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Isolation of *Euglena gracilis* Chloroplast 5S Ribosomal RNA and Mapping the 5S rRNA Gene on Chloroplast DNA†

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ABSTRACT: Ribosomal RNA (5S) from *Euglena gracilis* chloroplasts was isolated by preparative electrophoresis, labeled in vitro with ^{125}I, and hybridized to restriction nuclease fragments from chloroplast DNA or cloned chloroplast DNA segments. *Euglena* chloroplast 5S rRNA is encoded in the chloroplast genome. The coding region of 5S rRNA has been positioned within the 5.6 kilobase pair (kbp) repeat which also codes for 16S and 23S rRNA. There are three 5S rRNA genes on the 130-kbp genome. The order of RNAs within a single repeat is 16S–23S–5S. The organization and size of the *Euglena* chloroplast ribosomal repeat is very similar to the ribosomal RNA operons of *Escherichia coli*.

The 5S ribosomal RNAs from many organisms have been well characterized. At least 36 5S rRNAs[1] have been completely sequenced (Erdmann, 1978). This RNA is an integral component of both procaryotic and eucaryotic ribosomes. Its exact role in protein synthesis has not been determined, but it may serve to recognize and position transfer RNAs (Monier, 1974). The sequence of 5S rRNA has been well conserved throughout evolution. Sequence data have been used to estimate the evolutionary divergence of various species (Schwarz & Dayhoff, 1978). Prior to this report, the only chloroplast 5S rRNAs which have been mapped with respect to chloroplast DNA restriction nuclease site maps were in *Zea mays* (Bedbrook et al., 1977) and spinach (Whitfield et al., 1978).

Euglena is a versatile unicellular eucaryote which can grow on either phototrophic or organotrophic media. Chloroplasts from this organism contain DNA, which has been extensively studied. Depending on the growth conditions, 500–2000 copies of the 130-kbp genome are present in each cell (Rawson & Boerma, 1976; Chelm et al., 1977). This DNA codes for 16S and 23S rRNA (Scott & Smillie, 1967; Stutz & Rawson, 1970) and approximately 25 tRNAs (McCrea & Hershberger, 1976; Schwartzbach et al., 1976). In addition, 50 kb of RNA of yet unknown function is also transcribed from this genome in vivo (Chelm et al., 1978). The coding region for 16S and 23S rRNAs has been located on a 5.6-kbp DNA segment which is repeated three times in tandem (Gray & Hallick,

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[1] Abbreviations used: rRNA, ribosomal RNA; kbp, kilobase pair; tRNA, transfer RNA; kb, kilobase; G+C, guanine and cytosine; bp, base pair; nt, nucleotides.

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1978; Rawson et al., 1978; Jenni & Stutz, 1978). This ribosomal DNA has a much higher guanine and cytosine content (44% G+C) than the remaining 113 kbp of the chloroplast genome, which is 20% G+C (Gray & Hallick, 1979).

In order to extend previous mapping results on *Euglena* chloroplast rRNAs, we have isolated intact chloroplast 5S rRNA and determined its map position by hybridization of 5S rRNA to restriction fragments of chloroplast DNA and to cloned chloroplast DNA segments. This report represents the first isolation of *Euglena* chloroplast 5S rRNA and characterization of 5S coding sequences in the chloroplast genome. A preliminary report of these results has appeared (Hallick et al., 1978).

Materials and Methods

rRNA (5S) Isolation. rRNA (5S) could be isolated from either chloroplast ribosomes or total chloroplast RNA. Chloroplasts from phototrophically grown *Euglena gracilis* Kelbs strain Z Pringsheim were isolated as previously described (Hallick et al., 1976), except that the isolation buffer was 0.05 M Tris-HCl, pH 7.8, 0.37 M sucrose, 0.01 M $MgCl_2$, and 1 mM aurintricarboxylic acid (Sigma). Aurintricarboxylic acid is a potent inhibitor of ribonuclease activity (Hallick et al., 1977). Chloroplasts were lysed with isolation buffer containing 5% Triton X-100.

Total chloroplast RNA was prepared by extracting the lysate three times with phenol saturated with 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA. Alternatively, chloroplast ribosomes were prepared by centrifugation of the lysate through 1.37 M sucrose, 0.05 M Tris-HCl, pH 7.8, 5 mM $MgCl_2$, and 0.2 mM aurin tricarboxylic acid (Vasconcelos & Bogorad, 1970). The pelleted ribosomes were resuspended in 0.07 M sucrose, 0.01 M Tris-HCl, pH 7.8, 2 mM $MgCl_2$, and 0.2 mM aurintricarboxylic acid and centrifuged in a 10–34% sucrose gradient at 76000*g* for 12 h. Fractions could not be directly analyzed spectrophotometrically because of the high absorption of aurintricarboxylic acid. Aurintricarboxylic acid can be removed from samples by Sephadex G-25 chromatography, but gradient fractions were more easily analyzed by acrylamide gel electrophoresis (described below).

rRNA (5S) was purified by preparative gel electrophoresis. RNA was separated in 6% or 8% acrylamide (acrylamide:bisacrylamide was 29:1) gels with 7 M urea as described by Donis-Keller et al. (1977). RNA was subjected to electrophoresis in gels (12 × 14 × 0.3 cm) for 2 h at 150 V. The gels were soaked in 5 mg/mL ethidium bromide for 30 min and subsequently photographed under UV illumination as previously described (Gray & Hallick, 1977). In 6% acrylamide–7 M urea gels the electrophoretic mobility of *Euglena* chloroplast 5S rRNA (and *E. coli* 5S rRNA) was the same as a xylene cyanol FF dye marker. Therefore 5S rRNA could be located in nonstained preparative 6% gels by slicing out the region of the gel containing the dye band. RNA was eluted from gels by the crush and soak technique of Maxam & Gilbert (1977).

Purified chloroplast 5S rRNA was radioactively labeled in vitro with ^{125}I to a specific activity of 8×10^6 dpm/μg by the technique of Tereba & McCarthy (1973). ^{125}I-Labeled 5S rRNA was hybridized in 50% formamide, 0.75 M NaCl, 0.075 M sodium citrate at 43 °C to DNA transferred to nitrocellulose filters (Southern, 1975) by the method of Bedbrook et al. (1977).

A discrete RNA of electrophoretic mobility intermediate between that of 16S rRNA and 5S rRNA was routinely observed in ethidium bromide stained gels of chloroplast RNAs. This RNA was purified by preparative electrophoresis

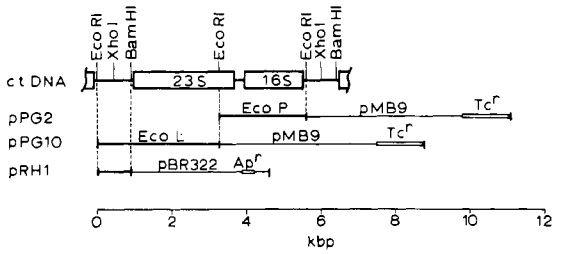

FIGURE 1: Linearized restriction endonuclease maps of plasmids pPG2, pPG10, and pRH1. The vehicle DNA is represented by a thin line, while the inserted DNA is shown with a heavy line. The origin on chloroplast DNA of the plasmid insert is indicated by dashed lines from the ribosomal RNA coding region of the chloroplast genome (top). Electrophoresis of digestion products of these plasmid DNAs is shown in Figure 4.

in a 6% acrylamide–7 M urea gel. The RNA was located by ethidium bromide staining and the appropriate gel region was excised. The RNA was eluted by the crush and soak method and labeled in vitro with ^{125}I as described above for 5S rRNA.

DNA Isolation and Cloning. Isolation of supercoiled *Euglena gracilis* chloroplast DNA, restriction endonuclease digestions, and electrophoresis of DNA fragments have been described (Gray & Hallick, 1978). pPG2 and pPG10 are plasmids containing the vehicle pMB9 (Rodriquez et al., 1976) and inserts of *Eco*RI chloroplast DNA fragments P (2.3 kbp) and L (3.2 kbp), respectively. They were isolated from a collection of *Eco*RI fragments of *Euglena* chloroplast DNA cloned in *Eco*RI digested pMB9 (Gray, Hall, & Hallick, manuscript in preparation).

The plasmid pRH1 was constructed by digesting a mixture of pPG10 and pBR322 (Bolivar et al., 1977) with restriction endonucleases *Eco*RI and *Bam*HI, ligating the products with T4 DNA ligase, and transforming *E. coli* HB101 (Rodriquez et al., 1976). Ampicillin-resistant, tetracycline-sensitive clones were then selected by replica plating. The size of the chloroplast DNA insert in plasmids of transformed cells was determined by gel electrophoresis (Barnes, 1977). pRH1 was selected because of its size (4.6 kbp). P2 and EK1 biohazard containment procedures were followed in experiments involving recombinant DNA. Restriction nuclease maps for pRH1, pPG2, and pPG10 were determined by using previously described methods (Gray & Hallick, 1978). The results are summarized in Figure 1.

Results

Characterization of 5S rRNA. Isolation of 5S rRNA from chloroplasts of *Euglena gracilis* has not been previously reported. RNA isolated from chloroplast ribosomes was fractionated on 8% acrylamide–7 M urea gels, as shown in Figure 2. Also shown for comparison are *Euglena* whole cell, low molecular weight RNAs. The chloroplast RNA preparations are notably free of detectable amounts of the major RNAs present in the whole cell RNA preparation. The three observed classes of chloroplast rRNA, 23S, 16S, and 5S, are approximately the same size as the corresponding *E. coli* ribosomal RNAs (not shown). In particular, chloroplast 5S rRNA has the same mobility as purified *E. coli* 5S rRNA (Figure 3). Consequently, *Euglena* chloroplast 5S rRNA must have a size of approximately 118–120 nucleotides, similar to other 5S rRNAs (Erdmann, 1978).

rRNA (5S) could also be identified as a discrete, well-resolved RNA following electrophoresis of total chloroplast lysates (Figure 3). In the studies which follow, 5S rRNA was isolated from whole chloroplast lysates instead of purified ribosomes. When this RNA was eluted from gels and sub-

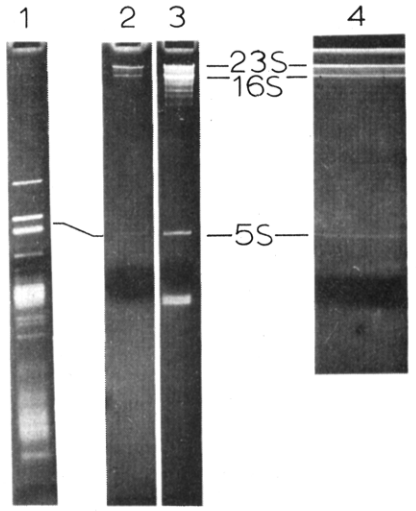

FIGURE 2: Eight percent acrylamide–7 M urea gels of *Euglena* chloroplast RNA samples. (Lane 1) *Euglena* whole cell, soluble RNA prepared by the method of von Ehrenstein (1967); (2) RNA from purified chloroplast ribosomes; (3) total chloroplast RNA; (4) preparative gel of total chloroplast RNA.

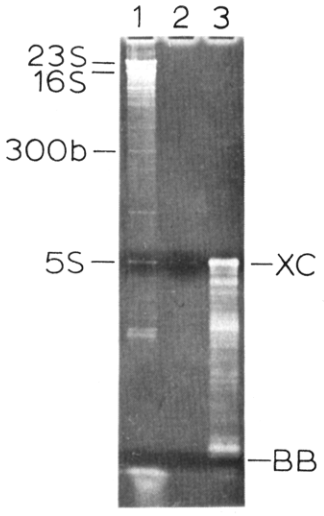

FIGURE 3: Six percent acrylamide–7 M urea gel of purified 5S rRNA. Samples are as follows: (1) total chloroplast RNA. The "300 b" RNA, described in the text, was purified by preparative electrophoresis for further study. (2) rRNA, 5S, purified by preparative electrophoresis from total chloroplast RNA. (3) A commercial sample of *E. coli* 5S rRNA. XC and BB refer to the dye markers xylene cyanol FF and bromophenol blue, respectively.

jected to reelectrophoresis, two bands were occasionally observed (Figure 3). The major RNA has the same mobility as 5S rRNA, while the minor species migrates slightly slower than 5S rRNA. This minor RNA has not been observed in every preparation of total chloroplast RNA and may represent a 5S rRNA precursor.

Characterization of the 5S rRNA Coding Region. Iodinated 5S rRNA labeled to a specific activity of 4×10^6 cpm/μg was hybridized to various restriction nuclease fragments of chloroplast DNA, which had previously been transferred to a nitrocellulose filter by the method of Southern. Fragments hybridizing with 5S rRNA were detected by autoradiography. The results are shown in Figure 4. ^{125}I-Labeled 5S rRNA hybridizes with fragments *Pvu*A (lane 8), *Bam*D and *Bam*E (lane 7), and *Eco*F and *Eco*L (lane 1, for nomenclature, see Gray & Hallick, 1977, 1978). These fragments map on the chloroplast DNA in the region previously shown to code for 16S and 23S rRNAs (Gray & Hallick, 1978). This region contains three identical rRNA

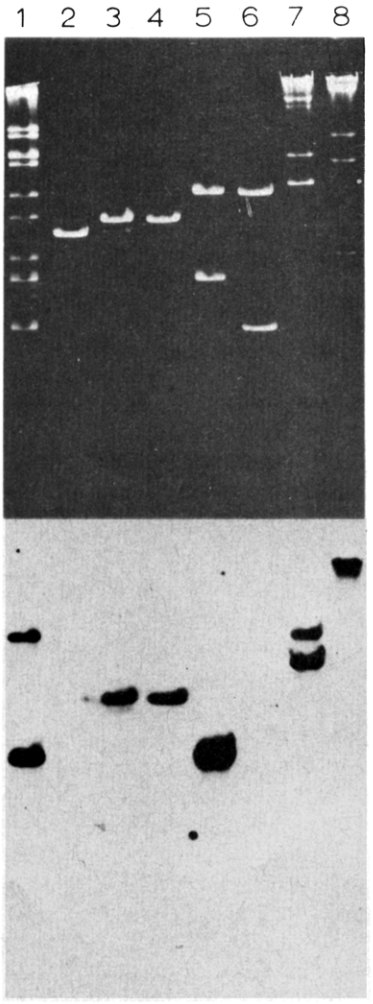

FIGURE 4: Hybridization of ^{125}I-labeled chloroplast 5S rRNA to restriction endonuclease digested chloroplast DNA and cloned chloroplast DNA fragments. (Top) Photograph of ethidium bromide stained, 0.7% agarose gel. (Bottom) Autoradiogram of Southern blot of the agarose gel, hybridized with ^{125}I-labeled 5S rRNA as described by Bedbrook et al. (1977). (Lane 1) *Eco*RI digest of chloroplast DNA; (2) *Bam*HI and *Xho*I double digest of pRH1 (partial *Xho*I digest); (3) *Eco*RI and *Xho*I double digest of pRH1 (partial *Xho*I digest); (4) *Eco*RI digest of pRH1; (5) *Eco*RI digest of pPG10 (*Eco*L insert); (6) *Eco*RI digest of pPG2 (EcoP insert); (7) *Bam*HI digest of chloroplast DNA; and (8) *Pvu*II digest of chloroplast DNA. The DNA samples contained either 1.5 μg of chloroplast DNA or 0.8 μg of plasmid DNA. The hybridization reaction, containing 4×10^6 dpm of [^{125}I]rRNA and yeast carrier tRNA, was incubated for 16 h at 43 °C in 50% formamide, 0.75 M NaCl, 0.075 M sodium citrate.

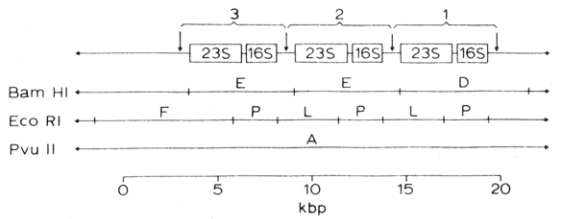

FIGURE 5: The *Euglena* chloroplast DNA ribosomal RNA region. The ribosomal RNA genes of *Euglena gracilis* chloroplast DNA are arranged as a triple tandem repeat (Gray & Hallick, 1978). The repeated DNA segments, labeled 1, 2, and 3, are each 5.6 kbp. Approximately 20 kbp of the 130-kbp genome is shown. ^{125}I-Labeled 5S rRNA hybridized to all restriction fragments shown except *Eco*P.

repeats, arranged in tandem (Figure 5). Each rRNA repeat is 5.6 kbp in length and codes for a 16S and a 23S rRNA.

It is possible to locate the 5S rRNA genes within each rRNA repeat. As shown in Figure 5, the two 5.6-kbp *Bam*E fragments arise from cleavage at the *Bam*HI sites near one

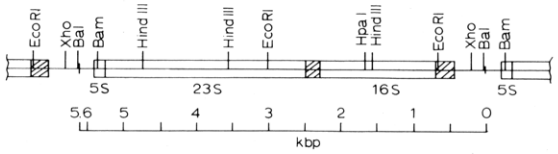

FIGURE 6: The position of 5S rRNA located on a single 5.6-kbp repeat. The cross-hatched areas represent precursor RNA which is processed to form mature 16S and 23S rRNA (Scott, 1976). The positions of *Hin*dIII and *Hpa*I sites are from Rawson et al. (1978); other restriction sites are from Gray & Hallick (1978). Cleavage of the 5S gene by *Bam*HI is based on an assumption of one 5S gene per 5.6-kbp rRNA repeat. The possibility of an intergenic spacer between the 23S and 5S genes, although not shown in the diagram, cannot be ruled out.

end of each 23S rRNA gene. The *Bam*E DNAs contain the entire DNA sequence content of a single rRNA repeat (Gray & Hallick, 1978). *Bam*D, which is 6.9 kbp, contains a complete 16S gene and 23S gene but lacks a DNA sequence of 0.4 kbp at one end of the 23S gene present in each of the three repeated segments (Figure 5). Since 5S rRNA hybridizes to *Bam*D, there must be 5S rRNA coding sequences prior to the terminal 0.4 kbp in each 5.6-kbp repeat. This coding region is on the side of the *Bam*HI cleavage site in each repeat proximal to the 23S rRNA gene.

Each rRNA repeat is cleaved twice by *Eco*RI (Figure 5) yielding fragments *Eco*L (3.2 kbp) and *Eco*P (2.3 kbp). The 5S rRNA hybridizes only to *Eco*L (Figure 4). *Eco*L contains 23S rRNA coding sequences and the DNA sequences from the end of the 23S gene of one repeat to a region near the start of the 16S gene in the adjacent repeat. *Eco*L contains the *Bam*HI cleavage site described above.

Hybridization studies were also carried out with cloned chloroplast DNAs. As shown in Figure 4, 5S rRNA hybridizes with a plasmid (pPG10; lane 5) which contains the cloned *Eco*L DNA but not with a plasmid (pPG2; lane 6) which contains the *Eco*P DNA. This result is consistent with the hybridization observed to *Eco*RI digested total chloroplast DNA (lane 1). To determine more precisely the location of 5S rRNA coding regions, the *Eco*L plasmid was subcloned as described in Materials and Methods. One of the resulting products was a plasmid (pRH1) containing 0.9 kbp of DNA derived from the side of the *Bam*HI site in EcoL *distal* to the end of the 23S rRNA gene (Figure 1). The 5S rRNA hybridizes to this plasmid (Figure 4, lane 4). Since it was shown above that the 5S rRNA hybridizes to *Bam*D, which contains DNA sequences on the side of the *Bam*HI cleavage site *proximal* to the end of the 23S gene, 5S rRNA coding sequences must be located on both sides of this *Bam*HI site. If there is a single 5S gene per rRNA repeat, this gene must be cleaved internally by *Bam*HI.

To further localize the 5S rRNA coding region, the plasmid pRH1 was digested with *Eco*RI and *Xho*I (Figure 4, lane 3). The products are a 4.2-kbp fragment and a 400-bp fragment with electrophoreses off the gel. The 4.2-kbp fragment contains approximately half of the 0.9-kbp cloned DNA of pRH1, specifically the region from the *Bam*HI to the *Xho*I site (see Figure 1). The 5S rRNA hybridizes to this 4.2-kbp fragment. Alternatively, pRH1 was digested with *Bam*HI and *Xho*I. In this treatment, a 4.2-kbp fragment is produced without the 400 bp between the *Bam*HI and *Xho*I sites. The 5S rRNA did not hybridize to this 4.2-kbp DNA (Figure 4, lane 2).

From the hybridization results described above, the loci on the 130-kb *Euglena* chloroplast DNA coding for 5S rRNA can be positioned. The genes are in a region of approximately 0.6 kbp in each rRNA repeat. As shown in Figure 6, this 0.6-kbp region is bounded on one end by the 23S rRNA gene

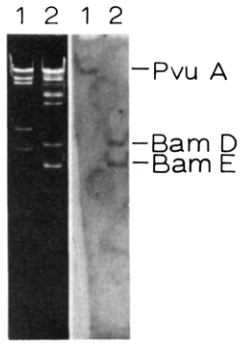

FIGURE 7: Mapping a small chloroplast RNA in the ribosomal RNA repeat. Agarose (0.7%) gels (left) of *Pvu*II (lane 1) and *Bam*HI (lane 2) digested chloroplast DNA and autoradiogram (right) of Southern blot of gels hybridized to ^{125}I-labeled 300 base RNA. Hybridization was carried out as described in the text and in the legend to Figure 4.

and on the other by a *Xho*I cleavage site. If we assume a single 5S rRNA gene is present in each rRNA repeat, this gene must be located as is shown in Figure 6.

A Small Chloroplast RNA Encoded in the rRNA Repeat. A prominent RNA of approximately 300 nt was observed as a discrete species in ethidium bromide stained gels of electrophoretically separated chloroplast RNAs. This RNA was present in the chloroplast RNA preparation from phenol extracted chloroplast lysates but not in chloroplast ribosomes. This RNA is also found in the RNA pellet when chloroplast DNA is isolated by centrifugation of chloroplast lysates to equilibrium in CsCl–ethidium bromide gradients. This RNA was purified by preparative electrophoresis, labeled in vitro with ^{125}I and hybridized to chloroplast DNA restriction fragments by the Southern method. As shown in Figure 7, this RNA hybridizes to the ribosomal repeat fragments *Bam*D and *Bam*E. In another experiment (not shown), this RNA was found to hybridize specifically to *Eco*P but not to either *Eco*F or *Eco*L. From these results it can be concluded that this RNA is a transcription product of the ribosomal RNA region. The function of the RNA is not known.

Discussion

The chloroplast genome of *Euglena gracilis* Klebs strain Z Pringsheim appears to contain three identical 5.6-kbp DNA segments arranged in tandem. The evidence for tandem sequence organization has previously been discussed (Gray & Hallick, 1978; Rawson et al., 1978; Jenni & Stutz, 1978). The conclusion that all three 5.6-kbp repeats contain identical DNA sequences is based on the interpretation of restriction nuclease cleavage data, summarized in Table I. All restriction nucleases that have been used to cleave *Euglena* chloroplast DNA recognize each repeated segment in an identical manner. To date this includes 12 cleavage sites of 8 enzymes. The identical cleavage sequences total 1.3% of the nucleotides of each 5.6-kbp repeat. The recognition sequence for four additional enzymes is absent in all three repeated DNA regions (Table I). It would be impossible to rule out minor sequences variation in individual repeats from this type of analysis, but it is reasonable to assume that the gene arrangement in all three repeats is identical.

From the results of the present study it may be concluded that 5S rRNA coding sequences are present in each 5.6-kbp repeat. The 5S rRNA gene(s) have been located in repeat 1 (Figure 5) based on hybridization to *Bam*D and in repeat 3 based on hybridization to *Eco*F. We cannot unequivocally show hybridization to repeat 2, but the two fragments *Bam*E from repeats 2, 3 (Figure 5) and *Eco*L from repeats 1, 2

Table I: Restriction Endonuclease Cleavage Sites within the Ribosomal Repeat of *Euglena gracilis* Chloroplast DNA[a]

enzyme	no. of cleavage sites within individual repeat	no. of cleavage sites within ribosomal DNA	size of cleavage products (kbp)
*Sal*I	0	0	
*Pst*I	0	0	
*Pvu*II[b]	0	0	
*Sma*I	0	0	
*Bam*HI	1	3	5.6
*Bal*I	1	3	5.6
*Xho*I	1	3	5.6
*Kpn*I[b]	1	3	5.6
*Hpa*I[c]	1	3	5.6
*Eco*RI	2	6	3.25, 2.35
*Bgl*II[d]	2	6	3.6, 2.0
*Hin*dIII[c]	3	9	2.4, 2.1, 1.0

[a] All results are from Gray & Hallick (1978) except for the following. [b] Hallick et al., 1978; Gray, Hall, and Hallick, unpublished results. [c] Rawson et al., 1978. [d] Jenni & Stutz, 1978.

hybridize with 5S rRNA and are identical by the criteria described above. We would therefore conclude that there are 5S rRNA gene(s) in each 5.6-kbp rRNA repeat in the *Euglena* chloroplast genome. There is most likely a single 5S rRNA gene per repeat, in equal stoichiometry with the number of 16S and 23S rRNA genes. However, from the hybridization results presented in this study, we cannot rule out the possibility of more than one 5S rRNA gene within each repeat, which would result in some integral multiple of three 5S rRNA genes in the chloroplast genome. The 5S rRNA hybridizes to either side of the *Bam*HI site near the end of the 23S rRNA gene. Assuming this hybridization is due to a single coding locus in each repeat, we can map the position of the coding sequence for 5S rRNA to within at least 100 base pairs. A map of repeat 2 with the exact position of the 5S gene, assuming one 5S gene per repeat, is shown in Figure 6. It is seen that the 16S rRNA gene, the intergenic spacer, and the 23S gene are contained completely within a DNA segment of only 4.8 kbp. This region is bounded by an *Eco*RI site at one end and a *Bam*HI site in the 5S gene at the other. The size of the intergenic spacer is estimated to be approximately 300 bp from three lines of evidence. First, 23S rRNA is produced from a precursor approximately 200 bases larger than the mature product (Scott, 1976). Second, we have isolated a chloroplast RNA of approximately 300 bases which hybridizes only to restriction fragments containing the region. Finally, tRNA(s) are encoded between the 16S and 23S gene, on the same DNA strand as these rRNAs (Hallick et al., 1978; E. M. Orozco, Jr., & R. B. Hallick, manuscript in preparation). Therefore the 16S and 23S rRNAs must be encoded in a region of approximately 4.4 kbp. Based on a comparison of the electrophoretic mobilities of *Euglena* chloroplast rRNAs and *E. coli* rRNAs (not shown), we estimate *Euglena* chloroplast 16S rRNA and 23S rRNA to be approximately 1500–1600 and 2700–2800 nt, respectively.

Chloroplast rRNAs have been located in the chloroplast genomes of two higher plants. In *Zea mays*, the order of genes in the rRNA region is 16S rRNA–2.1 kbp spacer–23S rRNA–5S rRNA (Bedbrook et al., 1977). A very similar arrangement has been reported for spinach, but the spacer between the 16S and 23S rRNA genes is only 1.4 kbp (Whitfield et al., 1978). In both cases there are only two sets of rRNA coding regions, and these are present in the genome as inverted repeats, separated by a large spacer. A similar gene arrangement has been described for *Chlamydomanas reinhardii* (Rochaix & Malnoe, 1978), with the additional findings that, in this organism, 7S and 3S RNAs are encoded in the region between the 16S and 23S rRNA genes. Furthermore the 23S gene is interrupted by a 940-bp intervening sequence. It is evident that the rRNA gene arrangement in *Euglena gracilis* is quite distinct from these organisms. *Euglena* has three sets of chloroplast rRNA genes rather than two, a tandem gene arrangement rather than inverted repeats, contiguous rather than widely spaced rRNA coding regions, and a spacer between 16S and 23S genes of only 0.3 kbp rather than 1.4 to 2.1 kbp.

The similarity between the *Euglena* chloroplast rRNA coding region and that of the *E. coli* rRNA operons is noteworthy. In *E. coli* there are thought to be seven rRNA operons, each with a gene arrangement of 16S rRNA–tRNA(s)–23S rRNA–5S rRNA–tRNA(s) (Lund et al., 1976; Morgan et al., 1978). The number of distal tRNAs ranges from 0 to 3. The size of these operons is 5.6 kbp. A further similarity between the *Euglena* and procaryotic rRNAs is in the primary RNA sequences. Ribonuclease T_1 oligonucleotides of *Euglena* chloroplast 16S rRNA have been characterized and compared with the oligonucleotides of several procaryotic 16S rRNAs. It has been concluded (Zablen et al., 1975) that the *Euglena* chloroplast 16S rRNA oligonucleotide pattern is most similar to that of a procaryotic 16S rRNA. Based on these comparisons, it is interesting to speculate that the clustered genes in the 5.6-kbp repeats of *Euglena* chloroplast DNA may be single transcription units, analogous to the *E. coli* rRNA operons. The gene sequence in *Euglena* of 16S rRNA–tRNA(s)–23S rRNA–5S rRNA may be initially transcribed into a primary RNA of >5000 nt and subsequently matured to rRNAs and tRNAs (Hallick et al., 1978). This will be an interesting subject for further study.

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Photoreductive Titration of the Resonance Raman Spectra of Cytochrome Oxidase in Whole Mitochondria†

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ABSTRACT: A photoreductive titration of the resonance Raman (RR) spectra of cytochrome *c* oxidase in whole mitochondria was recorded by exploiting the preferential enhancement of the Raman signals of reduced cytochrome oxidase excited at 441.6 nm. When the sample was cooled to about −10 °C, it was possible to slow down the photoreductive effect of the laser and to record RR spectra at various states of reduction. Compared to the earliest recorded scan (most oxidized), the dithionite-reduced sample shows the appearance of new bands at 216, 363, 560, and 1665 cm^{-1}. At intermediate stages of photoreduction, the 216- and 560-cm^{-1} bands appear before the 363- and 1665-cm^{-1} bands; photoreduction induces full intensity in the former bands, whereas the latter bands are photoreduced to 50% of the dithionite-reduced intensity. The relative intensities of a doublet at 1609–1623 cm^{-1} are affected by reduction: the band at 1609 cm^{-1} is weaker in the earlier scans; in later scans this band has grown to equal intensity with the 1623-cm^{-1} band. We conclude that this reductive titration of the RR spectrum of cytochrome *c* oxidase reflects three states in its reduction. The behavior of the doublet at 1609–1623 cm^{-1} suggests that the two hemes are nonequivalent but interacting. The band at 216 cm^{-1} may be indicative of an iron–copper interaction that is affected by the presence of external ligands.

Cytochrome *c* oxidase is the terminal component in the electron transport chain whose function is to transfer four electrons to oxygen, thereby reducing it to water, and to convert the energy released to chemical energy by phosphorylating ADP to ATP. The enzyme contains four prosthetic groups that are active in its function: two *a*-type hemes and two copper ions. In spite of much activity in the investigation of this system, a detailed picture of its molecular organization and mechanism of activity has yet to emerge.

Resonance Raman (RR) spectra of many heme proteins [see Warshel (1977), for example] have been examined with the aim of relating the spectroscopic behavior of the hemes to their biological function. In the case of cytochrome *c* oxidase, it was recognized early that the polarization behavior of the RR spectra was different than that of other hemes and reflected differences in resonance enhancement by excitation in the Soret rather than the visible absorption bands (Salmeen et al., 1973; Nafie et al., 1973; Friedman & Hochstrasser, 1973).

Salmeen et al. (1973) reported that when exciting cytochrome oxidase at 441.6 nm, the strongest band appeared as a doublet at 1372 and 1358 cm^{-1} and suggested that this reflected contributions from the inequivalent hemes *a* and a_3. Subsequently, Adar & Yonetani (1977), using the 413.1-nm excitation, showed that the laser beam exciting the spectra induced photochemical events which produced reducing equivalents and caused reduction of the enzyme. Thus, if the oxidase preparation used by Salmeen et al. (1973) was not fully oxidized, the strongest band in their spectrum would appear as a doublet; in fact, the markers for oxidized and reduced hemes at 1372 and 1358 cm^{-1} did appear simultaneously. This is consistent with the data of heme *a* derivatives of Kitagawa et al. (1977) in which the RR bands of ferric and ferrous hemes fall in the expected regions.

In this work, we took advantage of the capability of the laser beam to produce reducing equivalents and carried out the photoreductive titrations of cytochrome *c* oxidase in whole mitochondria induced by the 441.6-nm HeCd laser line. Cytochrome oxidase accepts four reducing equivalents which reside on the two *a*-type hemes and two copper ions.

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