THE MULTIPLE COPIES OF THE *EUGLENA GRACILIS* CHLOROPLAST GENOME ARE NOT UNIFORM IN SIZE

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1. Introduction

The unicellular euglenophyte *Euglena gracilis* contains several chloroplasts each with multiple copies of a 44 μm circular DNA molecule [1]. From renaturation kinetics data [2] and analyses using numerous restriction enzymes [3,4] it was deduced that the genetic complexity of the chloroplast genome was equivalent to the size of a DNA molecule (~135–140 $\times$ 10^3 basepairs). Therefore, the multiple copies, by and large, should be identical both in size and base sequence. This, however, does not exclude a priori the existence of minor structural differences. In fact, one would anticipate such differences to exist due to mutational events occurring with time, in particular, since it is known that all DNA molecules can independently undergo replication [1].

Minute divergences in base sequences between molecules (e.g., point mutations) will be difficult to detect contrary to more extended size variations (deletions or insertions). In the following we describe observations which show *Euglena gracilis* chloroplast DNA molecules to contain within the fragment *Eco*RI-B a region which is not uniform in length. The size variation extends over several hundred base pairs and is probably due to a variable number of clustered DNA units (e.g., repeats).

2. Material and methods

2.1. *Preparation of chloroplast DNA*

DNA was isolated from purified chloroplasts of *Euglena gracilis* (Z-strain, culture collection of Algae, Indiana University, no. 753) as in [3].

2.2. *Preparation of* Bgl*II-Z*

Chloroplast DNA (~50 μg) was digested to completion with endoR.*Bgl*II [3] and the fragments were separated electrophoretically in a 0.9% low melting agarose gel (Bio-Rad), at 20 mA, 4°C, 20 h, with 0.04 M Tris, 0.02 M Na-acetate (pH 7.8) as electrophoresis buffer. The broad band was cut from the ethidium bromide stained gel, the agarose pieces were diluted with 3 vol. DEAE-loading buffer [3], melted at 70°C and the sample was chromatographed through a 1 ml DEAE-Sephacel (Pharmacia) column at 45°C. The eluted DNA was recovered by ethanol precipitation.

2.3. *Labelling of DNA fragments*

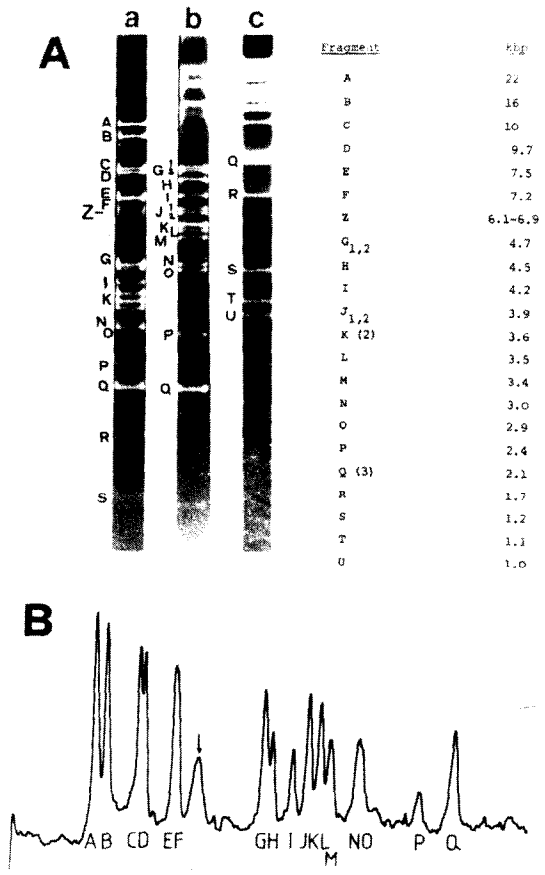
DNA fragments were labelled by nick-translation [5] using d-[α-^{32}P]ATP. Complementary labelled RNA was obtained by in vitro transcription of DNA templates using [α-^{32}P]ATP [6].

2.4. *Enzymes and radioisotopes*

EndoR.*Bgl*II was provided by L. Graf. Other enzymes were purchased from Boehringer Biochem., Mannheim. Radioisotopes were from Radiochemical Center, Amersham.

3. Results and discussion

A first indication that *Euglena gracilis* chloroplast DNA might not be uniform in size stems from endoR.*Bgl*II restriction analysis. In fig.1A, we show the ethidium bromide stained gels of electrophoretically separated *Bgl*II fragments. A total of 23 discrete bands can be resolved in the 3 different kinds of gels (a,b,c). In addition 1 broad band Z with av. M_r =



Fragment	kbp
A	22
B	16
C	10
D	9.7
E	7.5
F	7.2
Z	6.1–6.9
$G_{1,2}$	4.7
H	4.5
I	4.2
$J_{1,2}$	3.9
K (2)	3.6
L	3.5
M	3.4
N	3.0
O	2.9
P	2.4
Q (3)	2.1
R	1.7
S	1.2
T	1.1
U	1.0

Fig.1. Gel electrophoresis of *Bgl*II fragments. (A) ~1 μg chloroplast DNA was hydrolysed with endoR.*Bgl*II and electrophoresed [3]: (a) 0.75% agarose, 18 mA, 15 h; (b) 2% agarose, 18 mA, 40 h; (c) 2.5% acrylamide + 1% agarose, 40 mA, 20 h. The nomenclature of fragments is as in [3] except for *Bgl*II-U and *Bgl*II-Z which were not described before. The size of the fragments (recalibrated with *Hae*III fragments of pBR322) are given in the table in kilobase pairs (kbp). Stoichiometries of fragments are indicated if different from unity. (B) Densitometer scanning of a film negative of pattern A,a. The bands G and J can be resolved into 2 bands, while bands K and Q have stoichiometries of 2 and 3, respectively [3].

6.5×10^3 basepairs is seen in gel fig.1Aa. The densitometer scan (fig.1B) of gel Aa also reveals the unusual broad shape of *Bgl*II-Z (arrow). By integration of peak areas and computation of the approximate stoichiometries of each band (considering the M_r of the respective fragments), we obtain for the broad *Bgl*II-Z band a value of 0.92, while, e.g., *Bgl*II-Q has a stoichiometry of 2.9 (*Bgl*II-Q is known to be repeated 3 times/DNA circle [3]). A possible interpretation of this observation is that the *Bgl*II-Z band contains a population of DNA fragments having a combined stoichiometry of one and a size heterogeneity between $6.1–6.9 \times 10^3$ basepairs. If so, the sum of M_r values of all *Bgl*II fragments should match, e.g., the sum of all *Eco*RI fragments which is $\sim 139 \times 10^3$ basepairs [7]. In fact, the sum of all discrete *Bgl*II fragments shown in fig.1 reaches this size only if we take the av. M_r of *Bgl*II-Z into account (see inserted table). Therefore, it seems reasonable to consider *Bgl*II-Z as integral component of the circular chloroplast genome.

Assuming this to be correct, *Bgl*II-Z should show the usual properties of restriction fragments; it should be susceptible to cleavage by other restriction enzymes and should have its equivalences in DNA fragment patterns obtained with other restriction enzymes. We already know that *Bgl*II-Z is not hydrolysed by endoR.*Eco*RI, endoR.*Bam*HI and endoR.*Sal*I [3,7]. On the other hand, we see in fig.2d that *Bgl*II-Z is cleaved by endoR.*Hae*III, generating a new *Hae*III–*Bgl*II broad band of $\sim 5.4 \times 10^3$ basepairs. In order to

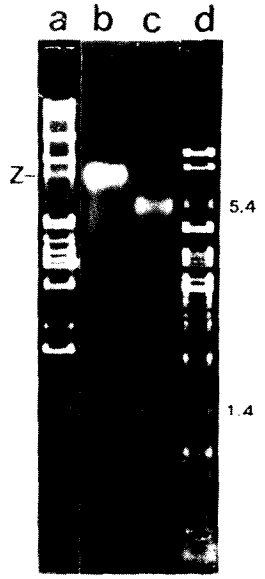


Fig.2. Hydrolysis of *Bgl*II-Z with endoR.*Hae*III: (a) *Bgl*II fragments of total chloroplast DNA; (b) *Bgl*II-Z, isolated from low melting agarose gels, nick-translated; (c) aliquot from (b) digested with endoR.*Hae*III; (d) *Hae*III–*Bgl*II fragments of total chloroplast DNA. Samples (a–d) were electrophoresed together in 1% agarose, 20 mA, 15 h; (a,d) are UV photographs of the ethidium bromide-stained gels, while (b,c) are autoradiographs. Numbers indicate the fragment size in kilobase pairs.

prove that this observation was not due to an analytical artefact, *Bgl*II-Z was eluted from gels, nick-translated and rerun on a gel before and after hydrolysis with endoR.*Hae*III. The corresponding autoradiographs for undigested (fig.2b) and endoR.*Hae*III digested *Bgl*II-Z (fig.2c) are compared with the fragment patterns from total chloroplast DNA digested with endoR.*Bgl*II (fig.2a) and endoR.*Bgl*II–*Hae*III (fig.2d). It is evident that undigested nick-translated *Bgl*II-Z has the same mobility as the *Bgl*II-Z band in the *Bgl*II pattern and digestion with endoR.*Hae*III yields in a faster moving broad band (av. 5.4×10^3 basepairs) identical to the one seen in pattern d. A second band of $\sim 1.4 \times 10^3$ basepairs shows up in panel c which corresponds in size to a sharp band in the stained gel pattern d. The combined size of the two DNA fragments ($5.4 + 1.4 \times 10^3$ basepairs) matches in size the original *Bgl*II-Z band and therefore we may conclude that all *Bgl*II-Z fragments have one *Hae*III site in common which is located $\sim 1.4 \times 10^3$ basepairs proximal to one of the terminal *Bgl*II sites.

In order to check the existence of equivalences between *Bgl*II-Z and DNA fragments obtained with other restriction enzymes, we constructed ^{32}P-labelled RNA complementary to isolated *Bgl*II-Z and hybridized it to Southern blots [8] from endoR.*Eco*RI, endoR.*Eco*Ri–*Bam*HI, endoR.*Hae*III, endoR.*Hin*dIII, endoR.*Bgl*II–*Hae*III and endoR.*Bgl*II–*Hin*dIII digests.

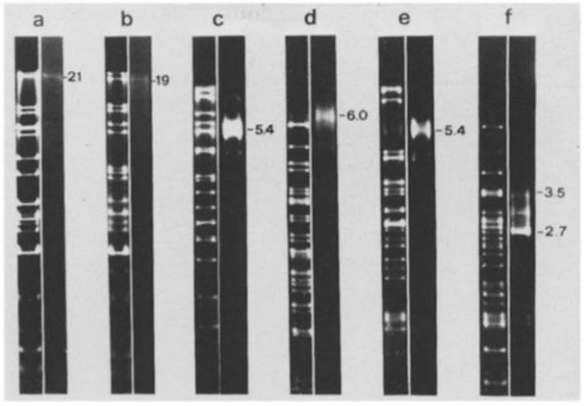


Fig.3. Hybridization of ^{32}P-labelled RNA complementary to *Bgl*II-Z with different restriction fragment patterns. Ethidium bromide-stained gels (left) are aligned with the corresponding autoradiographs (right) of the filter imprints [8] which were hybridized with labelled RNA complementary to *Bgl*II-Z. (a) *Eco*RI; (b) *Eco*RI–*Bam*HI; (c) *Hae*III; (d) *Hin*dIII; (e) *Bgl*II–*Hae*III; (f) *Bgl*II–*Hin*dIII. Electrophoresis conditions: (a,b) 1% agarose, 20 mA, 18 h; (c–f) 1.8% agarose, 20 mA, 38 h. Numbers indicate the fragment size in kilobase pairs.

In fig.3 the ethidium bromide-stained gels are aligned with the corresponding autoradiographs (panels a–f). In case of *Eco*RI and *Eco*RI–*Bam*HI the second largest fragment hybridizes to *Bgl*II-Z. In all other cases a rather broad zone interacts, in the size region of 5.4×10^3 basepairs (*Hae*III), 6.0×10^3 basepairs (*Hin*dIII), 5.4×10^3 basepairs (*Bgl*II–*Hae*III) and 3.0×10^3 basepairs (*Bgl*II–*Hin*dIII). In case of *Hin*dIII (d) and *Bgl*II–*Hae*III (e) a broad band is clearly apparent in the stained gels while in case of *Hae*III (c) the diffuse zone is less apparent and masked by two strong bands. In case of *Bgl*II–*Hin*dIII (f) the hybridizing zone is not diffuse but resolved into several distinct bands, as seen in the autoradiograph. In the stained gel the fine striations are not seen, because the concentration for detectability was not reached.

These hybridization data can be interpreted as follows: *Bgl*II-Z is located in *Eco*Ri-B (21×10^3 basepairs) and more precisely in its subfragment *Eco*RI–*Bam*HI-2 (19×10^3 basepairs), since endoR.*Bam*HI cleaves one terminal piece of $\sim 2 \times 10^3$ basepairs

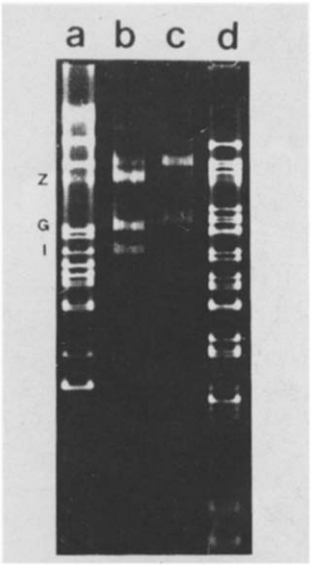


Fig.4. Hydrolysis of fragments *Eco*RI–*Bam*HI-1 (*Eco*RI-A) and *Eco*(RI–*Bam*HI-2 with endoR.*Bgl*II. The fragments *Eco*RI–*Bam*HI-1 and *Eco*RI–*Bam*HI-2 were each cut out from low melting agarose gels. The DNA was digested while still in the agarose with endoR.*Bgl*II [9], layered on a 1.2% agarose gel and electrophoresed at 20 mA, 19 h: (a) *Bgl*II fragments from total chloroplast DNA (marker); (b) endoR.*Bgl*II digest of *Eco*RI–*Bam*HI-2; (c) endoR.*Bgl*II digest of *Eco*RI–*Bam*HI-1; (d) *Bgl*II–*Eco*RI chloroplast DNA fragments (marker). In patterns (b,c) an experimental artefact led unfortunately to a broadening of the bands.

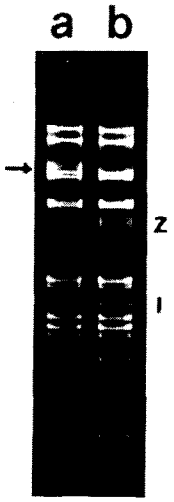


Fig.5. Partial and complete digestion of chloroplast DNA with endoR.*Bgl*II: 1 μg chloroplast DNA was digested with 1 unit endoR.*Bgl*II for 20 min (a) and 1 h (b) and electrophoresed on a 0.9% agarose gel, 20 mA, 16 h.

from *Eco*RI-B [3]. In both cases (fig.3a,b) a size variation of the fragments is not yet apparent. However, in all other cases (fig.3c–f) the size variation is apparent either as a broad diffuse band, if the hybridizing zone is in the region of $5–7 \times 10^3$ basepairs, or as a cluster of faint bands if the zone is in the region of 3×10^3 basepairs. These results suggest that the size variation of *Bgl*II-Z is due to differences in the number of short discrete units of DNA, which could be, e.g., repeats of ~30–50 basepairs, as estimated from fig.3f.

In order to obtain further evidence for placing *Bgl*II-Z within *Eco*RI-B, we analysed the endoR.*Bgl*II digestion products from *Bam*HI–*Eco*RI-2 fragment which is a large subfragment of *Eco*RI-B (fig.4b). We included in this experiment an endoR.*Bgl*II digestion of purified *Bam*HI–*Eco*RI-1 (equivalent to *Eco*RI-A) as control to exclude the possibility that *Bgl*II-Z was part of *Eco*RI-A (fig.4c). In pattern b, and contrary to pattern c, we definitely see a band migrating like *Bgl*II-Z. Furthermore, we see that also the fragments *Bgl*II-G and *Bgl*II-I appear in pattern b, as expected from previous work. As a matter of fact, we know [7] that *Eco*RI-B is split by endoR.*Bgl*II, and that *Bgl*II-G (4.7×10^3 basepairs) and *Bgl*II-I (4.2×10^3 basepairs) are positioned within *Eco*RI-B while *Bgl*II-H (4.5×10^3 basepairs) and *Bgl*II-J (3.9×10^3 basepairs) are terminal and overlapping fragments (see fig.6). Taking the length of *Eco*RI-B as ~21×10^3 basepairs

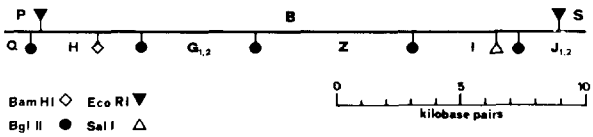


Fig.6. Relative position of *Bgl*II-Z in *Eco*RI-B. *Eco*RI-B has been mapped on the circular genome and is known to be found between *Eco*RI-P and *Eco*RI-S as indicated [10]. *Bgl*II-Z is placed in the gap between *Bgl*II-G and *Bgl*II-I, which were mapped in [7]. According to fig.1 A(b), *Bgl*II-G and *Bgl*II-J can be resolved into $G_{1,2}$ and $J_{1,2}$, respectively. We have not yet determined which of the G and J fragments (1,2) are positioned in *Eco*RI-B. The endoR.*Sal*I and endoR.*Bam*HI sites are positioned as published [4].

[4] there remains a gap of ~$6–7 \times 10^3$ basepairs, which could be the site of *Bgl*II-Z.

We have tested this possibility by studying the appearance of *Bgl*II-Z as function of digestion time. In fig.5a we show a fragment pattern from partial digestion where *Bgl*II-Z is not yet visible, instead a broad band of ~10.5×10^3 basepairs (see arrow) is apparent. This band totally disappears after complete digestion (pattern b) and the fragments *Bgl*II-Z and *Bgl*II-I become apparent. Since the combined size of the two fragments (*Bgl*II-Z + *Bgl*II-I is equal to the 10.5×10^3 basepairs of the broad band in pattern a, we may argue that the two fragments were originally linked as shown in fig.6.

In conclusion, we postulate that the multiple copies of the chloroplast genome of *Euglena gracilis*, Z-strain, are not uniform in size. They contain a variable number of short DNA units (e.g., repeats) which are clustered in fragment *Eco*RI-B between its subfragments *Bgl*II-G and *Bgl*II-I. We estimate the size fluctuation between the extreme values to be ~800 basepairs or <0.6% of the average genome length. Origin and level (inter or intra chloroplasts or cells) of the observed heterogeneity is not yet known.

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