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Comparative base compositions of chloroplast and cytoplasmic $tRNA^{Phe}$'s from Euglena gracilis

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ABSTRACT

The nucleoside compositions of chloroplast and cytoplasmic $tRNA^{Phe}$'s from Euglena gracilis have been determined. The modified nucleoside compositions of these two tRNAs indicate that $tRNA^{Phe}_{Chl}$ is more similar to procaryotic (E. coli) $tRNA^{Phe}$ than to either the Euglena cytoplasmic $tRNA^{Phe}$ or other eucaryotic cytoplasmic $tRNA^{Phe}$'s.

INTRODUCTION

It is a well-documented fact that the chloroplasts of Euglena gracilis and other plant systems contain a set of tRNAs that are chromatographically distinguishable from their cytoplasmic counterparts (1, 2, 3). It has also been shown that the structural genes for these Euglena chloroplast tRNAs reside in the chloroplast DNA (1, 4). The evolutionary origin of these chloroplast tRNA structural genes, and indeed the entire chloroplast genome, is considerably less well understood; however, one major school of thought is that the extant chloroplast and its genome evolved from a photosynthetic procaryotic endosymbiont (5).

In this presentation we report the first publication of an organelle tRNA and have taken advantage of the observation that there are certain distinguishing features, primarily in the minor or substituted base content, between procaryotic and eucaryotic $tRNA^{Phe}$'s (19). More specifically, we have purified the phenylalanine tRNAs from both the cytoplasm and chloroplasts of Euglena gracilis and have determined their nucleoside compositions. Evidence is presented that Euglena chloroplast $tRNA^{Phe}$ is more closely related to procaryotic $tRNA^{Phe}$ than to either Euglena cytoplasmic $tRNA^{Phe}$ or other eucaryotic $tRNA^{Phe}$'s.

MATERIALS AND METHODS

Isolation of chloroplasts

Chloroplasts were isolated as previously described (6) except that the zonal equilibrium centrifugation was shortened to 2.5 hr.

Preparation of aminoacyl-tRNA synthetases

Whole-cell synthetases were extracted in 0.2 M potassium phosphate (pH 7.5), 10% glycerol, 0.01 M 2-mercaptoethanol, 0.003 M sodium azide (azide) buffer solutions as previously described (7), and, to remove endogenous tRNA, were passed over DEAE-cellulose in the same buffer. Protein-containing fractions were precipitated by the addition of ammonium sulfate to saturation. The precipitates were then redissolved in a medium containing 0.05 M potassium phosphate (pH 7.5), 25% glycerol, 0.01 M 2-mercaptoethanol and azide and then passed through a Sephadex G-25 column equilibrated with the same buffer to remove salts and free amino acids. The enzyme-containing fractions were frozen in liquid nitrogen and stored at -80°C.

Chloroplast phenylalanyl-tRNA synthetase (EC 6.1.1.20) (Syn^{Phe}_{Chl}) was separated from cytoplasmic and mitochondrial Syn^{Phe}'s (6) by passage over hydroxylapatite C (Clarkson Chemical Co.) followed by DEAE-cellulose chromatography (Hecker and Barnett, unpublished). The partially purified enzyme preparation was made to 25% glycerol and stored at -80°C.

Preparation of tRNAs

Total nucleic acids were extracted from cells in the logarithmic phase of growth and prepared as previously described (6). Six kg of cells yielded about 10^5 A_{260} units of tRNA. The tRNA thus obtained was deacylated in 0.5 M Tris, pH 8.0, for 1-2 hr at room temperature. The deacylated tRNA was then chromatographed on BDC (benzoylated DEAE-cellulose) columns (8) to separate the chloroplast and cytoplasmic species of $tRNA^{Phe}$.

Purification of $tRNA^{Phe}$'s

Both chloroplast and cytoplasmic fractions of $tRNA^{Phe}$ from BDC were acylated and derivatized with phenoxyacetic acid N-hydroxysuccinimide ester (Schwarz-Mann) (8). Derivatized tRNAs were separated from unacylated tRNAs on BDC columns. The

phenylalanine-accepting fractions from these columns were deacylated and further purified by a combination of BDC and RPC-5 (9) column chromatography.

tRNA assays and charging reactions

The tRNA fractions from BDC and RPC-5 columns were assayed under the same conditions as previously described (6), except that each reaction mixture contained 0.3 A_{280} units of whole cell synthetases per ml of reaction mixture; 25-μl samples of tRNA fractions were added to 100 μl of enzyme containing reaction mixtures; assays were run for 30 min at 30°C. The amount of aminoacyl-tRNA formed was proportional to the amount of tRNA present.

The conditions for the charging of large quantities of tRNA or in the determination of maximal charging of purified tRNAs were the same as stated above with the following exceptions: (1) Unlabeled phenylalanine was added at either 100 or 200 times the concentration of the tritiated amino acid (59 Ci/mmole) to give a final assay concentration of 8.5 or 17 μM unlabeled phenylalanine. (2) All reaction mixtures contained 1.2 A_{280} units of whole cell synthetase per ml of reaction mixture except for $tRNA^{Phe}_{Chl}$ reaction mixtures, which contained 0.19 A_{280} units per ml of partially purified Syn^{Phe}_{Chl}. Charging reactions for large tRNA fractions were stopped by the addition of 1/20 of the volume of 5 M sodium acetate buffer, pH 4.5, and immediate immersion in an ice bath. Protein was then removed by extraction with phenol.

Nucleoside compositions

The compositions of the tRNAs were determined after hydrolysis to free nucleosides. Three A_{260} units were used for each analysis. Nucleosides were formed by phosphatase treatment of alkaline hydrolysates (10) at 37°C as well as by the combined action of RNase T_1 (30 min in 0.2 M ammonium acetate, pH 7.2) followed by venom diesterase and phosphatase (10). The molar extinctions used for each compound were taken from the Handbook of Biochemistry (11). The nucleosides were separated on a column of uniform-particle-size cation-exchange resin (12) (70 X 0.6 cm). In addition to the pH 4.65 solvent, a second solvent was passed through the column to elute the nucleosides following cytidine (13). All nucleosides were identified by their spectral ratios (11) as well as positions of elution. This column was not calibrated to detect wye ("Y base"), but this nucleoside has been previously identified in preparations of _Euglena_ $tRNA^{Phe}_{Cyt}$ (15).

RESULTS

Figure 1A shows the chromatographic behavior of deacylated whole cell tRNA on a BDC column. The isoacceptors for phenylalanine are easily separated from one another. The chloroplast species of $tRNA^{Phe}$ has previously (1) been identified by its enrichment in chloroplast preparations and its absence in W_3BUL, a mutant lacking both detectable chloroplast structure and chloroplast DNA. The chromatographic profile of tRNA extracted from isolated chloroplasts is shown in Figure 1B. Only one species is found for $tRNA^{Phe}$. Cytoplasmic tRNAs are present in extremely small

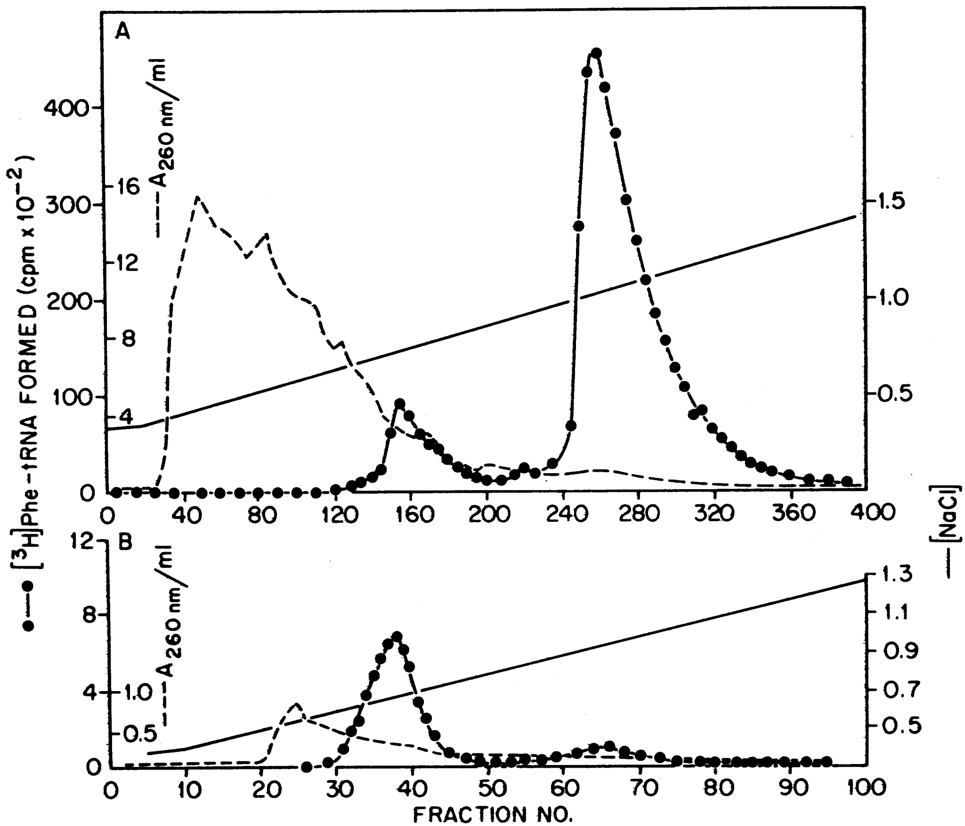

Figure 1. BDC chromatography of deacylated Euglena $tRNA^{Phe}$'s. (A) ~30,000 A_{260} units of whole cell RNA were applied to a 5 X 80 cm column and eluted with an 8-liter linear gradient from 0.33-1.5 M NaCl. (B) 200 A_{260} units of RNA from purified chloroplasts were applied to a 1 X 21 cm column and eluted with an 800-ml linear gradient from 0.33-1.25 M NaCl. In addition to NaCl, all BDC solutions contained 0.01 M Tris, pH 7.5, 0.01 M magnesium acetate, and 0.003 M sodium azide.

amounts or not at all for phenylalanine and all other tRNAs tested for (Hecker, Schwartzbach, and Barnett, unpublished).

In whole cell preparations of tRNA, chloroplast $tRNA^{Phe}$ is contaminated with a minor species of cytoplasmic $tRNA^{Phe}$, which may be a modified form of the main $tRNA^{Phe}_{Cyt}$ peak (1). This is clearly seen in Figure 2; $tRNA^{Phe}_{Chl}$ acceptor activity from 10^5 A_{260} units of whole cell tRNA was pooled and rerun on another BDC column with a shallower gradient. When fractions were assayed with whole cell synthetases, the $tRNA^{Phe}$ peak had a distinctive trailing shoulder; assaying the same fractions with Syn^{Phe}_{Chl}, which does not acylate _this_ $tRNA^{Phe}_{Cyt}$, resulted in one symmetrical peak of chloroplast phenylalanine-acceptor activity. Thus Syn^{Phe}_{Chl} was used to acylate

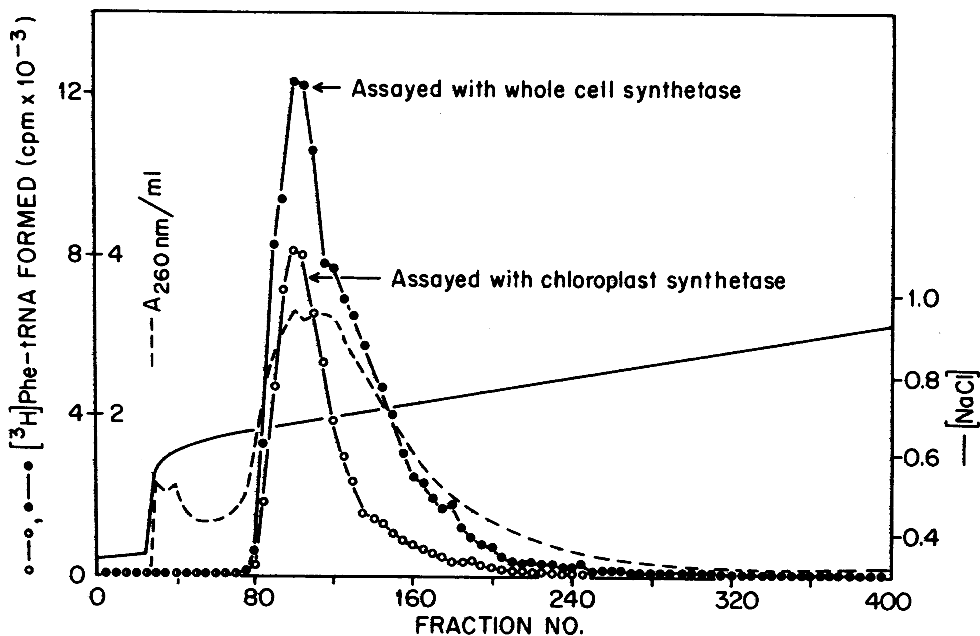

Figure 2. Rechromatography of $tRNA^{Phe}_{Chl}$ fractions from whole cell RNA (Fig. 1A) on a 5 X 80 cm BDC column with an 8-liter linear gradient from 0.6-0.95 M NaCl.

$tRNA^{Phe}_{Chl}$ in the next steps of purification. Figure 3 shows the final step in the purification of $tRNA^{Phe}_{Chl}$; fractions 11-19 were pooled yielding 54 A_{260} units of tRNA. The purified $tRNA^{Phe}_{Chl}$ accepts 1.35 nmoles of phenylalanine per A_{260} unit.

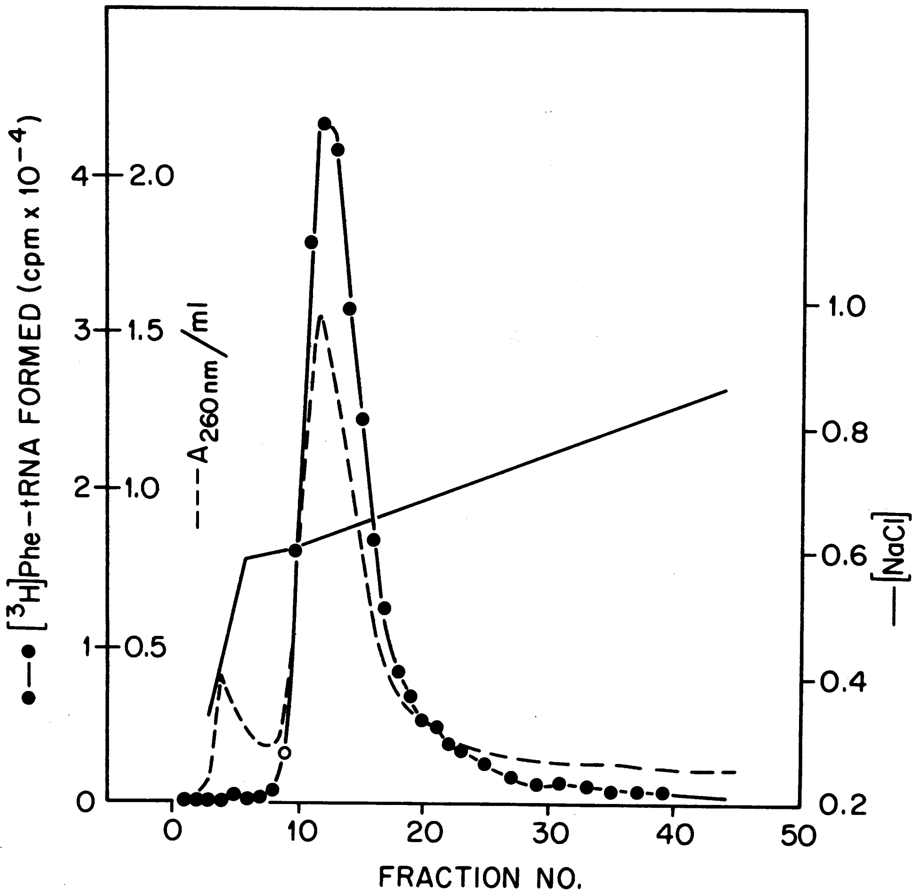

Figure 3. Final step in the purification of $tRNA^{Phe}_{Chl}$. A 1 X 20 cm BDC column was eluted with a linear gradient of 250 ml from 0.6-0.9 M NaCl.

The final step in the purification of $tRNA^{Phe}_{Cyt}$ is shown in Figure 4. The indentation in the center of the peak is highly reproducible, but the specific activity through both peaks is the same and it is likely that they are very similar. The yield of this tRNA was 190 A_{260} units and it accepts 1.30 nmoles of phenylalanine per A_{260} unit. There are several possible explanations for the duality of the $tRNA^{Phe}_{Cyt}$ peak. As previously stated, it has been shown by RPC-2 chromatography that the main $tRNA^{Phe}_{Cyt}$ is the source of the minor cytoplasmic species. This shift in chromatographic behavior may involve differences in some modified nucleoside such as wye. Another possibility

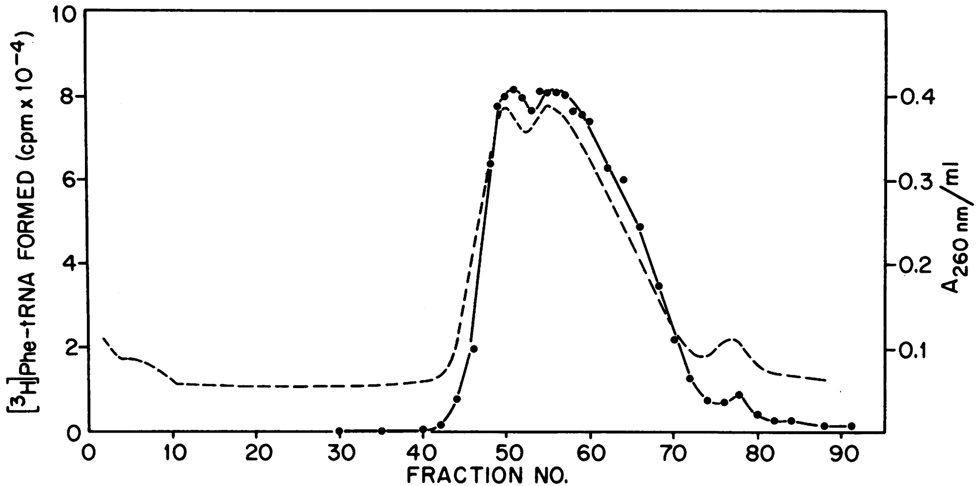

Figure 4. Final step in the purification of $tRNA^{Phe}_{Cyt}$. A 0.6 X 60 cm RPC-5 column was eluted with an 800-ml gradient from 0.475-0.6 M NaCl containing 0.01 M Tris, pH 7.0, 0.01 M magnesium chloride, 0.001 M 2-mercaptoethanol, and 0.003 M sodium azide. The column was run at 37°C with a pressure of 300 psi.

is that one of the two peaks observed may be due to the lack of a 3' terminal A (16).

Table 1 lists the nucleoside compositions of both chloroplast and cytoplasmic $tRNA^{Phe}$'s. Both tRNAs appear to be more than 90% pure if one compares the moles of phenylalanine acceptor activity per A_{260} unit to the moles of tRNA/A_{260} unit obtained in the compositional analysis as shown in Table 1. The level of purity is further supported by the fact that most of the modified nucleosides are present in the amount of approximately one residue or an integral multiple for each molecule of tRNA. In both $tRNA^{Phe}$'s, m^7G is the only modified nucleoside not present at the level of at least one residue. m^7G is known to be labile (17, 18), and it is therefore probable that the methods of isolation and analysis used cannot quantitate this nucleoside without loss. Because of the reasons stated above and due to the fact that m^7G has been found in each $tRNA^{Phe}$ so far sequenced (19), its presence is probably not the result of contaminating species of tRNAs in the *Euglena* $tRNA^{Phe}$ preparations.

Table 1. Nucleoside Composition of Euglena $tRNA^{Phe}$'s

Nucleoside	moles nucleoside/mole tRNA [a]	
	$tRNA^{Chl}$	$tRNA^{Cyt}$
— [b]	1	2
ψ	1.3	5
$4(abu)^3U$ [c]	0.1	0
U	13	8
T	1.1	1
N [c]	1.1	0
G [d]	21	21
m^2_2G	0	1.2
Gm	0.9	0.9
A	19	16
m^1A	0	1.1
C	18	18
Am	0	0.9
Cm	0	2
m^5C	0	1
m^7G [e]	0.5	0.3
ms^2i^6A [f]	+	0
Y	0	1
nmole tRNA [h] / A_{260}	1.32	1.29

[a] (1) moles nucleoside/mole tRNA was calculated by first dividing the total number of moles of individual nucleosides by an assumed chain length of 76 (to obtain the number of moles of tRNA), and then dividing the number of moles of each nucleoside by the number of moles of tRNA. (2) Dihydrouridine cannot be detected in these assays. (3) The data shown are a composite of the results obtained with both alkaline hydrolysis and RNase T_1 digestion (see Methods).

[b] All unknown nucleosides were assumed to have a molar extinction of 10,000 at 260 nm. These compounds have a negative charge and are excluded from the resin matrix. Those peaks appearing in this region after alkaline hydrolysis are due to the formation of such compounds as dinucleoside phosphates from the O-methylated nucleosides and their neighbors.

[c] This tRNA was prepared by phenoxyacetylation and BD-cellulose chromatography. This procedure may acylate the amino group of $4(abu)^3U$ (14). The compound N may be this derivative.

[d] The value of 21 for $tRNA^{Phe}_{Cyt}$ is averaged from an alkaline hydrolysate analysis (19 residues) and an enzymatic analysis (22.5 residues).

[e] Low values of m^7G may be due to the lability of this compound (see Discussion).

[f] Identification of ms^2i^6A is tentative.

[g] Demonstrated in previous publication (15).

[h] Calculated from the sum of moles of individual nucleosides divided by the total A_{260} units in the analysis.

DISCUSSION

The most striking difference between the nucleoside compositions of Euglena cytoplasmic and chloroplast $tRNA^{Phe}$'s (shown in Table 1), is their modified nucleoside content. The chloroplast $tRNA^{Phe}$ contains about six modified base residues whereas the cytoplasmic species contains 15. We included in this latter value one mole of a derivative of the fluorescent nucleoside wye, since we have shown earlier that it is present in the cytoplasmic but not the chloroplast $tRNA^{Phe}$ (15). It should also be noted that the present analyses do not permit detection and quantitation of dihydrouridine residues, and thus this base is not included in our calculations.

The compositions of both Euglena tRNAs may also be compared with published sequences for the $tRNA^{Phe}$'s from E. coli, yeast, wheat germ, and rabbit liver (19). On the basis of modified nucleoside content, these $tRNA^{Phe}$'s may be divided into two groups. The first, Group I, which consists of the eucaryotic cytoplasmic $tRNA^{Phe}$'s, contains many methylated nucleosides including at least one residue each of m^7G, m^1A, Gm, Cm, m_2^2G, and a form of wye. All $tRNA^{Phe}$'s in this group except wheat germ contain m^5C and all except Euglena cytoplasmic contain m^2G. Euglena cytoplasmic $tRNA^{Phe}$ is particularly rich in O-methyl nucleosides and is the only $tRNA^{Phe}$ containing one residue of Am. Group II, on the other hand, which contains $tRNA^{Phe}$'s from Euglena chloroplasts and E. coli, contains fewer minor nucleosides, in particular, methylated nucleosides. Both members of Group II contain ms^2i^6A as well as another derivatized base, referred to (19) as "X" in E. coli and "N" (which is probably quite similar to phenoxyacetylated-"X" (14) in chloroplasts).

Although the function of modified bases in tRNAs is at best poorly understood, their importance is emphasized by their conservation in evolutionary diverse organisms. It is also apparent that, based on nucleoside composition, chloroplast $tRNA^{Phe}$ is more closely related to the procaryotic $tRNA^{Phe}$. Such an observation is consistent with the notion that the structural genes for chloroplast tRNAs evolved from those of a photosynthetic procaryotic endosymbiont, but may also be consistent with other theories of chloroplast origin (20).

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