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

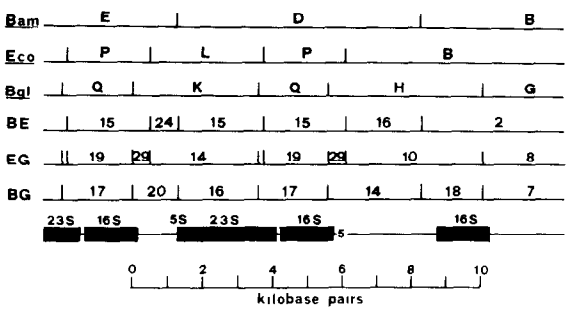

Fig 1 Segment of the rDNA region of the chloroplast genome The 7 horizontal lines symbolize a segment of the double stranded chloroplast DNA The vertical lines indicate the position of the cleavage sites of restriction enzymes The abbreviations *Bam, Eco* and *Bgl* stand for endo R *bam*HI, endo R *Eco*RI, 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, Kossel, 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

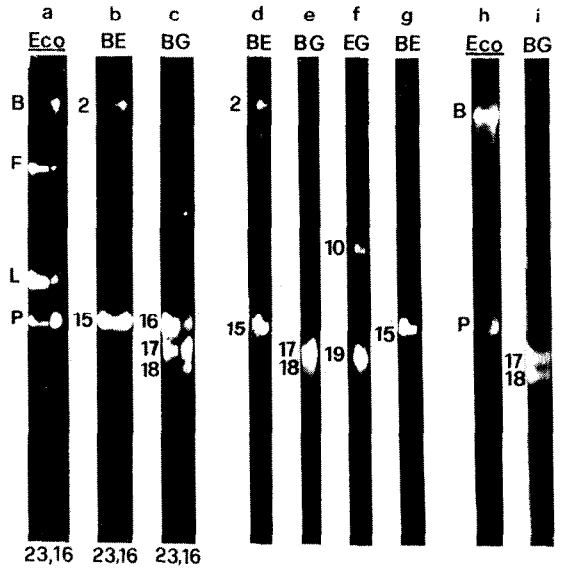

Fig.2. Autoradiographs of DNA : RNA and DNA : DNA hybridization experiments. Panels a,b,c show, respectively, the hybridization of labelled 23 S and 16 S rRNA to *Eco* fragments, BE fragments and EG fragments from total chloroplast DNA. Solutions of ^{32}P-labelled 23 S or 16 S rRNA (4.5×10^5 cpm. μg 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.

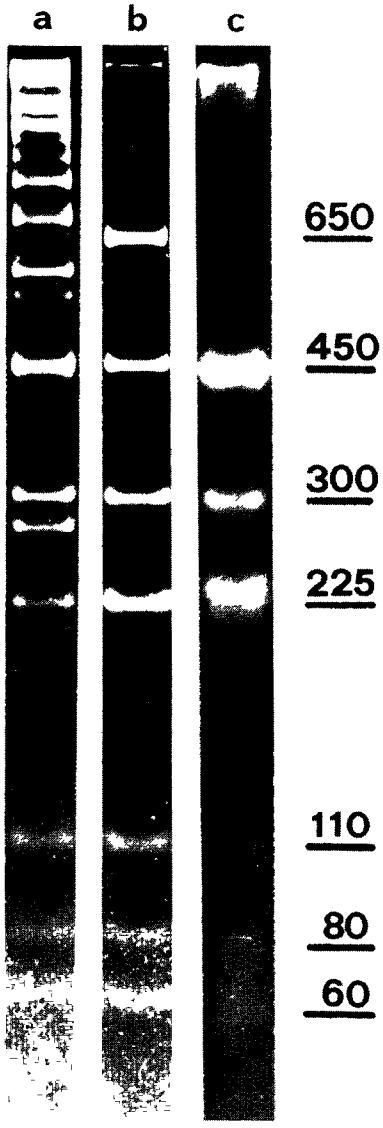

Fig 3 *Hae*III fragment patterns of total chloroplast DNA and the DNA fragments BG 17 and BG 18 Panels a and b show, respectively, an ultraviolet photograph of ethidium bromide stained gels with *Hae*III fragments of total chloroplast DNA and *Hae*III digestion products of fragment BG 17 Panel c shows 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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