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Transfer RNA gene mapping studies on cyanelle DNA from *Cyanophora paradoxa*

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Summary. The 4S RNA of cyanelles from *Cyanophora paradoxa* strain LB 555 UTEX was fractionated by two-dimensional gel electrophoresis. Individual tRNA species were identified by aminoacylation, labeled in vitro and hybridized to restriction endonuclease fragments of cyanelle DNA. Hybridization experiments, using individual tRNA species, have revealed the location of two tRNA genes, coding for $tRNA^{Ala}$ and $tRNA^{Ile}$, in each of the two spacer segments separating the 16S and 23S rRNA genes on the two inverted repeats (10 kbp each) and three tRNA genes in the small single-copy region (17 kbp) separating the two inverted repeats. A minimum of 14 tRNA genes in the large single-copy region (88.5 kbp) has also been found.

Heterologous hybridization studies, using cyanelle tRNAs and chloroplast DNA from spinach, broad bean, or maize, indicate a high degree of homology between some tRNAs from cyanelles and chloroplasts.

Although cyanelles are often considered as having evolved from endosymbiotic cyanobacteria, the organization of tRNA genes on cyanelle DNA and the results of heterologous hybridization studies show that cyanelles are related to higher plant chloroplasts.

Introduction

Cyanophora paradoxa is a biflagellated protist containing photosynthetic organelles, called cyanelles, which function physiologically like chloroplasts. Cyanelles are generally considered to represent endosymbiotic, degenerated cyanobacteria (for a review, see: Trench 1982) because of the nature and composition of their photosynthetic pigments and the presence of peptidoglycan in their cell-wall remnant. Recent evidence demonstrated, however, that the cyanelle genome is too small for a cyanobacterium (Herdman and Stanier 1977). By the complexity of their DNA, which is equivalent to 127 kbp (Bohnert et al. 1982a), cyanelles have to be placed among plastids (Edelman 1981; Bohnert et al. 1982b). The similarity of cyanelle DNA and the DNA of many plastids is further illustrated by the anatomy of the circular chromosome (Bohnert and Löffelhardt 1982; Bohnert et al. in preparation): cyanelle DNA contains two inverted repeat segments (10 kbp in size) separated by two single-copy regions (a large one and a small one).

To compare tRNAs and tRNA gene organization in cyanelles and chloroplasts, individual cyanelle tRNA species were isolated, identified, labeled in vitro, and hybridized to cyanelle DNA fragments generated upon cleavage of the cyanelle genome with various restriction endonucleases. The results of these homologous hybridizations allowed the mapping of tRNA genes on the physical map of cyanelle DNA. Heterologous hybridization studies revealed extensive sequence homologies between some cyanelle and the corresponding chloroplast tRNA genes.

Materials and methods

Isolation of DNA. Strain LB 555 UTEX of *Cyanophora paradoxa* was obtained from the culture collection of algae of the University of Texas at Austin. Cells were grown as described (Mucke et al. 1980). Cyanelles and cyanelle DNA were isolated as already described (Bohnert et al. 1982a). Chloroplast DNA from spinach (*Spinacia oleracea* var. Monopa), broad bean (*Vicia faba*) and maize (*Zea mays*) was isolated as previously described (Koller and Delius 1980; Bohnert and Crouse 1981).

Isolation, fractionation and identification of transfer RNA. Cyanelles were suspended in 100 mM Tris-HCl pH 8.3, 10 mM MgCl, 10 µg/ml guanidinium isothiocanate (Merck, Darmstadt, FRG) and lysed during 2 h at 0° C by phenol saturated with 150 mM Tris-HCl pH 8.3, 150 mM NaCl, 2% sodium dodecylsulfate and 2% sodium sarcosyl, immediately before use. The total cyanelle tRNAs were then purified as previously described for chloroplast tRNAs (Mubumbila et al. 1980, Burkard et al. 1982). Fractionation of the total tRNAs was accomplished by two-dimensional polyacrylamide gel electrophoresis, either by method N° 1, using a 10% polyacrylamide gel containing 4 M urea in the first dimension for 40 h at 450 V and then using a 20% polyacrylamide gel containing 4 M urea in the second dimension for 140 h at 350 V (Burkard et al. 1982), or by method N° 2, using a 20% polyacrylamide gel containing 5 M urea in the first dimension for 60 h at 14 mA and then using a 25% polyacrylamide gel containing 7 M urea for 100 h at 18 mA in the second dimension (J. Tittgen, Universität Düsseldorf, personal communication). The individual tRNAs were recovered from the gels (for details,

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see Burkard et al. 1982) and identified using *Escherichia coli* aminoacyl-tRNA synthetases.

Hybridization of labeled tRNAs to DNA fragments. Each identified tRNA was individually treated with snake venom phosphodiesterase (Worthington, Freehold, NJ, USA) to remove the terminal nucleotide(s) of the -CCA end and enzymatically labeled at the 3′-end in the presence of α^{32}-P)ATP, CTP (Silberklang et al. 1977) and yeast tRNA nucleotidyl-transferase (Rether et al. 1974).

Cyanelle DNA and chloroplast DNAs were digested with restriction endonucleases (Boehringer, Mannheim, FRG; New England Biolabs, Schwalbach; PL Laboratories, St. Goar) according to recommended procedures and DNA fragments were separated by electrophoresis on horizontal 0.6%–1.5% SeaKem agarose gels (MCI, Rockland). DNA fragments were visualized by ethidium bromide, photographed, transferred to nitrocellulose filters (BA85, Schleicher and Schull, Dassel) and hybridized in 2 × SSC (0.3 M NaCl, 0.03 M Na-citrate), 50% formamide at 37° C with the labeled tRNAs as already described (Driesel et al. 1979).

Results

Isolation and identification of cyanelle tRNAs

Fractionation of the cyanelle 4S RNAs by two-dimensional polyacrylamide gel electrophoretic method No. 1 yielded about 40 RNA species. Of these, 27 RNAs have been identified as tRNAs by aminoacylation using *E. coli* aminoacyl-tRNA synthetases (Fig. 1). These tRNAs correspond to 16 amino acids (Table 1, first column). To improve the resolution of the RNA species present in the upper left part of the gel (Fig. 1), the cyanelle 4S RNAs were separated by two-dimensonal polyacrylamide gel electrophoretic method N° 2 (not shown). Although less RNA could be recovered after this fractionation, two species of $tRNA^{Phe}$ and two of $tRNA^{Met}$ could be resolved, which brought the number of identified cyanelle tRNAs to 29. The aminoacylation of tRNAs purified on both types of gel revealed that there are single tRNA species for seven amino acids (alanine, asparagine, aspartic acid, histidine, isoleucine, lysine and threonine), two isoaccepting species for six amino acids (arginine, methionine, phenylalanine, proline, tryptophan and valine), three species for two amino acids (serine and tyrosine) and four species for leucine (Table 1, first column). For four amino acids (cysteine, glutamic acid, glutamine and glycine) no tRNA species has yet been identified.

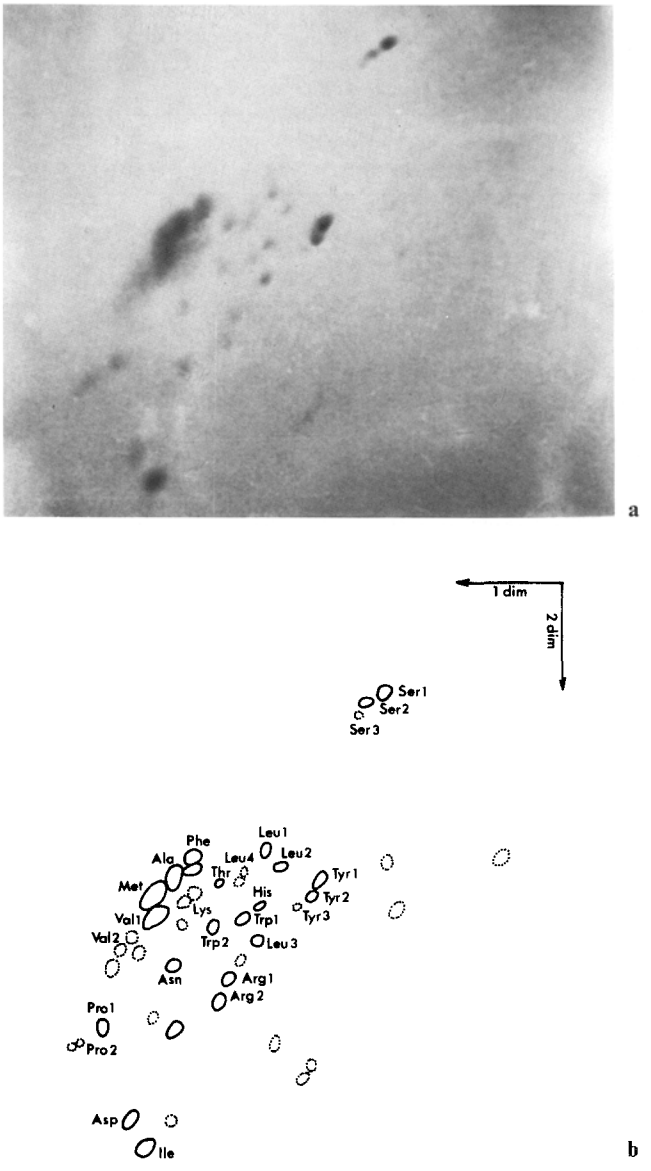

Fig. 1a, b. Fractionation of 4S RNAs from *Cyanophora paradoxa* by two-dimensional polyacrylamide gel electrophoresis. **a** Photograph of the gel after staining with methylene blue. The cyanelle 4S RNAs were separated in a 10% polyacrylamide gel (4 M urea) in the first dimension an 20% polyacrylamide gel (4 M urea) in the second dimension. **b** Diagrammatic representation of **a** indicating the amino acid accepted by each identified tRNA, as revealed by aminoacylation of the tRNA after extraction from the gel. Weak spots are indicated by dotted circles

Mapping of tRNA genes on the restriction endonuclease cleavage site map of cyanelle DNA

DNA prepared from purified cyanelles was digested with various restriction endonucleases and the DNA fragments were separated electrophoretically in horizontal agarose slab gels and transferred to nitrocellulose sheets. The endonucleases *Bgl*II, *Xho*I, *Eco*RI and *Pst*I cleaved the cyanelle DNA into 19, 9, >40, and >30 fragments (Bohnert et al. in preparation), respectively. The sum of the sizes of the fragments generated by each restriction endonuclease was approximately 127 kbp (Löffelhardt et al. 1983; Bohnert et al. 1982a). Of the 29 identified tRNAs, 25 have been hybridized to cyanelle DNA fragments (Table 1), allowing the location of their genes on the circular cyanelle DNA map (Fig. 2).

Two tRNAs ($tRNA^{Ala}$ and $tRNA^{Ile}$) hybridized to a 3.4 kbp *Eco*RI-generated fragment of double stoichiometry, which also contains part of both the 16S and the 23S rRNA gene sequences and the spacer DNA separating these two rRNA genes (Löffelhardt et al. 1980, 1983). The fact that $tRNA^{Ala}$ and $tRNA^{Ile}$ hybridized to PstI fragments 14.0 and 11.2 showed that both the *trnA* and *trnI* genes are present in each of the two copies of the inverted repeat segment (10 kbp) of cyanelle DNA (Fig. 2).

Most of the tRNA genes (21) have been located in the large single-copy region (88.5 kbp) of cyanelle DNA

Table 1. Hybridization of cyanelle tRNAs to cyanelle DNA fragments obtained upon action of various restriction endonucleases

tRNA	Gene symbol	Cyanelle DNA fragments				
		*Bgl*II	*Bgl*II/ *Xho*I	*Xho*I	*Eco*RI	*Pst*I
Ala	*trnA*	16.1; 12.5	NT	NT	3.4	14.0; 11.2
Arg1	*trnR1*	16.5	13.9	21.6	8.8	17.2
Arg2	*trnR2*	16.5	13.9	21.6	8.8	17.2
Asn	*trnN*	11.5	NT	NT	NT	14.0
Asp	*trnD*	5.7	NT	NT	5.5	NT
His	*trnH*	16.5	NT	NT	8.8	17.2
Ile	*trnI*	16.1; 12.5	NT	NT	3.4	14.0; 11.2
Leu1	*trnL1*	12.5	2.7	4.45	5.7	8.0
Leu2	*trnL2*	11.5	11.5	25.0	4.3	14.0
Leu3	*trnL3*	12.5	2.7	4.45	5.7	8.0
Leu4	*trnL4*	NT	NT	NT	NT	NT
Lys	*trnK*	10.3	NT	NT	7.9	10.3
Met1	*trnM1*	4.7	NT	NT	13.7	9.1
Met2	*trnM2*	NT	NT	NT	NT	NT
Phe1	*trnF1*	NT	NT	NT	NT	NT
Phe2	*trnF2*	NT	NT	NT	NT	NT
Pro1	*trnP1*	6.8	4.2	8.8	9.4	4.3
Pro2	*trnP2*	6.8	4.2	8.8	9.4	4.3
Ser1	*trnS1*	10.3	NT	NT	7.9	10.3
Ser2	*trnS2*	1.25	0.9	20.6	13.7	4.7
Ser3	*trnS3*	6.8	4.2	8.8	9.4	4.3
Thr	*trnT*	5.7	NT	NT	NT	9.1
Trp1	*trnW1*	16.5	NT	NT	8.8	17.2
Trp2	*trnW2*	16.5	NT	NT	8.8	17.2
Tyr1	*trnY1*	10.3	8.25	25.6	7.9	10.3
Tyr2	*trnY2*	10.3	8.25	25.6	7.9	10.3
Tyr3	*trnY3*	10.3	NT	NT	7.9	10.3
Val1	*trnV1*	5.7	NT	NT	13.7	9.1
Val2	*trnV2*	11.5	NT	NT	4.3	14.0
X	*trnX*	1.25	0.9	20.6	13.7	4.7

NT = Not Tested

The numbers specify the size (in kbp) of the restriction fragments to which the various individual tRNAs have been found to hybridize

(Fig. 2). This number includes the gene of a tRNA which has not yet been identified and has been tentatively called *trnX*. But as some tRNAs, such as $tRNA_1^{Leu}$ and $tRNA_3^{Leu}$, hybridized to the same region of the chromosome, it is not possible to distinguish whether two genes (e.g., *trnL1* and *trnL3*) are present, or whether two populations of the same primary transcript were resolved (for instance because they differ in their post-transcriptional modifications). As a consequence, a minimum of 14 tRNA genes are located in the large single-copy region.

In the small single-copy region (17 kbp), three identified tRNA species are encoded: *trnN*, *trnV2* and *trnL2*. It should be pointed out that two populations of cyanelle DNA exist, which differ in the orientation of the small single-copy region (Bohnert and Löffelhardt 1982), as shown in Fig. 2a, b.

Heterologous hybridization of cyanelle tRNAs to chloroplast DNA fragments

From the 29 identified cyanelle tRNAs, 12 species were hybridized to higher plant chloroplast DNA (not shown).

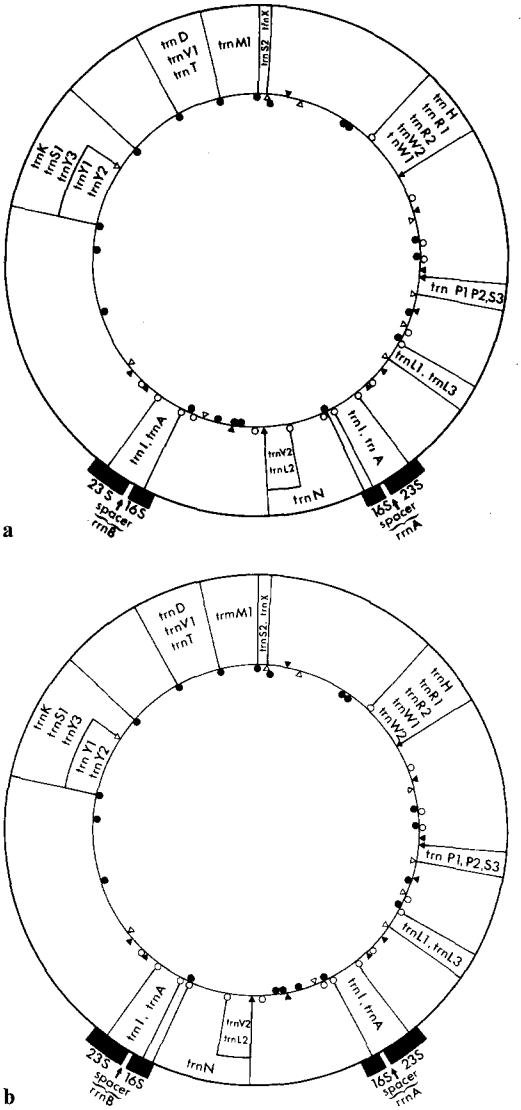

Fig. 2a, b. Location of tRNA genes on the restriction map of the cyanelle genome. The smallest DNA segment to which hybridization was observed is indicated for each cyanelle tRNA. The arrangement of rRNA genes in each of the two ribosomal RNA gene units is: 16S-spacer-23S. ● *Bgl*II cleavage site; ○ *Eco*RI cleavage site; ▼ *Pst*I cleavage site; ▽ *Xho*I cleavage site. For tRNA gene nomenclature, see Table 1, second column. All cleavage sites for *Bgl*II, and *Xho*I are shown, while only some sites for *Pst*I and *Eco*RI are included. The two populations of cyanelle DNA (**a**, **b**) differ in the orientation of the small single-copy region (Bohnert and Löffelhardt 1982)

The conditions used for these heterologous hybridizations were very stringent, as shown by the fact that maize chloroplast $tRNA^{Ile}$ (encoded in the ribosomal spacer) did not hybridize to *Euglena* chloroplast DNA (Mubumbila et al. 1980), although the $tRNA^{Ile}$s encoded in the ribosomal spacer of *Euglena* and maize chloroplasts exhibit an 85% sequence homology (Orozco et al. 1980; Koch et al. 1981; Guillemaut and Weil 1982).

The $tRNA^{Ala}$ and $tRNA^{Ile}$ from cyanelles both hybridized to maize and spinach chloroplast DNAs. Of the other ten tRNAs tested, six ($tRNA^{Asn}$, $tRNA^{His}$, $tRNA^{Lys}$, $tRNA_3^{Ser}$, $tRNA_2^{Trp}$, and $tRNA_3^{Tyr}$) hybridized to broad bean and spinach chloroplast DNAs. It is interesting to point out that when two cyanelle tRNAs hybridized to the same

cyanelle DNA fragment (such as for instance $tRNA^{Lys}$ and $tRNA_3^{Tyr}$ on one hand, or $tRNA^{His}$ and $tRNA_2^{Trp}$ on the other), they also hybridized to a single fragment of chloroplast DNA from spinach and broad bean. Four tRNAs ($tRNA_1^{Met}$, $tRNA^{Thr}$, $tRNA_1^{Trp}$ and $tRNA_1^{Val}$) did not show any hybridization with chloroplast DNA from spinach or broad bean.

Discussion

Two-dimensional electrophoretic fractionation of cyanelle 4S RNAs has revealed approximately 40 distinct molecules. Of these, 29 have been identified as tRNAs since, after elution from the gel, they could be charged specifically with radioactive amino acids (Table 1, first column) using *E. coli* aminoacyl-tRNA synthetases. It is interesting to note that most cyanelle tRNAs can be charged using a bacterial enzyme, which is also the case for most chloroplast tRNAs (Weil 1979). The number of 29 tRNA species (or 33 if the tRNAs specific for Cys, Gln, Glu and Gly are added) is close to the number of 32 tRNAs which are needed to read the 61 sense code words according to the wobble hypothesis (Crick 1966).

With respect to the number of tRNA species and of tRNA genes, cyanelles from *Cyanophora paradoxa* appear to be very similar to chloroplasts (Driesel et al. 1979; Kuntz et al. 1982; Mubumbila et al. 1983; Selden et al. 1983) and to contain a complete set of tRNA genes. From the codon usage in several sequenced genes of higher plant chloroplast DNAs (McIntosh et al. 1980; Zurawski et al. 1981, 1982a, b; Krebbers et al. 1982; Sugita and Sugiura 1983; Deno et al. 1983; Alt et al. 1983), it appears that the genetic code in chloroplasts is identical to that in *E. coli* and that all codons, although not necessarily in each gene, are used (for reviews see Bohnert et al. 1982b; Gillham et al. 1984).

Comparing the arrangement of tRNA genes on the cyanelle chromosome with that in higher plant and *Euglena* chloroplasts, there appears to be no constraint to keep certain tRNA genes together (which would indicate an operon). However one clear example for positional conservation is the location of genes *trnA* and *trnI* in the cyanelle ribosomal spacer, between the 16S and 23S rRNA genes, a feature observed in several chloroplasts, in three out of seven rRNA operons in *E. coli,* in *Bacillus subtilis* (for a review, see Bohnert et al. 1982b), and more recently in a cyanobacterium, *Anacystis nidulans* (Williamson and Doolittle 1983).

Further examples of common features in the organization of cyanelle and chloroplast tRNA genes are provided by the experiments of heterologous hybridization: For instance cyanelle $tRNA^{Lys}$ and $tRNA_3^{Tyr}$ hybridized to a single fragment from cyanelle DNA and from broad bean or spinach chloroplast DNA. This is also true in the case of $tRNA^{His}$ and $tRNA_2^{Trp}$. The heterologous hybridization experiments have revealed a high degree of homology between 8 cyanelle tRNAs (out of the 12 tRNAs tested) and the corresponding higher plant chloroplast tRNAs, whereas Mubumbila et al. (1980) have shown that out of 14 *Euglena* chloroplast tRNAs tested only $tRNA^{Phe}$ hybridizes to higher plant chloroplast DNAs.

Morphological and biochemical data (especially the nature of the photosynthetic pigments and the presence of peptidoglycan in the cell-wall) have been used to suggest that cyanelles are derived from endosymbiotic cyanobacteria. A recent report (Heinhorst and Shively 1983), showing that the genes for both subunits of ribulose bisphosphate carboxylase are encoded in the cyanelle genome, seems to confirm the procaryotic origin of cyanelles. However, our results, concerning the organization of the tRNA genes on cyanelle DNA and the high degree of homology revealed by cross-hybridization experiments performed under stringent conditions, suggest that cyanelles should be considered as a class of organelles related to higher plant chloroplasts. A more precise and definite evaluation of the relationship between cyanelles and chloroplasts would require gene sequencing data from cyanelles, plastids (including red-algal plastids) and cyanobacteria.

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