Vol. 95, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1980 Pages 47-54

TRANSFER RNA GENES ASSOCIATED WITH THE 16S AND 23S rRNA GENES OF EUGLENA CHLOROPLAST DNA

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Received May 8,1980

SUMMARY

Hybridization studies of Euglena chloroplast ^{125}I-labeled tRNAs to restriction fragments of Euglena chloroplast DNA have shown that the spacer between the 16S and 23S rRNA genes, in two and possibly all three of the ribosomal DNA units, contains genes for tRNA^{Ile} and tRNA^{Ala}, whereas a tRNA gene (for either tRNA^{Trp} or tRNA^{Glu}) is located before probably all four 16S rRNA genes present on the chloroplast DNA molecule.

INTRODUCTION

Three complete sets of rRNA genes are present on each 130 kbp circular molecule of Euglena chloroplast DNA (1, 2, 3). Within each reiterated rDNA unit (5.6 kbp), the rRNA genes are clustered in the following order : 16S-spacer-23S-5S (2, 3). The three rDNA units, which are arranged in tandem, are separated from each other by DNA stretches of about 800 base pairs in the Euglena Z-strain (2) and by about 700 and 250 base pairs in the Euglena B-strain (4). The spacer between the 16S and 23S rRNA genes within a ribosomal unit is about 250 base pairs long. A set of genes for the 16S, 23S and 5S rRNAs is transcribed into a precursor RNA molecule which has a molecular weight of about 1.8 megadaltons (5), equivalent to the size of the rDNA unit. In addition the existence of a fourth 16S rRNA gene has been reported (6).

Apart from the chloroplast rRNAs, the only other identified RNA molecules which have been shown to hybridize to Euglena chloroplast DNA are the chloroplast-specific 4S RNAs (3, 7, 8, 9). Saturation hybridization of *in vitro* labeled 4S RNAs to chloroplast DNA reveals that up to 26 tRNA genes exist on the Euglena chloroplast DNA molecule. Fractionation of this 4S RNA population by two-dimensional polyacrylamide gel electrophoresis yields about 30 RNA species, 23 of which were identified by aminoacylation as tRNAs specific for 18 amino acids (10). At present, all but two of the 23 identified chloroplast tRNA species hybridize to the chloroplast DNA (unpublished data). Studies on the

0006-291X/80/130047-08$01.00/0

hybridization of total chloroplast 4S RNAs to restriction endonuclease digests of Euglena chloroplast DNA showed that (a) tRNA gene(s) must be located near the 16S and 23S rRNA genes (3). Most of the identified Euglena tRNA genes localized so far map in the single-copy region of the chloroplast DNA molecule whereas some identified tRNAs hybridize to DNA fragments containing the rDNA units (11, 12). In this paper we report the presence of genes for Euglena chloroplast $tRNA^{Ile}$ and $tRNA^{Ala}$ in the "spacer" between the 16S and 23S rRNA genes and the presence of another tRNA gene at the other end of the 16S rRNA gene. As Euglena chloroplast $tRNA^{Glu}$ and $tRNA^{Trp}$ could not be separated by the two-dimensional gel electrophoretic method used (10), it has not been established which of the two tRNAs has its gene located before the 5'-end of the 16S gene.

MATERIALS AND METHODS

Total chloroplast DNA and chloroplast rDNA were prepared from *Euglena gracilis* Z-strain (Culture Collection of Algae, Indiana University, N° 753) as described by Kopecka *et al.* (1). Restriction endonucleases Bal I, Bam H I, Bgl II, Eco R I, Hind II, Hind III, Hpa II, Xba I and Xho I were obtained from BioLabs (Beverley, MA) or Boehringer (Mannheim) and used as recommended by the suppliers. Restriction endonuclease cleavage products of the Euglena chloroplast rDNA were separated on 0.5 to 2.0% agarose gels (Marine Colloids, Rockland), stained with ethidium bromide, photographed under ultraviolet light (Polaroïd 665 film), and transfered from the gels to nitrocellulose filters (13).

Spinach chloroplast 16S, 23S and 5S rRNAs were prepared as previously described (14). Chloroplast 4S RNAs, from *Euglena gracilis* Z-strain, were fractionated by two-dimensional gel electrophoresis and identified by amino-acylation (10). The individual rRNAs and tRNAs were labeled with ^{125}I (15) and hybridized to chloroplast rDNA fragments using the conditions as described for the rRNAs (14) and for the tRNAs (16). Hybrid bands were visualized by auto-radiography (Kodak XR-1 or XR-5 film) after exposure from 2 days to 1 month.

RESULTS

In Table I are listed the restriction fragments obtained upon cleavage of Euglena chloroplast DNA enriched for the rDNA sequences. The positions of these DNA fragments have been located on the physical map of the rDNA region of the 130 kbp circular molecule. Maps of the restriction sites in the rDNA region and in one of the 5.6 kbp rDNA units are shown in Fig. 1. From their map positions in Fig. 1, the large rDNA fragments, listed in Table I, generally include DNA sequences from the two terminal rDNA units (units I and III, Fig. 1a) and adjacent single-copy segments. The multiple DNA fragments, as indicated in Table I, are totally derived from within the rDNA region. The double 5.6 kbp fragments from digestions using Xba I, Bam H I, Bal I, Xho I and Hind II are each equivalent in size to one complete ribosomal unit. Hybridization of chloroplast rRNAs was used to identify fragments derived from the rDNA,

Table I. Hybridization[1] of labeled rRNA and tRNA to restriction fragments of Euglena chloroplast rDNA

Restriction endonuclease[2]	Fragment size (kbp)[3]	rRNA 16S	rRNA 23S	$tRNA^{Ile}$	$tRNA^{Ala}$	$tRNA^{Glu}$ + $tRNA^{Trp}$
Xba I	6.4	x	x	x	x	NT
	5.6 (2x)	x	x	x	x	NT
	4.5	x				NT
Bam H I	45	x				x
	6.9	x	x	x	x	x
	5.6 (2x)	x	x	x	x	x
Hind III	2.5	x				x
	2.2 (2x)	x	x			x
	2.0 (4x)	x	x	x	x	
	1.8 (2x)	x	x			x
	1.0 (3x)		x			
	0.5 (3x)		x			
Bgl II	7.5		x			
	4.5	x				x
	3.6 (2x)	x	x			x
	2.0 (3x)	x	x	x	x	
Eco R I	21	x				x
	7.2		x			
	3.2 (2x)		x			
	2.4 (3x)	x	x	x	x	x
Bal I	12	x				NT
	6.3	x	x	x	x	NT
	5.6 (2x)	x	x	x	x	NT
	2.0					NT
Xho I	49	x		NT	NT	NT
	28	x	x	x	x	NT
	5.6 (2x)	x	x	x	x	NT
Hind II	6	x	x	x	x	
	5.6 (2x)	x	x	x	x	x
	4.5	x				x
	1.1	x				x
Hpa II	2.0	x				
	1.9 (3x)		x			
	1.6 (3x)	x	x			x
	1.0 (3x)	x	x	x	x	
	0.6 (4x)	x				
	0.5 (4x)	x				

(1) x denotes hybridization ; NT denotes not tested.
(2) Only fragments which hybridize to 16S and/or 23S rRNAs are listed (for maps and references see Fig. 1).
(3) Fragment multiplicity is given in brackets.

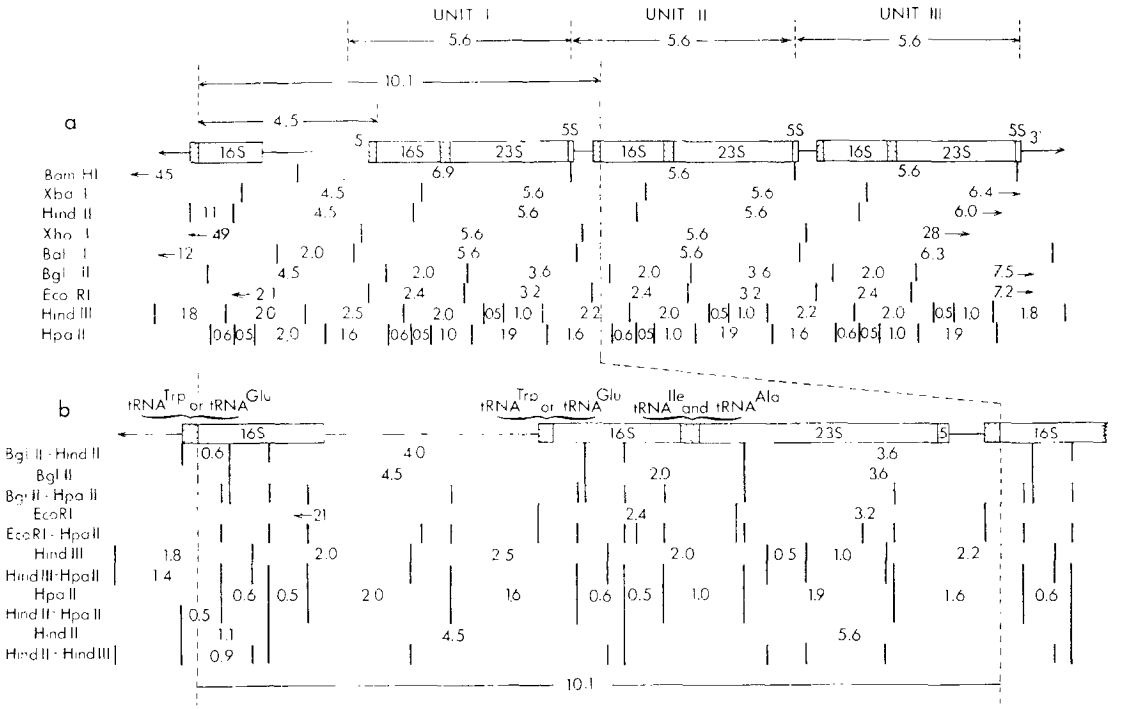

Fig. 1. Physical map of the rDNA region of the Euglena chloroplast DNA molecule. The size of the repeating rDNA unit is taken to be 5.6 kbp (see reference 3). The Bam H I, Eco R I and Hind III cleavage sites are from Rawson and coworkers (2) ; the Bal I, Bam H I, Eco R I, Xba I and Xho I sites are from Hallick and coworkers (3) ; the Bam H I, Bgl II and Eco R I sites are from Stutz and coworkers (6). DNA fragments are indicated by their size in kbp. A horizontal arrow (⟵ or ⟶) means that the other cut by that particular enzyme is beyond the region of the molecule shown in the figure. The lower map (b) is an expansion of the left part of the rDNA region shown in (a) and includes the fourth 16S gene and rDNA unit 1. In (a), restriction sites of the nine endonucleases used in this study are given. The three complete rDNA units are marked I, II and III as shown, starting from the unit nearest the fourth 16S rDNA sequence. The previously unpublished Hind II and Hpa II sites are mapped relative to the sites of the other endonucleases. The relative position of the Hind II cut to the central Hpa II cut within the 16S genes has not been established. An additional Hind III fragment (0.5 kbp) is located within the 23S rRNA gene. In (b), the minimum DNA segment which contains the genes for $tRNA^{Ile}$ and $tRNA^{Ala}$ is indicated by the larger shaded segment (▨) between the 16S and 23S genes. The minimum DNA segment which contains the gene for either $tRNA^{Trp}$ or $tRNA^{Glu}$ is shown by the smaller shaded segments (▩) before the 16S rRNA genes.

confirming their mapped locations,in order to provide a basis for the analysis of tRNA hybridizations. These rRNAs also enabled the further mapping of restriction sites for Hind II and Hpa II within the rDNA relative to the previously determined Xba I, Bam H I, Hind III, Bgl II, Eco R I, Bal I and Xho I cleavage site maps. An additional Hind III site was located within the 23S rRNA gene (Fig. 1).

Two types of "spacer " DNA segments are associated with the rRNA genes : the 0.8 kbp segments separate the 3'-end of the 5S rRNA gene from the 5'-end

of the 16S gene and the 0.25 kbp segments separate the 3'-end of the 16S gene from the 5'-end of the 23S gene within each rDNA unit. At present, it has not been determined if another, very small, spacer sequence exists between the 23S and 5S rRNA genes. The 0.8 and 0.25 kbp spacer segments may be obtained on different DNA fragments within a given restriction digest (see Fig. 1). Cleavage of the 5.6 kbp ribosomal unit by Bgl II, Hind III or Hpa II generates the shorter (0.25 kbp) "spacer" in a DNA band (2.0 , 2.0 or 1.0 kbp, respectively), whereas the larger (0.8 kbp) "spacer" is located within a separate DNA band (3.6, 2.2 or 1.6 kbp, respectively). For all of these enzymes, two tRNA species ($tRNA^{Ile}$ and $tRNA^{Ala}$) hybridize to the DNA fragments containing only the 0.25 kbp spacer (cf. Table I and Fig. 1). The tRNA sample containing both $tRNA^{Glu}$ and $tRNA^{Trp}$ hybridizes to Bgl II, Hind III and Hpa II fragments containing only the larger (0.8 kbp) "spacer". Although both the Eco R I 2.4 and 3.2 kbp fragments contain parts of the larger "spacer", only the 2.4 kbp fragment hybridizes with the $tRNA^{Glu}$ + $tRNA^{Trp}$ mixture while being also the only fragment to hybridize both with $tRNA^{Ile}$ and with $tRNA^{Ala}$. A gene for either $tRNA^{Glu}$ or $tRNA^{Trp}$ must therefore be located within about 200 bases from the 5'-end of the 16S rRNA gene and the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes are located within the 250 base pair spacer following the 3'-end of the 16S gene (see Fig. 1). By the use of other restriction endonucleases, the location of these tRNA genes was confirmed (see Fig. 1).

It remains to be determined in which of the three rDNA unitsthese tRNA genes are located since all fragments discussed so far come from each of these units. As far as the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes are concerned, it is concluded that they must be present in at least the first and third rDNA units, and possibly the central unit, since hybridization of both tRNAs is observed to the Bam H I fragments 6.9 and 5.6 kbp and to the Bal I fragments 5.6 and 6.3 kbp.

On the basis of hybridization using the $tRNA^{Glu}$ + $tRNA^{Trp}$ mixture to Bam H I fragment 5.6 kbp, Bgl II fragment 3.6 kbp and the 3.6 kbp fragment in a Bgl II · Hind II double digestion, it is concluded that at least one, and possibly both, of the two latter rDNA units (II and III) possess a gene for the same tRNA (either $tRNA^{Glu}$ or $tRNA^{Trp}$) preceding the 5'-end of the 16S rRNA gene. Hybridizations of these tRNAs to a 4.5 kbp Xba I fragment, the 4.5 kbp Bgl II fragment and a 4.0 kbp fragment in the Bgl II · Hind II double digestion suggest that a gene for $tRNA^{Glu}$ or $tRNA^{Trp}$ is also located before the 16S rRNA gene of rDNA unit I. Hybridization has also been observed to the 45 kbp Bam H I fragment and the 21 kbp Eco R I fragment.

Evidence was also obtained to suggest that a gene for either $tRNA^{Glu}$ or $tRNA^{Trp}$ is located preceeding the fourth 16S rRNA sequence present on Euglena chloroplast DNA. This sequence is located 4.5 kbp from the 5'-end of the

rDNA unit I, in the 21 kbp Eco R I fragment (Fig. 1b). Hybridization of $tRNA^{Glu}$ + $tRNA^{Trp}$ was indeed observed to this fragment and also to a 1.8 kbp Hind III fragment which is shown to be located here by hybridization of 16S rRNA (Table I). Using double digestions with Hind II + Hind III, Hind III + Hpa II and Hind II + Hpa II, hybridization was observed to small fragments of 0.9 kbp, 1.4 kbp and 0.5 kbp respectively. These fragments also hybridize to 16S rRNA and therefore the tRNA gene must be located, as within rDNA units I, II and III, preceeding this 16S rRNA gene.

Another Eco R I fragment located in the single-copy region hybridizes with $tRNA^{Glu}$ + $tRNA^{Trp}$ (data not shown). Similarly, in digestions using other enzymes, hybridization was observed to fragments not associated with the rDNA, and so it is concluded that a gene for only one of the two tRNAs is present in the rDNA units, with that for the other tRNA being in the single-copy region.

DISCUSSION

Cleavage of Euglena chloroplast rDNA using restriction endonucleases in single or double digestions generates, among several DNA fragments, a single DNA band which contains part of the 16S rRNA gene, part of the 23S rRNA gene and the entire 0.25 kbp "spacer" between these genes. Hybridization of individual chloroplast tRNA species to this DNA band shows that genes for two tRNAs, namely $tRNA^{Ile}$ and $tRNA^{Ala}$, are present in two (unit I and unit III) and probably all three of the 0.25 kbp spacers.

A different tRNA gene (either $tRNA^{Trp}$ or $tRNA^{Glu}$) is shown to be located before at least two, and probably all four, of the 16S rDNA sequences which have been located on the physical restriction site map of Euglena chloroplast DNA. Since it was not possible to separate $tRNA^{Trp}$ and $tRNA^{Glu}$ using the two-dimensional gel electrophoretic method, hybridization with the individual tRNA species could not be carried out.

An unidentified 4S RNA gene has been mapped in the rDNA spacer of Chlamydomonas chloroplast DNA (17). In both rDNA units of spinach chloroplast DNA, a gene for $tRNA^{Ile}$ has been localized within the central third of the spacer sequence between the 16S and 23S rRNA genes (18). A gene for $tRNA^{Ala}$ has been localized on the spinach chloroplast DNA map, but its position is not in the rDNA unit (16). At present, a tRNA species accepting glutamic acid has not been identified (10).

The results presented in this paper show that specific tRNA genes are associated with Euglena chloroplast rDNA units and they provide further information on the similarities and differences between the rDNA units of chloroplast DNA and *E.coli* DNA. The considerable level of homology between

higher plant and Euglena chloroplast 16S and 23S rRNAs on one hand and *E.coli* rRNAs on the other has been recently described in several independent studies (14, 19, 20). In the *E.coli* genome, several copies of two types of rRNA operons are known (for a review see 21). One type contains the genes for $tRNA^{Ile}$ and $tRNA^{Ala}$ in the spacer and the other type has only the gene for $tRNA^{Glu}$ in its spacer. Other tRNAs ($tRNA^{Asp}$ and $tRNA^{Trp}$) have their genes associated with the *E.coli* rDNA at the distal end of some operons and these two tRNAs, as well as $tRNA^{Ile}$ and $tRNA^{Ala}$, are co-transcribed with the rRNAs (21, 22). Among the Euglena and spinach chloroplast tRNAs, $tRNA^{Asp}$ has not yet been identified (10). In Euglena the map position of the $tRNA^{Trp}$ gene has not been clearly established and in spinach the $tRNA^{Trp}$ gene is not associated with the chloroplast rDNA units (16). As with the bacterial rRNA operons, the chloroplast rDNA unit of Euglena and spinach are transcribed as a single precursor RNA molecule (5, 23, 24). It is not known whether any of these chloroplast tRNA genes shares a common promoter with the rRNA genes and is co-transcribed as a part of a single precursor.

In Euglena and in *E.coli*, the sequences coding for $tRNA^{Ile}$ and $tRNA^{Ala}$ form a major portion of the spacer sequence within the rDNA unit. The corresponding chloroplast rDNA spacer of spinach and maize is approximately 8-fold larger in size (25). As recently revealed by sequence analysis of one of the rDNA spacers of maize (W. Koch, K. Edwards and H. Kössel, in preparation) and Euglena (E. Orozco and R. Hallick, personal communication ; L. Graf, H. Kössel and E. Stutz, personal communication) these maize and Euglena chloroplast rDNA spacers contain a gene for $tRNA^{Ile}$ and a gene for $tRNA^{Ala}$, but each maize tRNA gene contains a very large intron, and these two introns account for most of the size difference between maize and Euglena rDNA spacers.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Deutsche Forschungsgemeinschaft (to H.J.B.) and a fellowship of the European Molecular Biology Organization (to E.J.C.). The expert technical assistance of Miss Christine Michalowski is gratefully acknowledged.

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