ELECTRON MICROSCOPIC ANALYSIS OF THE EXTRA 16 S rRNA GENE AND ITS NEIGHBOURHOOD IN CHLOROPLAST DNA FROM *EUGLENA GRACILIS* STRAIN Z

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Received 11 January 1982

1. Introduction

The ribosomal RNA genes in the chloroplast DNA (cpDNA) of *Euglena gracilis* strain Z are organised in 3 tandem repeats [1–3]. The genes for the 16 S rRNA, $tRNA^{Ile}$, $tRNA^{Ala}$, 23 S rRNA, and 5 S rRNA are arranged in the same order as in the *Escherichia coli rrnD* operon [4–7]. Apart from the normal rRNA operons an extra gene for the 16 S rRNA was found in the cpDNA of *Euglena gracilis* strain Z using filter hybridizations [8].

We have used electron microscopy to determine the size and the coding strand of the extra 16 S rRNA gene and to look for possible 16 S–23 S spacer sequences near the 3′-end of this gene.

For this analysis we prepared heteroduplexes between *Euglena gracilis* strain Z cpDNA and DNA restriction fragments containing different heterologous rRNA operons: (a) with the *E. coli rrnD* operon; (b) with the *E. coli rrnB* operon; and (c) with the *Sal*I fragment 3b of *Vicia faba* chloroplasts. It could be demonstrated that the extra 16 S rRNA gene has the same size and the same orientation as the other 16 S rRNA genes. No sequence was found adjacent to the 3′-end of this gene which had a homology with the $tRNA^{Ile}$-, the $tRNA^{Ala}$-, or the $tRNA^{Glu}$-genes from the *E. coli* 16 S–23 S spacers or with the *Vicia* chloroplast spacer. Two small inverted repeats (125 base-pairs) were detected and localised relative to the positions of the rRNA genes.

2. Materials and methods

2.1. *Preparation of supercoiled DNA from* Euglena *chloroplasts*

Euglena gracilis strain Z was grown in a vitamin B12-deficient medium [9]. The cells of a 5-day-old culture (200 ml) were pelleted at 2000 rev./min in a Beckman J6 centrifuge, suspended in 100 ml medium A (300 mM sorbitol, 50 mM Tris–HCl (pH 8.0), 3 mM EDTA) and centrifuged again. The pellet (~8 g) was resuspended in 20–30 ml medium A, 50 mg solid trypsin (Worthington) were added, and the mixture was incubated in an ice bath for 45–60 min. The cells were spun down and resuspended twice with 100 ml medium A, and then broken with a homogenizer, made of 10 razor blades fixed onto a vertical rotating axis. The cpDNA was isolated as described for *Vicia faba* cpDNA [10].

2.2. *Preparation of self-annealed DNA*

Of the fraction from the CsCl gradient containing the supercoiled DNA, 20 μl were passed through a Sephadex G-100 column (45 × 2 mm), which was equilibrated with the spreading mixture (30% formamide, 0.1 M Tris–HCl (pH 7.5), 1 mM EDTA). The DNA was denatured by boiling for 2 min, incubated for 15 min at 37°C to allow self-annealing, and then spread with cytochrome *c* [11].

2.3. *Preparation of heteroduplexes*

Isolation of DNA fragments from pBK8, λ $rif^{d}18$, *Vicia faba* chloroplasts was done by electroelution from agarose gels [12]. The preparation of heteroduplexes, the electron microscopy and the length measurements were done as in [10,13].

3. Results and discussion

Intact supercoiled *Euglena gracilis* strain Z cpDNA was prepared. The supercoils were nicked and denatured by heating, then self-annealed and spread with cytochrome to visualize possible inverted repeats. Fig.1 shows an intact single-stranded circle with 2 small

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

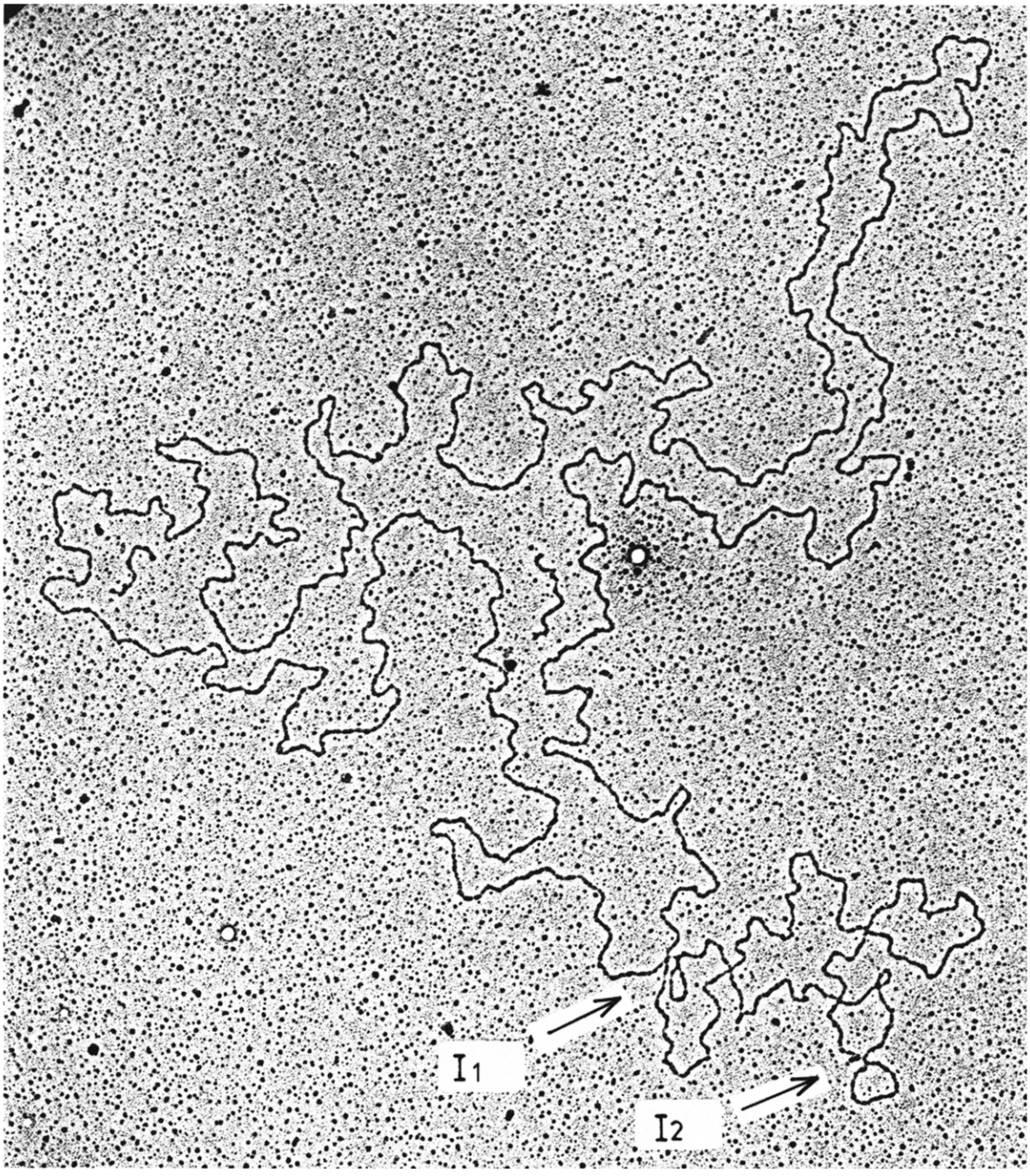

Fig.1. Cytochrome spreading of a single-stranded, self-annealed circular molecule of *Euglena* cpDNA. The arrows point to the inverted repeat I1, forming a smaller loop, and I2, forming a larger loop.

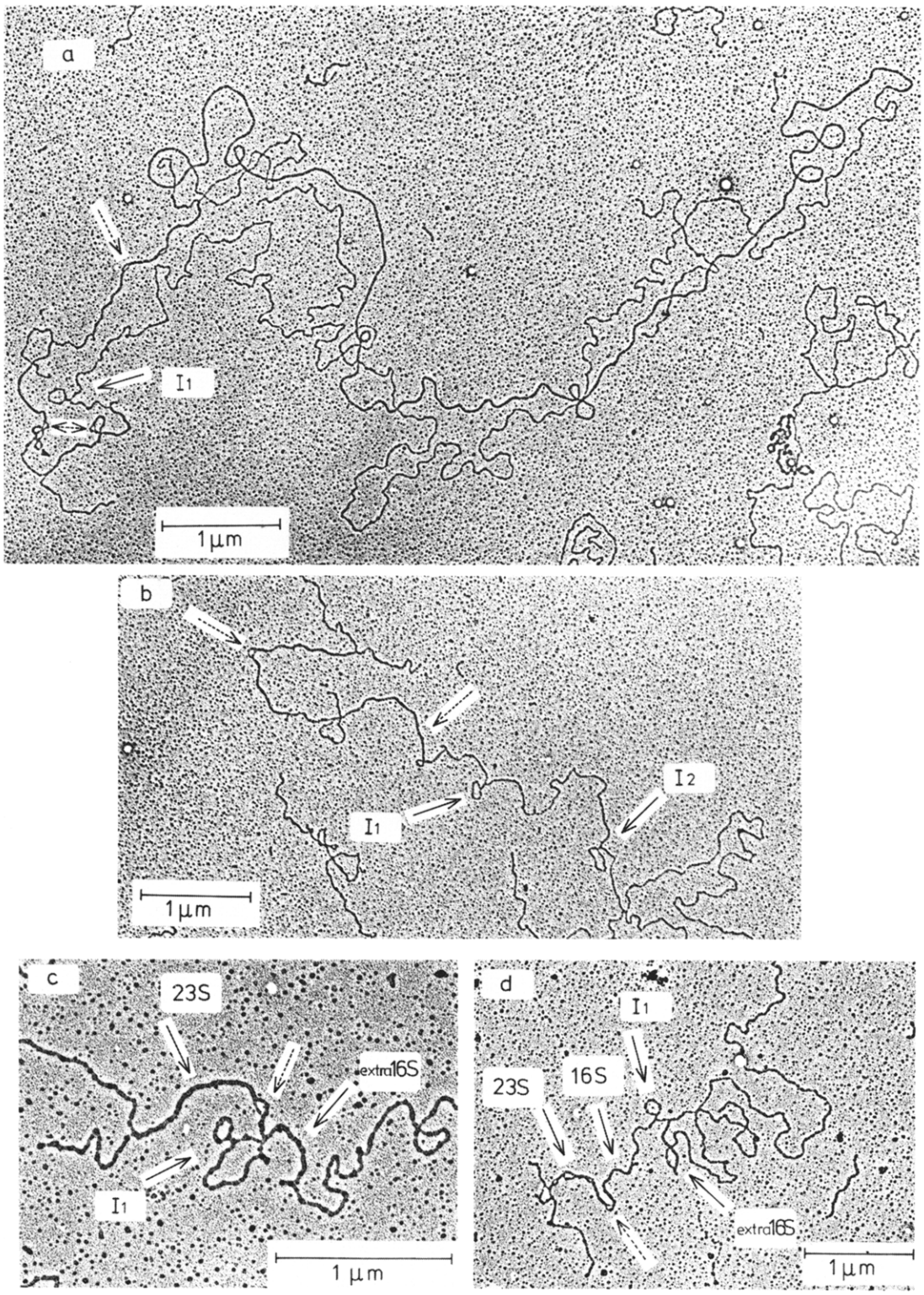
a
I1
1 µm
b
I2
I1
1 µm
c
23S
extra16S
I1
1 µm
d
I1
23S
16S
extra16S
1 µm

Fig.2.

inverted repeats labeled I1 and I2. The stem formed by I1 has a size of ~125 basepairs. I2 is varying in length between a simple crossing and a stem of ~125 basepairs. In cytochrome preparations spread from 30% formamide it is displayed by a minority of the circles. In preparations spread from 60% formamide this loop is absent contrary to I1 which remains stable.

To analyse the structure of the extra 16 S rRNA gene [8] and to determine the position of these inverted repeats on the cpDNA circle heteroduplexes were prepared between *Euglena* cpDNA and restriction fragments containing heterologous rRNA genes. The plasmid pBK8 [14] which contains the *E. coli rrnD* operon with the $tRNA^{Ile}$- and $tRNA^{Ala}$-genes in the 16 S–23 S spacer [7,14], was cut with restriction endonuclease *Bam*HI and denatured. To increase the yield of heteroduplexes with the cpDNA without excessive formation of pBK8 homoduplexes the complementary strands of the fragment were isolated by agarose gel electrophoresis [12]. The slower migrating strand was reannealed with circular *Euglena* cpDNA. The position of the rRNA genes on this fragment were described in [13]. The 5′-end of the 16 S rRNA gene is situated ~300 basepairs from the end of the fragment. Fig.2a shows 3 pBK8 fragments annealed to one *Euglena* circle. The 16 S–23 S spacer in the pBK8 fragment is ~200 basepairs longer than the *Euglena* spacer, but it includes the same tRNA genes [7,6,15]. Therefore a small asymmetrical loop is formed in that region. The inverted repeat I1 is visible upstream of all 3 operons. Another molecule of the same heteroduplex preparation (fig.2b), with 2 pBK8 fragments annealed to the cpDNA, shows that I2 is located upstream of I1, and, therefore, upstream of all 3 rRNA operons with respect to the direction of rRNA transcription. A pBK8 fragment which annealed to the extra 16 S rRNA gene first and then to the 16 S–23 S spacer and the 23 S rRNA gene of the first complete operon is shown in fig.2c. A large single-stranded loop is formed, which includes the 16 S rRNA gene of the first complete operon and the inverted repeat I1. No molecules were found, in which the pBK8 spacer annealed close to the extra 16 S gene. It always annealed to the homologous spacer sequences within the complete operon.

The same heteroduplex preparation was spread from 60% formamide instead of the normally used 30% to analyse the degree of base mismatching. In the example shown in fig.2d one pBK8 fragment has annealed to the extra 16 S rRNA gene alone, while a second fragment has annealed to the first complete operon. Under these spreading conditions the 23 S rRNA heteroduplex is unstable and forms a series of loops, but the normal as well as the extra 16 S rRNA heteroduplexes remain stable.

Heteroduplexes of *Euglena* cpDNA with the λ $rif^{d}18$ *Bam*HI fragment B which includes the *E. coli rrnB* operon with the $tRNA^{Glu}$ gene in the 16 S–23 S spacer [16,17] were prepared. The positions of the rRNA genes on this fragment were described in [13]. The heteroduplex shown in fig.3a is formed with the first complete rRNA operon. Heteroduplexes with the second, and third operon were distinguished by their distance from the inverted repeat I1 (not shown). The asymmetrical loop in the 16 S–23 S spacer region is larger and accordingly, the length of the duplex in the region of the 16 S rRNA genes is shorter, as compared with the pBK8 heteroduplexes (see fig.4). In fig.3b a λ rif fragment has annealed to the extra 16 S rRNA gene and to the 23 S rRNA gene of the first complete operon but not to the 16 S–23 S spacer. The heteroduplex structure is similar to the one shown in fig.2c, but in this case the spacer region

Fig.2. Cytochrome spreadings of heteroduplexes between *Euglena* cpDNA and the fragment of the plasmid pBK8, which contains the *E. coli rrnD* operon [14]. (a) A cpDNA circle to which 3 pBK8 fragments have reannealed. Part of the circle is double-stranded since it has reannealed with a homologous cpDNA fragment. I1 is visible upstream of all 3 rRNA operons with respect to the direction of rRNA transcription. (b) The same preparation with 2 pBK8 fragments annealed to the cpDNA, showing I2 upstream of I1. (c) A heteroduplex from the same preparation showing a pBK8 fragment which annealed first to the *Euglena* extra 16 S rRNA gene, and then to the spacer and the 23 S rRNA gene of the first complete operon. A single-stranded loop is formed, which includes the inverted repeat I1 and the 16 S rRNA gene of the first operon. (d) The same heteroduplex preparation was spread from 60% formamide instead of the normally used 30%. One pBK8 fragment has annealed to the first complete operon showing loops within the 23 S rRNA heteroduplex, which reveal regions of partial non-homology, but none in the 16 S rRNA gene. A second fragment has annealed to the extra 16 S rRNA gene alone, forming a duplex which is as intact as the 16 S rRNA duplex within the complete operon. I1 is located between the two heteroduplexes. The dashed arrows indicate the 16 S–23 S spacer.

remained completely open, showing no homology, either to the spacer of the complete operon, or to the sequence downstream of the extra 16 S rRNA gene.

Fig.2c shows a heteroduplex of *Euglena* cpDNA with *Vicia* cpDNA *Sal*I fragment 3b, which carries the chloroplast rRNA genes with a long 16 S–23 S spacer of 2300 basepairs [10]. This spacer forms 3 loops with the *Euglena* spacer. Fig.3d shows a *Vicia* fragment which annealed to the extra 16 S rRNA gene and to the first complete operon. The long single-stranded loop connecting the two double-stranded regions, represents *Euglena* cpDNA, while the short

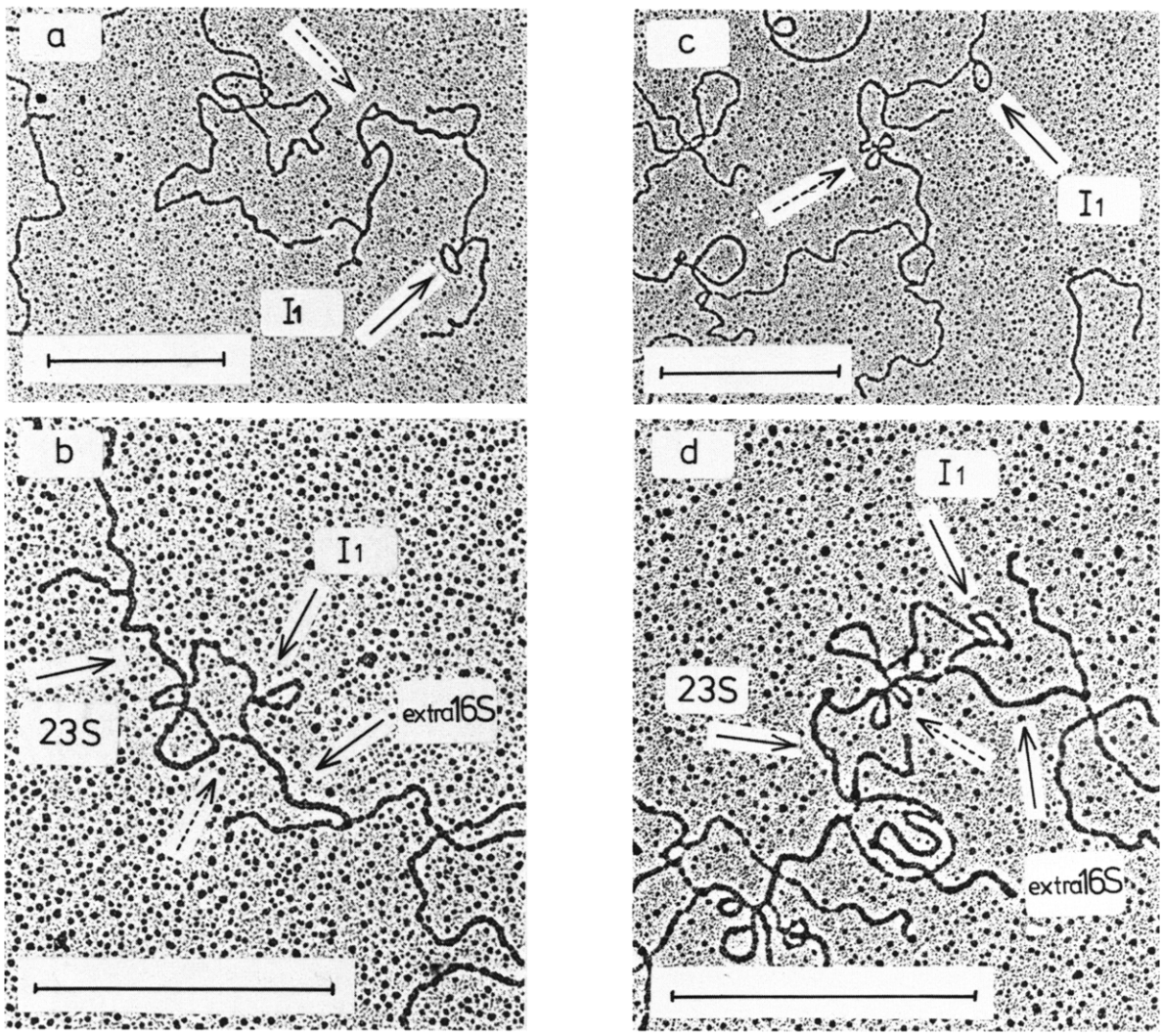

Fig.3. Cytochrome spreadings of heteroduplexes between *Euglena* cpDNA and the λ rif^{d}18 *Bam*HI B fragment, which contains the *E. coli rrnB* operon [16], and the *Vicia faba* cpDNA fragment *Sal*I 3b, which contains the chloroplast rRNA genes [10]. (a) One λ rif fragment has annealed to the first complete rRNA operon, as can be deduced from its distance to I1. The dashed arrow points to the non-homologous 16 S–23 S spacer. (b) The same preparation showing a λ rif fragment which annealed to the extra 16 S rRNA gene and to the 23 S rRNA gene of the first complete operon. The 16 S–23 S spacer of the λ rif fragment did not anneal either to the complete operon, or to the region downstream of the extra 16 S rRNA gene (– – →). (c) A heteroduplex between the *Vicia* fragment and the first rRNA operon. The long *Vicia* spacer (2300 basepairs [13]) formed 3 loops with the *Euglena* spacer (– – →). (d) A heteroduplex from the same preparation with the extra 16 S rRNA gene, corresponding to fig.2c and 3b. The smallest of the 3 loops next to the 16 S rRNA duplex in fig.3c is extended and connects the duplex of the extra 16 S rRNA gene region with the duplexes formed with the spacer (– – →) and the 23 S rRNA gene of the first complete operon.

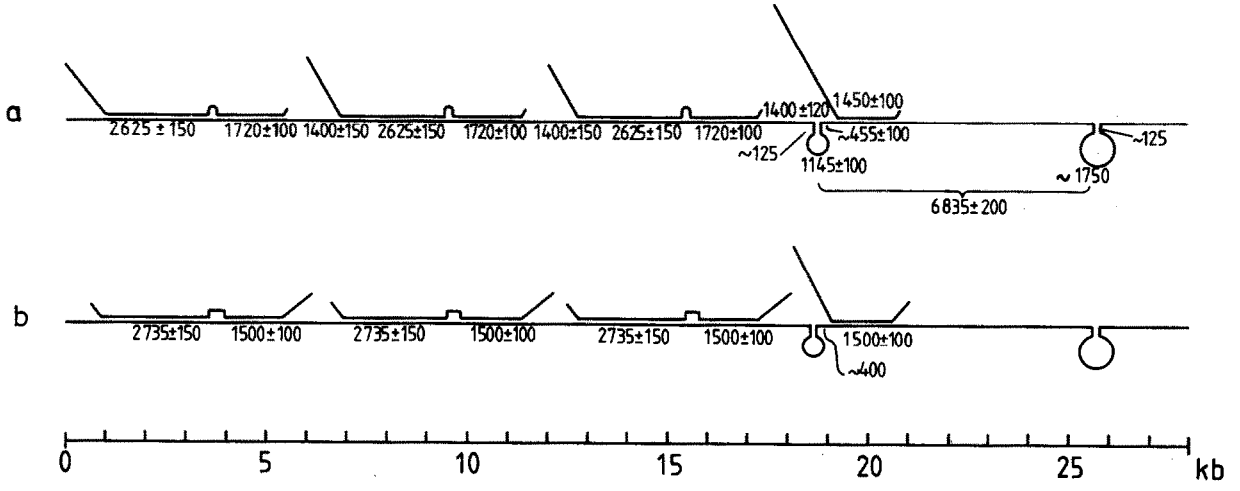

Fig.4. Length measurements on the heteroduplexes between *Euglena* cpDNA and (a) the pBK8 fragment, (b) the λ rif fragment (as in fig.2,3). Double-stranded T7 (39 400 basepairs [20]) DNA and single-stranded M13 DNA (6400 basepairs [21]) was used as internal length standard. All values are given in basepairs.

single-stranded connection is formed by one of the 3 small *Vicia* cpDNA loops, seen in the spacer region in fig.2c. The other 2 loops are part of the heteroduplex within the 16 S–23 S spacer of the first complete operon. Again the *Vicia* spacer did not anneal near the extra 16 S rRNA gene, but always to the complete operon.

The absence of any larger inverted structures in the self-annealed circular molecules (fig.1) as well as the heteroduplexes combining the 23 S rRNA gene of a complete operon with the extra 16 S rRNA gene, demonstrate that the latter has the same orientation as the other 3 operons.

Length measurements were done on the pBK8 and the λ rif heteroduplexes to determine the size and the exact position of the extra 16 S rRNA gene. A schematic representation of these measurements is given in fig.4. The length of the duplex formed with the extra 16 S rRNA sequence in the pBK8 as well as in the λ rif heteroduplexes is 1450–1500 basepairs which is identical to the size of the *E. coli* 16 S rRNA [18] and to the length of the duplexes between the 16 S rRNA genes of the complete operons and the λ rif gene (1500 basepairs). In both types of heteroduplexes the distance from the 3′-end of the extra 16 S rRNA gene to the inverted repeat I1 is identical. Measurements of the distance between the 2 inverted repeats place I2 at the position where a region of variable size (Z-region) was found [19]. It remains to be seen whether there is any correlation between these structural features. In summary we conclude that the extra 16 S rRNA gene in *Euglena gracilis* strain Z has the same orientation, the same length, and probably the same sequence as the 16 S rRNA genes in the complete operons. No sequence homology to the $tRNA^{Ile}$–$tRNA^{Ala}$-spacer nor to the $tRNA^{Glu}$-spacer is present downstream of the 3′-end of this gene.

Acknowledgements

We thank Jill Clarke for technical assistance, and E. Crouse for helpful discussions.

References

[1] Jenni, B. and Stutz, E. (1978) Eur. J. Biochem. 88, 127–134.
[2] Rawson, J. R. Y., Kushner, S. R., Vapnek, D., Kirby, N., Boerma, A. and Boerma, C. L. (1978) Gene 3, 191–209.
[3] Gray, P. W. and Hallick, R. B. (1978) Biochemistry 17, 284–289.
[4] Orozco, E. M., Rushlow, K. E., Dodd, J. R. and Hallick, R. B. (1980a) J. Biol. Chem. 255, 10997–11003.
[5] Gray, P. W. and Hallick, R. B. (1979) Biochemistry 18, 1820–1825.
[6] Graf, L., Koessel, H. and Stutz, E. (1980) Nature 286, 908–910.
[7] Young, R. A., Macklis, R. and Steitz, J. A. (1979) J. Biol. Chem. 254, 3264–3271.
[8] Jenni, B. and Stutz, E. (1979) FEBS Lett. 102, 95–99.

[9] Ortiz, W., Reardon, E. M. and Price, C. A. (1980) Plant Physiol. 66, 291–294.
[10] Koller, B. and Delius, H. (1980) Mol. Gen. Genet. 178, 261–269.
[11] Davis, R. W., Simon, M. and Davidson, N. (1971) Methods Enzymol. 21, 413–428.
[12] Koller, B., Delius, H., Buenemann, H. and Mueller, W. (1978) Gene 4, 227–239.
[13] Delius, H. and Koller, B. (1980) J. Mol. Biol. 142, 247–261.
[14] Boros, I., Kiss, A. and Venetianer, P. (1979) Nucleic Acids Res. 6, 1817–1830.
[15] Orozco, E. M., Gray, P. W. and Hallick, R. B. (1980b) J. Biol. Chem. 255, 10991–10996.
[16] Lund, E., Dahlberg, J. E., Lindahl, L., Jaskunas, S. R., Dennis, P. P. and Nomura, M. (1976) Cell 7, 165–177.
[17] Lindahl, L., Yanamoto, M., Nomura, M., Kirschbaum, J. B., Allet, B. and Rochaix, J. D. (1977) J. Mol. Biol. 109, 23–47.
[18] Brosius, J., Palmer, M. L., Kennedy, P. J. and Noller, H. F. (1978) Proc. Natl. Acad. Sci. USA 75, 4801–4805.
[19] Jenni, B., Fasnacht, M. and Stutz, E. (1981) FEBS Lett. 125, 175–179.
[20] Stueber, D. and Bujard, H. (1977) Mol. Gen. Genet. 154, 299–303.
[21] Van Wezenbeek, P. M. G. F., Hulsebos, T. J. M. and Schoenmakers, J. G. G. (1980) Gene 11, 129–148.