ANALYSIS OF *EUGLENA GRACILIS* CHLOROPLAST DNA

Mapping of a DNA sequence complementary to 16 S rRNA outside of the three rRNA gene sets

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1. Introduction

It is accepted now that *Euglena gracilis* chloroplast DNA contains 3 rRNA gene sets (16 S, 23 S, 5 S RNA) which are located in 3 tandemly repeated DNA segments of 5600 base pairs each [1–3]. Both the repeats and the rRNA genes were mapped on the circular DNA molecule in relation to numerous restriction enzyme cleavage sites and in particular it was shown that the DNA fragments *Eco*F, *Eco*L and *Eco*P (for DNA fragment nomenclature see legend to fig.1 and [3]) hybridized to rRNA. It was also shown that the 16 S rRNA hybridized exclusively to fragment *Eco*P [2] and that the three sets of genes were arranged from 5′ to 3′ end in the order of 16 S–23 S–5 S [4].

However, according to [3,5–8] and contrary to others [1,2,4] total rRNA hybridized also to the fragment *Eco*B which according to present mapping data [1,2,4] should not carry any structural rRNA genes. We have investigated this problem and give in this report unequivocal experimental evidence that the fragment *Eco*B does have a short DNA segment complementary to 16 S but not to 23 S rRNA, suggesting that *Euglena gracilis* chloroplast DNA contains in addition to three complete rRNA gene sets a supplementary 16 S rRNA cistron.

2. Methods

2.1. *Preparation of chloroplast DNA and rRNA*

Chloroplast DNA was isolated from purified chloroplasts of *Euglena gracilis* (Z. strain, culture collection of Algae, Indiana University, no. 753), as in [3]. Chloroplast 16 S and 23 S rRNA were obtained from purified chloroplast ribosomes [9]. The 23 S and 16 S rRNA were purified by twice centrifuging in a 5–20% linear, 5 ml sucrose gradient in a SW50.1 Beckman rotor, 50 000 rev./min, 3 h, 4°C.

2.2. *Preparation of DNA fragments BG 16, BG 17 and BG 18*

The DNA fragments BG 16 and 17 were prepared from cloned DNA fragments *Bam*D and/or *Bam*E [10]. The respective recombinant DNA was digested first with endo R. *Bgl*II and the resulting BG 17 fragment (equivalent to *Bgl*Q ~2000 base pairs) was separated on a 5–20% linear 5 ml sucrose gradient (10 mM Tris–HCl (pH 7.9), 1 mM EDTA) in a SW50.1 Beckman rotor, 50 000 rev./min, 3 h, 15°C from the rest of the recombinant DNA consisting of the DNA from the plasmid pBR322 (4362 base pairs [11]) plus the sequences remaining from either *Bam*E (3600 base pairs) or *Bam*D (5000 base pairs). The fragment BG 16 was obtained by a second digestion of this remaining DNA with endo R. *Bam*HI and by centrifugation in an identical sucrose gradient for 5 h. Fragment BG 18 was obtained by double limit digestion of total chloroplast DNA with endo R. *Bgl*II and endo R. *Bam*HI, separation by electrophoresis [3] and electrophoretic elution from a 1% agarose gel.

2.3. *Labelling of DNA fragments and rRNA*

DNA fragments were labelled by nick-translation [12] using d-[α-^{32}P]ATP. Ribosomal RNA was labelled by terminal phosphorylation using [γ-^{32}P]ATP

essentially as reported for DNA labelling [13]. Complementary labelled RNA was obtained by in vitro transcription of DNA templates using [α-^{32}P] ATP [14].

2.4. *DNA : RNA and DNA : DNA hybridization*

DNA fragments were transferred from gels into millipore HAWP 304 FO filter strips according to [15]. The filters were wetted with the labelled RNA in 50% formamide, 5X NaCl/Cit, (1X NaCl/Cit, 0.15 M NaCl and 0.015 M sodium citrate) then wrapped in Saran foil and incubated for 20 h, at 42°C. The filters were washed 4–6 times in 2X NaCl/Cit, 65°C, treated with RNase A (20 μg/ml), 1 h, 20°C and rewashed in 2X NaCl/Cit at 65°C. For DNA : DNA hybridization the conditions were the same as for DNA : RNA hybridization with the exception that the filters were treated before and after hybridization according to [16].

2.5. *Electrophoresis and autoradiography*

Gel electrophoresis and autoradiography were carried out as in [3] and as specified in the respective legends.

2.6. *Enzymes and radioisotopes*

Endo R. *Bgl*II was a kind gift from Dr T. Bickle, University of Basle. The following enzymes were purchased: endo R. *Eco*RI and DNA polymerase (*E. coli*), from Boehringer Biochem., Mannheim, RNA polymerase (*E. coli* K-12) and polynucleotide kinase (T_4-infected *E. coli* B) from Miles Labs, Elkart IN 46514; endo R. *Hae*III, from Biolabs, Beverly, MA 01915; endo R *Bam*HI, from Bethesda Res. Labs, Rockville, MD 20850. Radioisotopes were from Radiochemical Center, Amersham.

3. Results and discussion

In fig.1 a short segment of the *Euglena gracilis* chloroplast genome map is presented. It shows part of the rDNA region with one complete gene set for 16 S + 23 S + 5 S rRNA located in fragment *Bam*D, the beginning of another gene set in *Bam*E, left hand side, and a small part of the fragment *Bam*B with the crucial segment BG 18 at the right-hand side which contains, according to this report, the supplementary 16 S rRNA cistron. Details of the mapping procedure

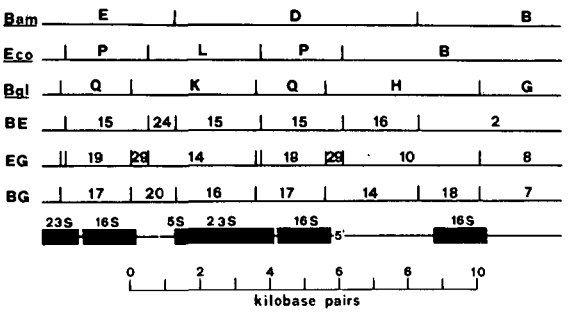

Fig.1. Segment of the rDNA region of the chloroplast genome. The 7 horizontal lines symbolize a segment of the double stranded chloroplast DNA. The vertical lines indicate the position of the cleavage sites of restriction enzymes. The abbreviations *Bam*, *Eco* and *Bgl* stand for endo R. *bam*HI, endo R. *Eco*RI, and endo R. *Bgl*II, respectively. BE, EG and BG stand for endo R. *Bam*HI plus endo R. *Eco*RI, endo R. *Eco*RI plus endo R. *Bgl*II, and endo R. *Bam*HI and endo R. *Bgl*II. The numbers and letters above the lines refer to restriction fragments described in [3]. Restriction enzyme nomenclature is according to [17]. On the bottom line the ribosomal RNA genes are positioned in scale with respect to the various cleavage sites. The 5' end of the 16 S rRNA is 170 base pairs apart from the endo R. *Bgl*II cleavage site BG 17/14 and BG 17/20 as determined by base sequence analysis (Schwarz, Kössel, Graf, E. S., in preparation). The sizes of the 16 S and 23 S structural genes are taken as 1500 and 2800 base pairs [18], respectively. The 5 S rRNA is placed according to [4]. The supplementary 16 S rRNA cistron in fragment BG 18 is positioned in accordance to the position of the 16 S rRNA gene in fragment *Bam*D (E) taking as reference point the endo R. *Bgl*II cleavage site BG 18/7 as discussed here.

have been published [1–3] and the fragment nomenclature is given in the legend.

Total chloroplast DNA was digested with endo R. *Eco*RI (*Eco* fragments), endo R. *Bam*HI and endo R. *Eco*RI (BE fragments), endo R. *Bam*HI and endo R. *Bgl*II (BG fragments) or endo R. *Eco*RI and endo R. *Bgl*II (EG fragments) and the respective fragments were separated by gel electrophoresis. The fragments were hybridized to ^{32}P-labelled 16 S or 23 S rRNA according to [15]. The respective autoradiographs are shown in fig.2 (panels a–c). We can see that only the 16 S rRNA hybridized to *Eco*B (a), BE 2 (b) or BG 18 (c). Otherwise the hybridization data were as expected and published [2,3]. As shown in [3] and drawn up in fig.1 these 3 DNA fragments are located outside of a complete rRNA gene set.

Although we had carefully purified the rRNA

sample we could not totally exclude the possibility that, e.g., some contaminating precursor rRNA or mRNA hybridized with the fragments *Eco*B, BE 2 and BG 18, respectively. In order to eliminate this possibility we prepared complementary ^{32}P-labelled RNA from purified fragments BG 16 and BG 17 (equivalent to *Bgl*Q). The autoradiographs in fig.2 (panels d–f) show the results when complementary RNA of fragment BG 17 was hybridized with the BE, BG and EG fragments of total chloroplast DNA and panel g represents the autoradiograph obtained when complementary RNA of BG 16 was hybridized with the BE fragments. An essential result is that complementary RNA from the fragment BG 17 but not from fragment BG 16 hybridized with fragment BE 2. Furthermore we see that the fragments BG 18 (panel e) and EG 10 (panel f) also hybridized with complementary RNA of BG 17 proving that the fragment BG 17 from *Bam*D (or *Bam*E) contains DNA sequences complementary to sequences within fragment BG 18. Since we definitely know from base sequencing studies (Schwarz, Kössel, Graf, E. S., in preparation) that the fragment BG 17 carries the structural gene for 16 S rRNA, we can deduce that the fragment BG 18 also contains DNA sequences complementary to 16 S rRNA.

In a somewhat reciprocal experiment we hybridized nick-translated BG 18 DNA to *Eco* fragments (panel h) and to BG fragments (panel i) from total chloroplast DNA. It can be seen that the nick-translated BG 18 DNA hybridized to fragments *Eco*B and *Eco*P (panel g) and to BG 17 and BG 18 (panel i). This is in full agreement with all the results shown in fig.2.

In order to estimate the extent of sequence homologies between the fragments BG 17 and BG 18 we digested both kinds of fragments with endo R. *Hae*III, an enzyme known to cleave *Euglena gracilis* chloroplast DNA frequently [9]. The respective results are shown in fig.3. The *Hae*III fragment patterns as obtained after gel electrophoresis look almost identical for digests of fragments BG 17 (panel b) and

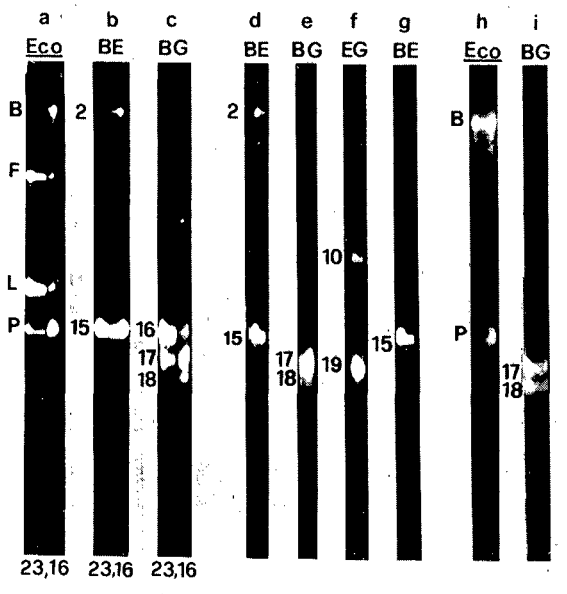

Fig.2. Autoradiographs of DNA : RNA and DNA : DNA hybridization experiments. Panels a,b,c show, respectively, the hybridization of labelled 23 S and 16 S rRNA to *Eco* fragments, BE fragments and EG fragments from total chloroplast DNA. Solutions of ^{32}P-labelled 23 S or 16 S rRNA (4.5×10^5 cpm. μg RNA^{-1}, 10 μg rRNA/ml) with a 5-fold concentration of cold competitor rRNA were used to separately incubate the halved DNA filter strips. Electrophoresis conditions were: 1% agarose, 18 mA, 20°C, 18 h; buffer, 0.04 M Tris, 0.02 M Na-acetate (pH 7.8). The filters were autoradiographed at room temperature for three days (Typon-X-ray film). Panels d,e,f show, respectively, the hybridization of labelled complementary RNA of BG 17 with BE fragments, BG fragments and EG fragments from total chloroplast DNA. Panel g shows the hybridization of labelled complementary RNA of BG 16 fragment with the BE fragments from total chloroplast DNA. Purified fragment BG 17 (*Bgl*Q) (~5 μg) was used as template to prepare complementary ^{32}P-labelled RNA according to [14]. Incubation volume for labelling was 80 μl in buffer Hepes–KOH (pH 7.9), 20 mM, Mg-acetate 10 mM, spermine 200 μM, KCl 200 mM, ATP, GTP, CTP, UTP, 0.5 mM each, [α-^{32}P]ATP, 10 μCi (10 Ci/mmol), dithiothreitol 5 mM, 5 units *E. coli* RNA polymerase, 37°C, 20 min. The reaction was stopped by adding formamide (final conc. 50%) and NaCl/Cit (final conc. 5 ×). Panels h,i show, respectively, the hybridization of nick-translated fragment BG 18 with *Eco* and BG fragments from total chloroplast DNA. Fragment BG 18 (~10 ng) was nick-translated [12] in buffer Tris–HCl, 50 mM (pH 7.8), MgCl 5 mM, 2-mercaptoethanol 10 mM, bovine serum albumin 50 μg/ml, dCTP, dTTP, dGTP, 10 μM each, d[α-^{32}P]ATP, 10 μCi (200 Ci/mmol), 5 units DNA polymerase, 20°C, 1 h. After passing through Sephadex G-75 the DNA containing fraction was treated with ethanol. The precipitated DNA was resuspended in Tris–HCl 10 mM, EDTA 1 mM (pH 7.8), heat denatured at 97°C, 10 min, chilled in ice, and the solution adjusted to 50% formamide and 5× NaCl/Cit. The hybridization conditions were as given in section 2.

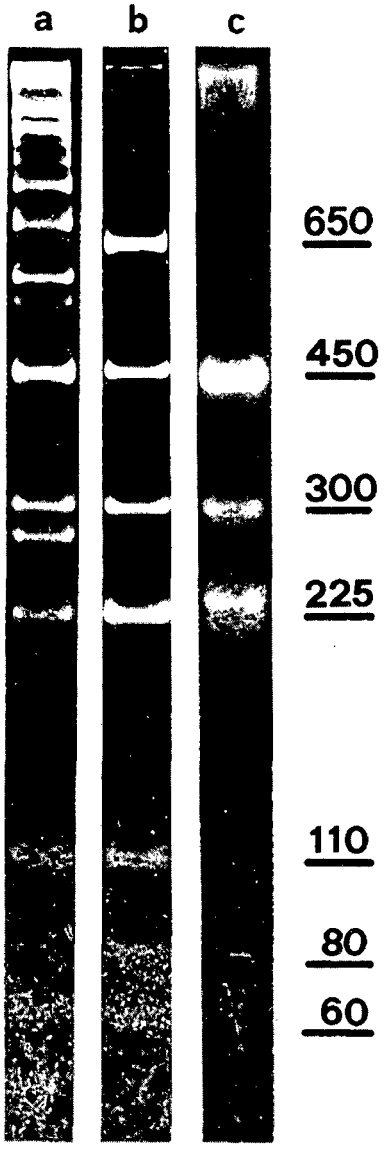

Fig.3. *Hae*III fragment patterns of total chloroplast DNA and the DNA fragments BG 17 and BG 18. Panels a and b show, respectively, an ultraviolet photograph of ethidium bromide stained gels with *Hae*III fragments of total chloroplast DNA and *Hae*III digestion products of fragment BG 17. Panel c shows the autoradiograph of *Hae*III fragments obtained by digestion of the fragment BG 18 labelled with ^{32}P by nick-translation. Gel electrophoresis conditions were as given in fig.2, except that the gel was 3% polyacrylamide–1% agarose. Under these conditions only DNA fragments of $M_r < 1000$ base pairs were clearly resolved. The molecular weights as indicated on the right side of panel c are expressed in number of base pairs and were determined by calibrating the gels with *Hae*III fragments from pBR322 [11].

BG 18 (panel c). In particular the fragments with sizes of 450, 300, 225, 110, 80 and 60 base pairs are present in both panels. The 650 base pair fragment seen in panel b, but not c, is a terminal piece, located adjacent to fragment BG 16 (B. J., E. S., unpublished). The corresponding terminal piece in BG 18 must be about 200 base pairs shorter, according to the fragment calibration data in [3] and most likely migrates along with the 450 base pair fragment, thereby generating an intensified band (panel c). The other terminal *Hae*III fragment in fragment BG 17 is known to be 225 base pairs long and it comigrates with the internal DNA fragment of equal size. The respective band, therefore, has a stoichiometry of ~2 (B. J., E. S., unpublished). In panel c the equivalent band is also strongly intensified suggesting that the fragment BG 18 also gives two kinds of *Hae*III fragments of 225 base pair length, i.e., the *Bgl*III cleavage site 18/7 is still part of the DNA segment homologous to the 16 S rRNA gene.

In panel a the relevant *Hae*III digestion products (< 1000 base pairs) from total chloroplast DNA are displayed. Most important is that those 6 bands which are apparent in both panel b and c are also present in panel a, but as expected, the 650 base pair fragment does not show up in panel a. This indicates that the fragment patterns b and c are not due to some artifacts introduced, e.g., through cloning or nick-translation procedures, but correlate with the *Hae*III fragment pattern of native chloroplast DNA.

In summary we can say that the *Eco*B fragment contains a DNA segment of ~1500 base pairs which is complementary to 16 S rRNA. Since the corresponding 23 S rRNA sequence is lacking we postulate that the circular *Euglena gracilis* (Z. strain) chloroplast DNA contains in addition to 3 complete rRNA gene sets a supplementary 16 S rRNA gene. It remains to be shown by base sequence analysis whether the 16 S rRNA cistron in fragment *Eco*B is fully identical to the 3 rRNA genes of the 3 repeat units.

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Physical Mapping of the Ribosomal DNA Region of *Euglena gracilis* Chloroplast DNA

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1. The relative positions of endo R · *Eco*RI and endo R · *Bgl*II cleavage sites are mapped within the linked DNA fragments *Bam*-E-E-D of the *Euglena gracilis* chloroplast DNA.

2. The DNA segment *Bam*-E-E-D contains three contiguous repeated segments of approximately 5600 base pairs.

3. Each repeated segment can code for an rRNA gene (16-S and 23-S).

Gray and Hallick [1] have recently reported that the restriction enzymes endo R · *Sal*I and endo R · *Bam*HI cleave the *Euglena gracilis* chloroplast DNA into three and six fragments, respectively. They tentatively mapped the relative positions of the nine cleavage sites on the circular DNA molecule which is known to have a molecular weight of approximately 9.2×10^7 [2]. Several laboratories have reported the use of various restriction endonucleases to study, for example, genome size and base compositional heterogeneity of *E. gracilis* chloroplast DNA [1,3–5] and to identify specific DNA fragments carrying rRNA genes [3–6]. Some *Eco*RI fragments have been spliced into plasmids and cloned in suitable bacterial strains [6].

It is accepted that *E. gracilis* chloroplast DNA contains the gene(s) for rRNA; however, the number of copies per genome remains uncertain, since the reported values vary from one [7] to three [4,8,9]. Results from very recent work suggests [1] that the rRNA gene(s) is(are) located exclusively in the linked *Bam*HI fragments D and E, F (for nomenclature see [1]). This prompted us to specifically analyze these DNA fragments by mapping the endo R · *Eco*RI and endo R · *Bgl*II cleavage sites within that DNA segment and to correlate these results with hybridization data using chloroplast rRNA and DNA.

A first account of these results was previously given elsewhere (International Conference on Regulations of Developmental Processes in Plants, Halle, July 4–9, 1977).

MATERIALS AND METHODS

Preparation of Chloroplast DNA

Euglena gracilis (Z strain, Culture Collection of Algae, Indiana University, no. 753) was grown, harvested and stored as published [4]. Chloroplasts were isolated as reported [4] and chloroplast DNA was isolated from purified chloroplasts following in essence the method of Kolodner and Tewari [10]. The average molecular weight of the DNA was in the range of $20-30 \times 10^6$.

Preparation and Iodination of Chloroplast rRNA

Chloroplast rRNA was obtained as reported [4]. In some cases the 16-S and 23-S rRNA were collected separately from sucrose gradients, and labelled with ^{125}I (The Radiochemical Center Ltd, Amersham, England) according to Orosz and Wetmur [11]. The labelled rRNA was processed as published [4]. The specific activities were between 10^5-10^6 counts min^{-1} μg RNA^{-1}. Radioactivity was measured in a Nuclear Chicago Isocap 300 liquid scintillation counter.

Restriction Endonucleases

Endo R · *Eco*RI, endo R · *Bam*HI and endo R · *Bgl*II were kind gifts from Dr T. Bickle, University of Basel.

Abbreviations. NaCl/Cit, 0.15 M NaCl and 0.015 M sodium citrate; restriction endonucleases and their fragments are abbreviated according to suggestions of Smith and Nathans [12]; *Bam*, *Bgl* and *Eco*, DNA fragments obtained from digestions with endo R · *Bam*HI, endo R · *Bgl*II, endo R · *Eco*RI, respectively; *BE*, *BG*, *EG*, DNA fragments resulting from double digestions with endo R · *Bam*HI and endo R · *Eco*RI, endo R · *Bam*HI and endo R · *Bgl*II, endo R · *Eco*RI and endo R · *Bgl*II, respectively; *BEG*, DNA fragments resulting from triple digestions with endo R · *Bam*HI, endo R · *Eco*RI and endo R · *Bgl*II.

Enzyme. Restriction endonuclease (EC 3.1.4.–).

The endo R · *Sal*I was kindly provided by Dr J. D. Rochaix, University of Geneva. As work progressed the endo R · *Eco*RI and endo R · *Bam*HI were purchased from Miles Research Products.

Restriction Enzyme Digestion of Chloroplast DNA

The enzyme nomenclature of Smith and Nathans [12] was used. The DNA samples were digested with an excess of enzyme. If not stated otherwise in the respective legends, the incubation conditions were as follows: for endo R · *Eco*RI: 0.01 M Tris-HCl, pH 7.9, 0.1 M NaCl, 0.01 M $MgCl_2$, 0.01 M 2-mercaptoethanol, 37 °C, 30 min; for endo R · *Bam*HI: 0.02 M Tris-HCl, pH 7.9, 0.02 M KCl, 0.01 M $MgCl_2$, 37 °C, 3 h; for endo R · *Bgl*II: 0.05 M Tris-HCl, pH 7.9, 0.05 M NaCl, 0.01 M $MgCl_2$, 0.01 M 2-mercaptoethanol; for endo R · *Sal*I: same conditions as for endo R · *Eco*RI. For double digestion we chose the buffer conditions given for endo R · *Eco*RI, incubation for 6 h.

Gel Electrophoresis

DNA digestion was stopped by adding 5 µl of 0.2 M EDTA, 2% Ficoll 400 and 0.05% bromophenol blue to 30 µl of DNA solution. This solution was directly used for electrophoresis in vertical gel slabs ($14 \times 18 \times 0.3$ cm). Agarose (Sigma type II) gels of various concentrations were used and electrophoresis conditions were as specified in the legends of the figures. The electrophoresis buffer was Tris-acetate, pH 7.8 (0.04 M Tris, 0.02 M sodium acetate).

Hybridization of rRNA to Chloroplast DNA Fragments

DNA fragments were transferred to Millipore HAWP 304 FO filter strips according to Southern [13]. The filter strips were incubated either in 23-S and 16-S ^{125}I-labelled rRNA or in 16-S-enriched or in 23-S-enriched ^{125}I-labelled rRNA. Several filters were placed into a single vial containing ^{125}I-labelled rRNA (5 µg rRNA/ml) in 2 × NaCl/Cit, 70 °C, 15 h.

Isolation of DNA Fragments from Gels

The DNA fragments were recovered from the gels using the freeze-squeeze method [14]. The eluted DNA samples (about 1 ml) were purified on a DEAE-cellulose column (DE32, approx. 1 ml). Loading buffer, 50 mM Tris-HCl, 10 mM EDTA (pH 7.6); washing buffer, 0.15 M NaCl, 20 mM Tris-HCl, 10 mM EDTA (pH 7.6); elution buffer, 1 M NaCl, 20 mM Tris-HCl, 10 mM EDTA (pH 7.6). The DNA-containing fraction (about 1 ml) was twice extracted with isoamylalcohol saturated with 0.01 M EDTA, then with chloroform/isoamylalcohol (24/1, v/v), dialyzed overnight at 4 °C against 0.1 NaCl/Cit and precipitated at − 20 °C in ethanol. The precipitated DNA was recovered by centrifugation at 16000 rev./min for 30 min in a Sorvall SS-34 rotor. The DNA samples were resuspended in 50 µl of 0.02 × NaCl/Cit and used for redigestion.

RESULTS

Identification of Bam, Eco *and* Bgl *Fragments Carrying rRNA Genes*

Aliquots of chloroplast DNA of average M_r approximately 20×10^6 (30×10^3 base pairs) were digested with endo R · *Bam*HI, endo R · *Eco*RI and endo R · *Bgl*II. The fragmented DNA was electrophoretically analyzed in agarose gels, stained with ethidium bromide and photographed as shown in Fig. 1. After this the DNA fragments were transferred to nitrocellulose filter strips according to Southern [13] and the imprints hybridized with ^{125}I-labelled rRNA to saturation. The filter strips were autoradiographed and are shown in Fig. 1 in parallel with the respective banding patterns.

The banding pattern obtained with endo R · *Eco*RI cleavage products (Fig. 1, strip 1a) and its autoradiograph (strip 1b) are in essence identical to those published and discussed previously [3]. The DNA in the bands *Eco*-F, L and P hybridized with rRNA. The fragment *Eco*-B also gave a faint signal. The fragment lengths for *Eco*-B, F, L and P were estimated from their relative mobility in various kinds of gels [15] to be about 19, 7.2, 3.2 and 2.4×10^3 base pairs, respectively. These values are in essence like those published previously [1, 3].

The banding pattern with the DNA products from digestion with endo R · *Bam*HI (Fig. 1, strip 2a) show the fragments C, D and E (F) (nomenclature according to [1]). The fragments *Bam*-A and B (larger than 40×10^3 base pairs) do not appear as two distinct bands in this gel since the initial DNA material was too small having an average size of only 30×10^3 base pairs. However, we see a diffuse zone above the *Bam*-C band which certainly contains randomly cut fragments in the size range of $25-30 \times 10^3$ bases which stem from the genome region equivalent to the fragments *Bam*-A and B. The small amount of contaminating nuclear DNA could not account for the observed zone in Fig. 1, strip 2a. As seen in strip 2b, only the fragments *Bam*-D and E (F) hybridized to rRNA, what is in line with reports from other laboratories (unpublished results from J. R. Y. Rawson). Obviously, our data alone would not suffice to exclude a priori that *Bam*-A and/or B could also carry rRNA genes.

Endo R · *Bgl*II cleaved the chloroplast DNA into 22 – 24 fragments (a detailed analysis of the *Bgl* fragments will be given elsewhere). From the 19 bands

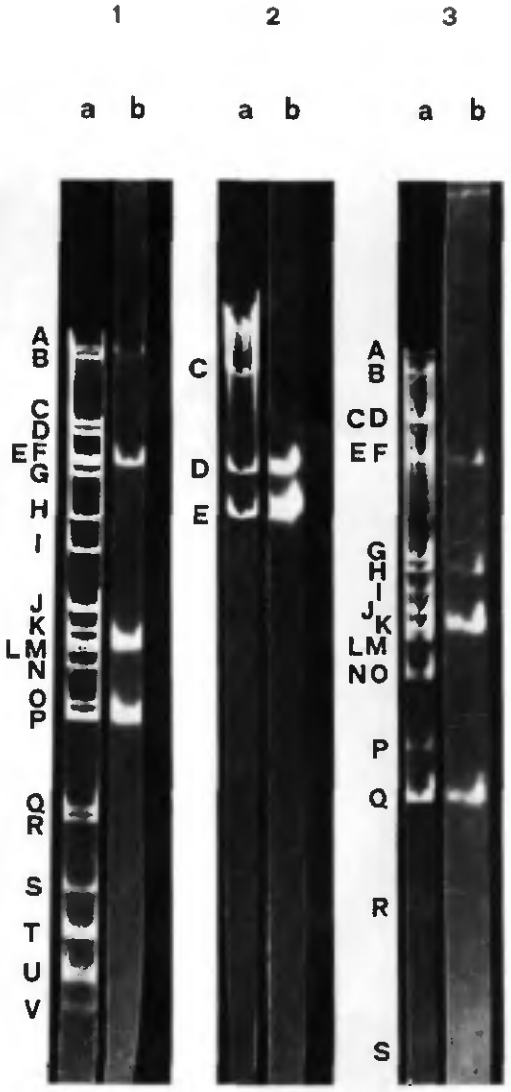

Fig. 1. *Gel electrophoresis and autoradiographs from DNA fragments obtained from digestion with endo R·EcoRI, endo R·BamHI and endo R·BglII.* Approximately 5 µg of chloroplast DNA were digested with endo R·*Eco*RI (strip 1) or endo R·*Bam*HI (strip 2) or endo R·*Bgl*II (strip 3) and the fragments separated electrophoretically in a 1% agarose gel, 20 °C, 18 mA, 15 h. The (a) strips are photographs of the respective ethidium-bromide-stained gels. According to this data and to Gray and Hallick [1], the fastest migrating *Bam* band has a stoichiometry of two. These DNA fragments were named E and F [1]. We omit the letter F, since it is shown in this paper, that the two fragments are identical repeats. The DNA fragments were transferred to nitrocellulose strips [13] and hybridized with ^{125}I-labelled rRNA. The processed filter strips were radiographed (Typon X-ray films) for 3 days. The (b) strips show the respective autoradiographs. In strip 3b the autoradiograph is composed of two lengthwise aligned filter strips. The left half stems from a filter strip hybridized to 16-S ^{125}I-labelled rRNA (10^5 counts min^{-1} µg RNA^{-1}), the right half stems from a filter strip hybridized to 23-S ^{125}I-labelled rRNA (5×10^5 counts min^{-1} µg RNA^{-1}). Strips 1b and 2b stem from filter strips hybridized to a mixture of 16-S and 23-S ^{125}I-labelled rRNA (approx. 8×10^5 counts min^{-1} µg RNA^{-1}). In order to unequivocally correlate bands in stained gels with bands in autoradiographs, where ambiguities existed (e.g. *Eco* A/B or *Eco* E/F), spacer gels were inserted before DNA transfer into filters (3) or the respective bands were resolved in gels of different agarose concentration (not shown)

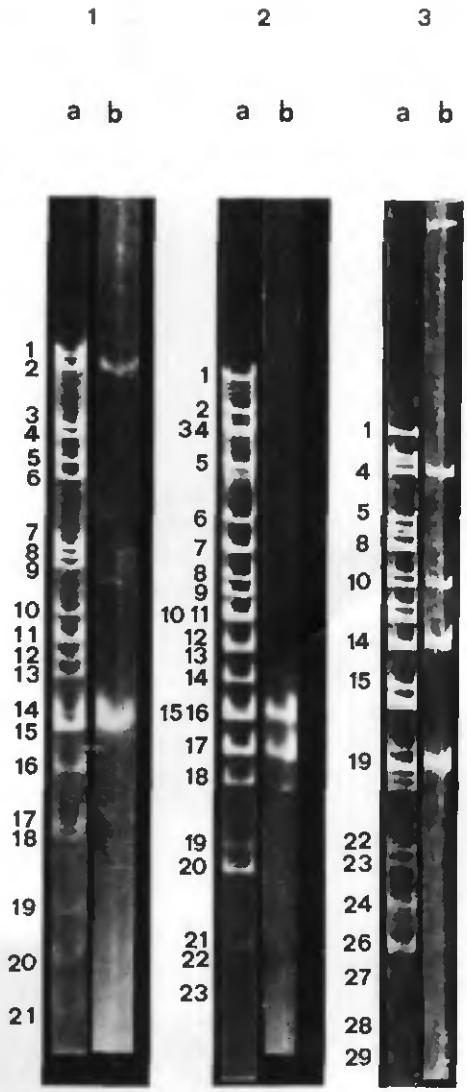

Fig. 2. *Gel electrophoresis and autoradiographs of DNA fragments obtained from double digestion with various restriction enzymes.* About 5 µg of chloroplast DNA were double digested with endo R·*Bam*HI and endo R·*Eco*RI (strip 1), endo R·*Bam*HI and endo R·*Bgl*II (strip 2) and endo R·*Eco*RI and endo R·*Bgl*II (strip 3). Electrophoresis conditions were as given in legends to Fig. 1. The (a) strips represent the photograph of the respective ethidium-bromide-stained gels, the (b) strips represent the respective autoradiographs. In all three cases the autoradiographs are composed of two aligned halves representing filters hybridized to 16-S rRNA (left) and 23-S rRNA (right) respectively (specific radioactivities as given in legends, Fig. 1)

visible in this gel (Fig. 1, strip 3a) the DNA in four bands hybridized to rRNA (*Bgl*-E, H, K, Q) as seen in strip 3b of Fig. 1. The fragments *Bgl*-K and Q gave stronger signals in the autoradiograph than *Bgl*-E and H. In this and some following experiments we had split the filter strips lengthwise and hybridized the left half (strip 3b) to 23-S rRNA and the right half to 16-S rRNA. (The 16-S rRNA had almost five times the specific radioactivity of the 23-S rRNA.) In all

four bands the autoradiographic signal (strip 3b) is stronger on the right side than on the left side. This, however, only reflects the different specific radioactivities of the two rRNA samples. No quantitative statement can be made from this result concerning a preferential position of 16-S or 23-S rRNA on any one of the four *Bgl* fragments.

The hybridizing chloroplast DNA fragments obtained by digestion with either endo R · *Eco*RI or endo R · *Bgl*II are, of course, related in some way to the fragments *Bam*-D and E (F). Double digestion of total chloroplast DNA with either endo R · *Bam*HI and endo R · *Eco*RI, endo R · *Bam*HI and endo R · *Bgl*II, or with endo R · *Eco*RI and endo R · *Bgl*II along with subsequent hybridization with rRNA allowed us to study this relationship. In Fig. 2, both the banding patterns and radioautographs are displayed for the limit double digestion experiments. Double digestion of total chloroplast DNA with endo R · *Bam*HI and endo R · *Eco*RI generated 21 bands resolvable in a 1% agarose gel (Fig. 2, strip 1a). From the autoradiograph (strip 1b) we can infer that the two hybridizing *Eco* fragments F and L have been cleaved by endo R · *Bam*HI. Most important, those fragments carrying rRNA genes comigrated in a single band with an electrophoretic mobility equal to the hybridizing fragment *Eco*-P (2.4×10^3 base pairs). The band *BE*-2, which must be a derivative of *Eco*-B having been cut by endo R · *Bam*HI, gave a faint autoradiographic signal.

The combined digestion of chloroplast DNA with endo R · *Bam*HI and endo R · *Bgl*II leads to the production of about 23 bands. The DNA in the two gel zones *BG*-15 + 16 and *BG*-17 strongly hybridized with rRNA. Also the band *BG*-18 gave a faint signal in the autoradiograph. The hybridizing fragments *Bgl*-E, H and K (see Fig. 1, strip 3a) disappeared upon digestion with endo R · *Bam*HI, while the fragment *Bgl*-Q, equivalent to *BG*-17, seemed to remain uncleaved.

Double digestion of chloroplast DNA with endo R · *Eco*RI and endo R · *Bgl*II lead to the production of 29 bands (Fig. 2, strip 3a), four of which hybridized with rRNA, namely, *EG*-4, 10, 14 and 19 (strip 3b). These fragments contain, respectively, 7.2, 4.1, 3.0, 1.9×10^3 base pairs. The *EG*-10 gave the faintest autoradiographic signal among those four bands.

Identification of Eco *and* Bgl *Fragments Stemming from the Fragments* Bam-*D and* Bam-*E (F)*

The DNA fragments *Bam*-D and E(F) were isolated in preparative amounts from agarose gels. The purified fragments were digested either with endo

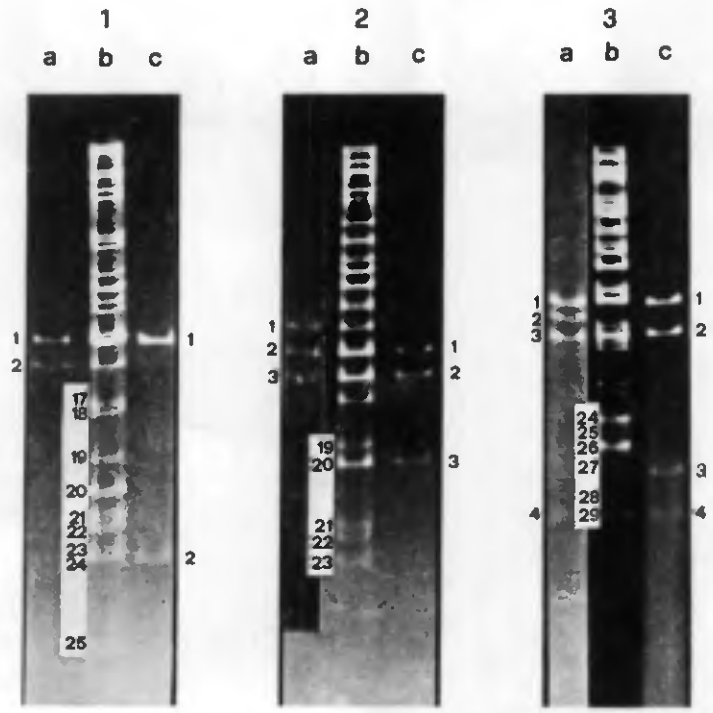

Fig. 3. *Gel electrophoresis of DNA fragments obtained from redigestion of fragments* Bam-*D and* Bam-*E and double digestions of total chloroplast DNA.* About 2 µg purified fragments *Bam*-D (a strips) or *Bam*-E (c strips) were redigested with endo R · *Eco*RI (strip 1), endo R · *Bgl*II (strip 2) or endo R · *Eco*RI plus endo R · *Bgl*II (strip 3). In order to identify the fragments each strip contains the fragment patterns from the respective double digestions: Endo R · *Bam*HI/endo · *Eco*RI (strip 1), endo R · *Bam*HI/endo R · *Bgl*II (strip 2), endo R · *Eco*RI/endo R · *Bgl*II (strip 3). Note that pattern 3b does not represent the fragments of the corresponding triple digestion. Electrophoresis: 1.5% agarose, 15–16 h, 18 mA

R · *Eco*RI, or endo R · *Bgl*II or both enzymes together. The digestion products were analyzed on gels as shown in Fig. 3. Endo R · *Eco*RI cleaved the *Bam*-E(F) fragments (strip 1c) into fragments of length 2.4×10^3 base pairs (1) and 0.8×10^3 base pairs (2). The fragments (1) and (2) in strip 1c correspond in sizes to the fragments *BE*-15 and *BE*-24, respectively. Only the top fragment (1) hybridized to rRNA (compare with Fig. 2, strip 1).

Redigestion of *Bam*-E(F) with endo R · *Bgl*II (Fig. 3, strip 2c) yielded three fragments of length 2.3×10^3 (fragment 1), 2.0×10^3 (fragment 2) and 1.3×10^3 base pairs (fragment 3); they correspond in size to the fragments *BG*-16, *BG*-17 and *BG*-20, respectively. Only the fragments (1) and (2) hybridized with rRNA (see Fig. 2, strip 2).

Redigestion of *Bam*-E(F) with both endo R · *Eco*RI and endo R · *Bgl*II yielded four bands in the gel (Fig. 3, strip 3c). The fragments (1), (3) and (4) in strip 3c correspond in size to the fragments *BG*-16 (2.3×10^3 base pairs), *BE*-24 (0.8×10^3 base pairs) and *EG*-29 (0.5×10^3 base pairs). Fragment (2) is slightly shorter than the fragment *BG*-17 and about 1.9×10^3 base pairs, as we verified by careful analysis of additional gels (not shown). Only the DNA in bands (1) and (2) hybridized to rRNA (autoradiograph not shown).

The size of the fragments *Bam*-E/F was estimated to be 5.9×10^3 base pairs [1]. The total length of *Bam*-E+F would therefore be in the range of 12×10^3 base pairs. The result from redigestion of the band *Bam*-E(F) indicates that the fragment *Bam*-E must be identical to *Bam*-F, both in size and base sequence. The total length is 5.6×10^3 base pairs according to the sum of the lengths of the fragments obtained $(2 \times 2.4 + 0.8) \times 10^3$ base pairs or $(2.3 + 2.0 + 1.3) \times 10^3$ base pairs or $(2.3 + 1.9 + 0.8 + 0.5) \times 10^3$ base pairs. A stoichiometry of two for the fragment (1) in Fig. 3 (strip 1c) was estimated from the staining intensity. According to the physical map published by Gray and Hallick [1] the two linked fragments E, F form a linkage group with *Bam*-D. According to our results this linkage group would now read E-E-D rather than E-(F)-D.

The *Bam*-D fragment was split by endo R · *Eco*RI (Fig. 3, strip 1a) into fragments of 2.4×10^3 base pairs

Table 1. Euglena gracilis *chloroplast DNA cleavage products from digestion with various restriction endonucleases*
The size of the fragments is given as the number of base pairs ($\times 10^{-3}$). Numbers in parentheses represent the stoichiometry of DNA fragments if different from unity

*Eco*RI		*Bgl*II		*Bam*HI-*Eco*RI			*Bam*HI-*Bgl*II			*Eco*RI-*Bgl*II	
fragment	size	fragment	size	fragment		size	fragment		size	fragment	size
Eco-A	24	*Bgl*-A	22	*Eco*-A	*BE*-1	24	*Bgl*-A	*BG*-1	22	*EG*-1	9.5
B[a,b]	19	B[a,b]	16		2	17		2	14	2	7.7
C	9.5	C	10	C	3	9.5	C	3	10	3	7.5
D	8.6	D	8.7	D	4	8.6	D	4	9.7	4	7.2
E[b]	7.3	E[a]	7.5	E	5	7.3	F	5	7.2	5	5.5
F[a]	7.2	F	7.2	G	6	6.9		6	5.4	6	5.2
G[b]	6.9	G[a]	4.7 (2)		7	4.9	G	7[c]	4.7 (2)	7	5.2
H[a]	5.4	H[a]	4.5	I	8	4.7	I	8	4.2	8[c]	4.6 (2)
I	4.7	I[b]	4.2		9	4.4	J	9[c]	3.9 (2)	9	4.15
J[a,c]	3.6 (2)	J[b,c]	3.9 (2)	J	10	3.6	L	10	3.5	10[c]	4.1 (2)
K	3.3	K[a]	3.6 (2)	K	11[c]	3.3 (2)	M	11[c]	3.4 (2)	11	3.55
L[a]	3.2 (2)	L	3.5	M	12	3.0	N	12[c]	3.0 (2)	12	3.5
M	3.0	M	3.4	N	13	2.9	O	13	2.9	13	3.25
N	2.9	N	3.0	O	14	2.5		14	2.7	14[c]	3.0 (3)
O	2.5	O	2.9	P	15[c]	2.4 (6)	P	15	2.35	15[c]	2.55(2)
P	2.4 (3)	P	2.35		16	2.1		16	2.3 (3)	16[c]	2.4 (2)
Q	1.7	Q	2.0 (3)	Q	17	1.7	Q	17	2.0 (3)	17	2.35
R	1.6	R	1.4	R	18	1.65		18	1.8	18	1.95
S	1.3	S	0.9	S	19	1.3 (2)	R	19	1.4	19	1.9 (3)
T[c]	1.1 (2)	T	0.85	T	20[c]	1.1 (2)		20	1.3 (2)	20	1.75
U[c]	0.9 (2)				21	0.95	S	21	0.9	21	1.7
V	0.82			U	22[c]	0.90 (2)	T	22	0.85	22	1.4
W	0.5			V	23	0.82		23	0.75	23	1.2
					24	0.8 (2)				24[c]	1.0 (2)
				W	25	0.5				25	0.9
										26[c]	0.8 (3)
										27	0.7
										28	0.65
										29	0.5 (3)

[a] Cleaved by endo R · *Bam*HI [b] Cleaved by endo R · *Sal*I [c] Bands containing a mixture of different fragments which comigrate

(fragment 1) and 2.1×10^3 base pairs (fragment 2). The fragments (1) and (2) correspond in size to the fragments *BE*-15 and *BE*-16, respectively. Only the fragment (1) hybridized to rRNA (autoradiograph not shown). Redigestion of fragment *Bam*-D with endo R · *Bgl*II (Fig. 3, strip 2a) yielded fragments (1), (2), and (3) equal in size to *BG*-14 (2.7×10^3 base pairs), *BG*-16 (2.3×10^3 base pairs) and *BG*-17 (2.0×10^3 base pairs). From these three bands only *BG*-16 and *BG*-17 hybridized to rRNA (see Fig. 2, strip 2b). It is to be noted that the faintly hybridizing fragment *BG*-18 did not show up either as part of fragment *Bam*-D or of *Bam*-E (see Discussion).

Redigestion of fragment *Bam*-D with both endo R · *Eco*RI and endo R · *Bgl*II yielded four bands as seen in Fig. 3, strip 3a. The smallest fragment is hardly visible. Three bands (1), (3) and (4) in strip 3a are equal in size to the three bands (1), (2) and (4) obtained by redigestion of *Bam*-E (strip 3c). Fragment (2) in strip 3a corresponds in size to the fragment *BE*-16 (2.1×10^3 base pairs).

Fragment *Bam*-D was reported to contain 7.3×10^3 base pairs [1]. According to our results this fragment contains 6.8 to 7.0×10^3 base pairs, i.e. $(2.7 + 2.3 + 2.0) \times 10^3$ or $(2 \times 2.4 + 2.1) \times 10^3$ or $(2.3 + 2.1 + 1.9 + 0.5) \times 10^3$ base pairs. A stoichiometry of two was estimated for fragment (1) shown in strip 1a (Fig. 3). Certainly, these results suggest base sequence similarities between fragments *Bam*-D and *Bam*-E.

In Table 1 all bands shown in Fig. 1–3 are listed and the respective molecular weights, stoichiometries and equivalences are given. The molecular weights were determined relative to the *Eco*RI fragments of λ phage DNA as published [4]. The stoichiometries of the fragments were estimated from the scanning profiles (not shown). The previous published nomenclature [3] for the *Eco* fragments was adapted to the results of this study and agrees in essence with that of Gray and Hallick [1]. In order to relate these results to the *Sal-Bam* cleavage map of Gray and Hallick [1] we marked in the Table 1 those *Eco* and *Bgl* fragments which carry a cleavage site for endo R · *Sal*I and endo R · *Bam*HI. The last column of Table 1 lists 29 bands obtained from double digestion of chloroplast DNA with endo R · *Eco*RI and endo R · *Bgl*II. The two enzymes together recognize about 54 sites, meaning that several of the 29 bands contain multiple fragments stemming from repeated sequences and/or fragments incidentally comigrating in the gel under the given experimental conditions. It was not possible to indicate equivalences between *EG* fragments and *Eco* or *Bgl* fragments. Hybridizing *EG* fragments, of course, relate (as discussed) to the hybridizing *Eco* and *Bgl* fragments.

Eco-Bgl *Cleavage Map of the Ribosomal DNA Region*

The results given in Fig. 1–3 and summarized in Table 1 allow one to construct a definite *Eco* and *Bgl* cleavage map of the linked *Bam* fragments E-E-D (Fig. 4). Ordering of the various *Eco* fragments is best started by placing the hybridizing fragment *Eco*-F,

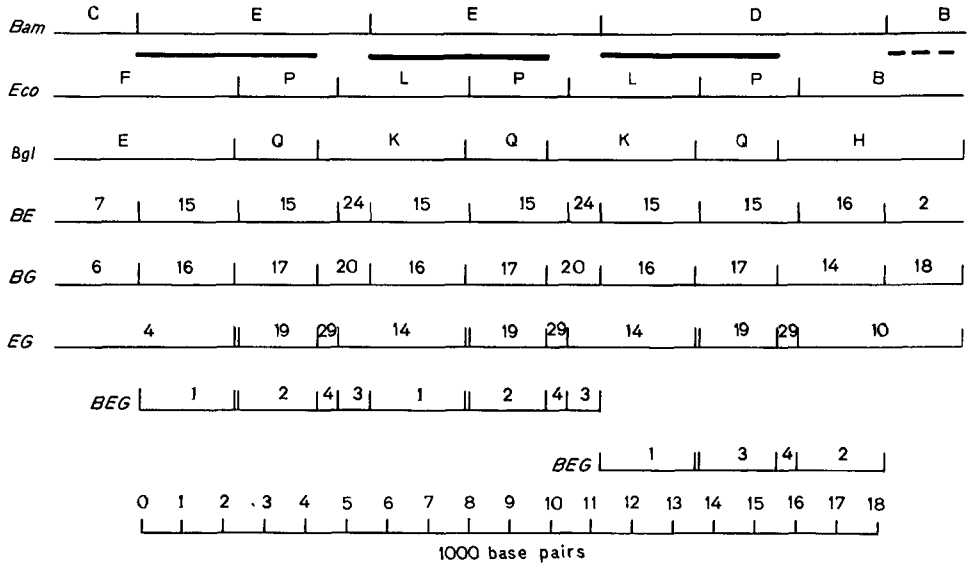

Fig. 4. *Endo R* · Eco*RI*/*endo R* · Bgl*II cleavage map of the linked fragments* Bam-*E-E-D*. The letters and numbers refer to the cleavage fragments or bands as given in Table 1 and in the text. Cleavage sites for the various restriction enzymes are marked with a vertical line. The top horizontal line (*Bam*) shows part of the revised *Bam* cleavage map according to Gray and Hallick [20]. The *BEG* fragments shown in Fig. 3 are separately numbered for the two repeated *Bam*-E and the *Bam*-D fragments. The unnumbered small *BEG* fragment (about 100 base pairs) was not detectable in Fig. 3. The fragments *BE*-7 and *BG*-6 are part of *Eco*-F and *Bgl*-E, respectively, as will be shown elsewhere. Below the top line (*Bam*) we indicate with heavy bars those three DNA stretches which can carry rRNA genes in *Bam*-E-E-D. With a dotted line we mark a region that hybridizes to the rRNA preparation external to the *Bam*-E-E-D segment

from which endo R · *Bam*HI splits a hybridizing fragment (*BE*-15) of length 2.4×10^3 base pairs. Fragment *Eco*-F could overlap either into *Bam*-D or *Bam*-E. However, it is obvious that *Eco*-F (7.2×10^3 base pairs) can only extend into the *Bam*-E fragment since the terminal piece in *Bam*-D, and therefore the fragment distal to *Bam*-E, must be the fragment *BG*-16 containing 2.1×10^3 base pairs. If not, there would exist an *Eco* fragment with an endo R · *Bam* cleavage site of either 4.5×10^3 base pairs ($2.1 + 2.4 \times 10^3$ base pairs) or 2.9×10^3 base pairs ($2.1 + 0.8 \times 10^3$ base pairs). Such an *Eco* fragment was not detected, either in this or in previous experiments [1, 3].

The fragment *Eco*-P (also equal to *BE*-15) is not cleaved by endo R · *Bam*HI. Therefore, it must be placed next to the terminal piece of 2.4×10^3 base pairs (*BE*-15) in fragment *Bam*-E and the piece of 2.1×10^3 base pairs (*BE*-16) in *Bam*-D. Considering the length of the repeat unit of 5.6×10^3 base pairs (*Bam*-E) a gap of about 3.2×10^3 base pairs remains which can only be filled by the hybridizing and repeated fragment *Eco*-L. This fragment has an endo R · *Bam*HI site and yields pieces of 0.8×10^3 base pairs (*BE*-24) and 2.4×10^3 base pairs (*BE*-15). All *Eco*RI sites are thereby placed within the linkage group *Bam*-E-E-D. A repetitive pattern is recognizable.

The endo R · *Bgl* cleavage sites within the same DNA sequence can be mapped by considering the following results. As shown in Fig. 1 and 2 and indicated in Table 1, the hybridizing fragment *Bgl*-E (7.5×10^3 base pairs) is slightly shortened by endo R · *Eco*RI to yield the hybridizing fragment *EG*-4 (7.2×10^3 base pairs), which is almost identical in size to *Eco*-F. Therefore, the fragment *Bgl*-E can only be placed on the same side as *Eco*-F and therefore overlaps into the *Bam*-E fragment. The overlapping piece from *Bgl*-E can only be the hybridizing fragment *BG*-16 (2.3×10^3 base pairs) since the only other hybridizing piece is the internal fragment *Bgl*-Q (equal to *BG*-17), having no endo R · *Bam*HI cleavage site. Since the two *Bam*-E fragments are contiguous the linking piece between the two fragments has to match the size of the repeat unit of 5.6×10^3 base pairs. Only the fragment *Bgl*-K (3.6×10^3 base pairs) fulfills this requirement. This fragment is split by endo R · *Bam*HI into the two pieces, *BG*-20 and *BG*-16. Fragment *Bgl*-K extends into the *Bam*-D fragment, similar to *Eco*-L. A third fragment, *Bgl*-Q (*BG*-17), makes up the third repeat unit of 5.6×10^3 base pairs. This leaves the non-hybridizing piece *BG*-14 (equal to *BEG*-2, 2.7×10^3 base pairs) as the terminal piece in the fragment *Bam*-D. Limit digestion of *Bam*-D and *Bam*-E with the combined enzymes endo R · *Eco*RI and endo R · *Bgl*II leads to the fragment *BEG*-4 of length about 0.5×10^3 base pairs. A fragment of this length is anticipated according to the cleavage map given in Fig. 4. We recognize from these results that the *Euglena gracilis* chloroplast DNA segment *Bam*-E-E-D contains three repeat units of 5.6×10^3 base pairs length.

In Fig. 4 we have marked those fragments within the linkage group *Bam*-E-E-D which hybridized to rRNA. The length of the fragments *BG*-16 plus *BG*-17, or twice *BE*-15, is 4.3×10^3 and 4.8×10^3 base pairs, respectively. About 4.5×10^3 base pairs, or 85% of the repeat unit, are required for the 16-S + 23-S rRNA cistrons [16]. This leaves the major part of the fragments *BE*-24 or *BG*-20 for precursor and spacer sequences.

In Fig. 4 we have marked with a dotted heavy bar an additional DNA stretch which, according to our results, hybridized with the rRNA preparation. This observation will be discussed below.

DISCUSSION

A few years ago [8] we reported that *Euglena* chloroplast DNA might carry two to three rRNA genes per genome. We also showed [17] that these genes were located in the (dG + dC)-rich segments of the chromosome and we suggested that values lower than three genes per genome might be due to selective loss of ribosomal DNA stretches during purification of chloroplast DNA in CsCl density gradients. The results of this report strongly suggest that there are indeed three rRNA genes [4] per circular DNA, mapping in a cluster in the segment *Bam*-E-E-D. Within each repeated unit a total stretch of about 4.3×10^3 base pairs could carry an rRNA cistron. This length seems marginal and about 200 base pairs too short to code for chloroplast 16-S plus 23-S rRNA [16]. One should, however, consider that the exact position of the 16-S and 23-S rRNA cistrons is not yet determined. Short hybridizing sequences in fragment *BE*-24, contiguous to the cleavage site *BE*-24/15, and in fragment *BG*-20, contiguous to the cleavage site *BG*-17/20, may have escaped autoradiographic detection.

The *Euglena* chloroplast DNA differs entirely from higher plant chloroplast DNA, where only two rRNA cistrons exist per circular chromosome [18]. In maize chloroplast DNA, the two genes are situated in two inverted repeats about 18.5×10^3 base pairs apart (shorter distance) [19]. Also spinach chloroplast DNA seems to carry two rRNA genes in two inverted DNA segments (R. G. Herrmann, unpublised work).

In a previous report [3] we have shown that the DNA fragment *Eco*-B faintly but consistently hybridized with total chloroplast ^{125}I-labelled rRNA (16-S + 23-S). We postulated that *Eco*-B might carry rRNA gene sequences. Other laboratories made similar observations [5, 6]. According to unpublished results from this and other laboratories (see acknowledgements) the fragment *Eco*-B does overlap into the *Bam*-D fragment (see Fig. 4) and is contiguous to the

repeat unit of 5.6×10^3 base pairs. One might argue therefore, that the faint autoradiographic signal of the *Eco*-B fragment obtained in our experiment and others [5] could be due to some terminal sequences of the rRNA gene or precursor RNA which might contaminate our RNA preparations; the piece *BE*-16 would be the hybridizing part of *Eco*-B. If so, one would, however, expect that the fragment *BE*-16 should also be visible in the autoradiograph, in our experiments, this was never the case. As a working hypothesis we postulate that the faint hybridization observed in experiments displayed in Fig. 1 and 2 is due to a DNA stretch within *Eco*-B, outside of the linkage group *Bam*-D-E-E. The following experimental data favor this hypothesis. (a) The fragment *Eco*-B, when cut by endo R · *Bam*HI, yields the fragments *BE*-2 (about 17×10^3 base pairs) which still hybridizes faintly to rRNA meaning that removal of the *BE*-16 piece did not cut off the hybridizing segment. (b) Among the *Bgl* fragments the fragment *Bgl*-H faintly, but consistently, hybridized. It is cleaved by endo R · *Bam*HI. Amongst the *BG* products we find the fragments *BG*-18 and *BG*-14 (Fig. 2 and Table 1). The fragment *BG*-14 was recognized as being part of *Bam*-D (Fig. 3). The faintly hybridizing fragment *BG*-18 (1.8×10^3 base pairs), which is not part of *Bam*-D nor of *Bam*-E, adds up together with the fragment *BG*-14 (2.7×10^3 base pairs) to the size of *Bgl*-H (4.5×10^3 base pairs). We postulate, therefore, that fragment *Bgl*-H is composed of the pieces *BG*-18 plus *BG*-14 and is next to *Bgl*-Q in fragment *Bam*-D. If so, we should find a hybridizing *EG* fragment of about 4×10^3 base pairs; this is indeed the case. The multiple band *EG*-10, containing fragments of length about 4.1×10^3 base pairs, gives a faint signal in the autoradiograph as seen in Fig. 2, strip 3b.

As a consequence, we have tentatively marked in Fig. 4 a DNA stretch, external to the repeat unit of 5.6×10^3 base pairs, which hybridized with the rRNA sample. We cannot exclude that the rRNA preparation contained other species of RNA (e.g. mRNA) and that this was the reason for the observed hybridization. However, we cannot exclude either that a fragmentary rRNA cistron is found outside of the repeat units of 5.6×10^3 base pairs.

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