were grown mixotrophically or phototrophically.

### Hybridization with total cellular tRNA

The hybridization to chloroplast DNA could be an underestimate if some species of tRNA were lost during the isolation of chloroplasts. Total cellular tRNA was isolated and hybridized to ct DNA to measure the level of hybridization to tRNA. Total cellular RNA was fractionated by chromatography on DEAE-cellulose. Chromatography on DBAE-cellulose in Buffer A was employed to

remove fragments of species of RNA with high molecular weights as described by McCutchen *et al.*<sup>17</sup> The purified tRNA was further separated into two fractions by chromatography on DBAE cellulose in Buffer I. The tRNA was used in three combinations for hybridization to ct DNA. The combinations were tRNA from Fraction I, tRNA from Fraction II and total tRNA including both Fractions I and II. Typical results are shown in Table III which includes results with

Hybridization of tRNA to ct DNA				
ng of tRNA	% of ct DNA Hybridized			
	Fraction I	Fraction II	Isolated Chloroplast	Total Cellular
100	0.11	0.06	0.19	----
250	0.17	0.19	0.26	----
500	0.55	0.21	0.49	0.42
1000	0.43	----	0.48	0.46
1500	----	----	----	0.70
2000	----	----	----	0.63
Number of cistrons	18.0	7.4	17.9	24.5

Specific activities of  $^{125}\text{I}$  tRNA were  $3.9 \times 10^6$  cpm/ $\mu\text{g}$  for Fraction I,  $7.5 \times 10^6$  cpm/ $\mu\text{g}$  for Fraction II,  $1.24 \times 10^6$  cpm/ $\mu\text{g}$  for isolated chloroplasts and  $2.4 \times 10^6$  cpm/ $\mu\text{g}$  for total cellular tRNA.

tRNA from isolated chloroplasts for comparison. The mean levels of hybridization of the ct DNA at saturation were 0.48% for Fraction I, 0.20% for Fraction II, 0.67% for total tRNA and 0.48% for tRNA from isolated chloroplasts. Several features of the results seem noteworthy. The levels of hybridization by tRNA in Fraction I and isolated chloroplasts are approximately equal. The level of hybridization by total cellular tRNA approximately equals the sum of hybridization by tRNA in Fractions I and II. The Fraction II-tRNA hybridizes to 0.20% of the ct DNA (Figure 4). The hybridization of Fraction II to nuclear DNA is the same as to the heterologous control, indicating that nuclear DNA does not contain cistrons for the tRNA in Fraction II.

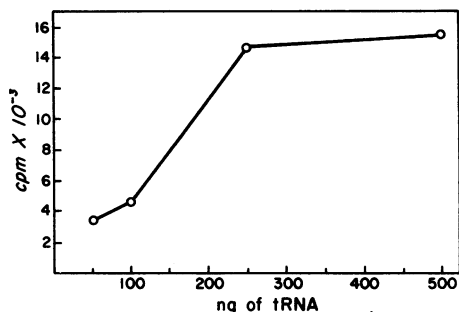


Figure 4. Hybridization of total cellular  $^{125}\text{I}$ -Fraction II-tRNA (specific activity of  $7.5 \times 10^6$  cpm/ $\mu\text{g}$  of tRNA) from mixotrophic cells to ct DNA (1  $\mu\text{g}$ ). The plateau represents hybridization to approximately 0.20% of the ct DNA. Corrections have been made for heterologous binding.

#### DISCUSSION

Experiments were conducted to determine if ct DNA contains genes for tRNA. tRNA was prepared from isolated chloroplasts and whole cells and hybridized to ct DNA. Chloroplast preparations contained tRNA that hybridized to both ct DNA and nuclear DNA. The tRNA was fractionated by hybridization to columns containing ct DNA linked to Sepharose 4B. The bound fraction of tRNA was specifically enriched for species that hybridize to ct DNA, indicating that ct DNA contains cistrons coding for unique species of tRNA. Hybridization-chromatography did not remove all tRNA hybridizing to nuclear DNA; however, a single passage through the column would not be expected to completely separate the fractions that hybridize differentially to ct DNA and nuclear DNA.<sup>32</sup> Nuclear-coded tRNA in the isolated chloroplasts could reflect the presence of nuclear-coded tRNA in the chloroplasts or contamination of the chloroplasts with cytoplasmic components. Chiu *et al.*<sup>33</sup> have reported the presence of three species of nuclear-coded tRNA in the mitochondria of *Tetrahymena pyriformis*. A similar situation could exist in the chloroplasts of *Euglena*; however, the observation does not justify a conclusion. It should be noted, however, that chloroplasts isolated by our procedure do not contain detectable levels of contamination by cytoplasmic rRNA (M. Kissil, unpublished observation).

The tRNA from whole cells was employed for measuring the number of cistrons on ct DNA because the isolated chloroplasts may not contain all of the species of tRNA coded by the ct DNA. Hybridization to 0.67% of the ct DNA was observed when tRNA from whole cells was used; however, hybridization to 0.48%

of the ct DNA was observed when tRNA from isolated chloroplasts was used. The hybridization levels should be interpreted cautiously because the results show interesting but unusual biphasic graphs. The biphasic hybridization curves may reflect different amounts of the tRNA-species. The levels of hybridization, however, suggest 25 cistrons for tRNA from the whole cells and 18 cistrons for tRNA from the isolated chloroplasts.

The difference in levels of hybridization seems to correlate with the presence or absence of tRNA in Fraction II from DBAE-cellulose columns. A fraction of tRNA from *E. coli*, which binds to DBAE cellulose under identical conditions, has been shown to contain the modified nucleoside Q.<sup>17</sup> Fraction II contains tRNA hybridizing to 0.20% of the ct DNA but hybridization to nuclear DNA is not detectable. The results do not exclude the possibility that unique-sequence nuclear DNA codes for tRNA in Fraction II because the reaction conditions only measure hybridization to repetitive-nuclear DNA. Other investigators have reported, however, that the nuclear cistrons for tRNA are part of the intermediate-repetitive nuclear DNA.<sup>34</sup>

Absence of Fraction II-tRNA from isolated chloroplasts is interesting but not explained by existing observations. The result could reflect location of tRNA coded by the ct DNA outside the chloroplasts; preferential loss of tRNA-species during isolation of the chloroplasts, or differential breakdown of tRNA during isolation of the chloroplasts which requires several hours.

This paper describes the use of RNA-DNA hybridizations to show that ct DNA codes for tRNA. The chloroplastic cistrons code for unique species of tRNA that are different than the species of tRNA hybridizing to nuclear DNA.

#### ACKNOWLEDGMENTS

Appreciation is expressed to B. Roe for helpful suggestions about isolation of tRNA, D. Söll for calling our attention to DBAE-cellulose before the procedure was published, and O. Uhlenbeck for answering many questions about techniques. This work was made possible by grants GB 35598 and GB 41928 from the National Science Foundation. Jan McCrea was a predoctoral trainee supported by National Institutes of Health Training Grant 00510.

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