Extrachromosomal circular nuclear rDNA in *Euglena gracilis*

Patrick Ravel-Chapuis, Paul Nicolas, Victor Nigon, Odile Neyret^{1*} and Georges Freyssinet¹⁺

UA CNRS 92, Département de Biologie Générale et Appliquée, Université Lyon I, 69622 Villeurbanne Cedex, and ¹Groupe de Recherche en Biochimie-Biologie Cellulaire et Applications, Université de Limoges, 87060 Limoges Cedex, France

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ABSTRACT

The presence of extrachromosomal nuclear ribosomal DNA (rDNA) in the unicellular alga *Euglena gracilis* has been established. This rDNA is circular. Each circle is 3.8 μ m long and contains one rDNA unit. Oligomers are rare. Extrachromosomal rDNA is present in large amounts during the exponential phase of growth and appears less abundant during the stationary phase. It was found in all wild-type and mutant strains of *Euglena* examined. Our estimations suggest that rDNA in *Euglena* is mainly extrachromosomal. Research of extrachromosomal rDNA in spinach and *Petunia* was negative.

INTRODUCTION

In Eukaryotes, nuclear ribosomal RNA genes are generally clustered on chromosomes, tandemly repeated often several thousand times in the nucleolar organizers (see Ref. 1 for a review). Ribosomal genes can also be extrachromosomal (1). This form of ribosomal DNA (rDNA) was observed during oogenesis of some animals like amphibians, fishes and certain insects, and occurs in many unicellular Eukaryotes. Extrachromosomal rDNA is circular in *Xenopus*, *Iriturus*, in the water beetles *Dityscus* and *Colymbytes*, in the cricket *Acheta* and in the yeasts *Saccharomyces* and *Torulopsis* (1, 2, 3). It is linear and circular in *Paramecium*, and exclusively linear in the ciliated protozoans *Tetrahymena*, *Oxytricha*, *Stylonichia*, and in the slime molds *Physarum* and *Dictyostelium* (1, 4). Although sometimes suggested (5-8), the presence of extrachromosomal rDNA was never demonstrated in photosynthetic cells. We report here the first evidence that there exist large amounts of extrachromosomal nuclear rDNA in a photosynthetic cell, the unicellular alga *Euglena gracilis*. This rDNA is circular and mainly monomeric. Extrachromosomal rDNA was not detected in the two higher plants studied, spinach and *Petunia*.

MATERIALS AND METHODS**Cultures and extraction of total DNA**

Euglena cells were grown under white light in *Euglena* Broth medium

Fig. 1 Characterization of extrachromosomal rDNA in *Euglena* cells.

A. Electrophoreses of undigested total DNA from *Y3BUD*. DNA was extracted from cells in the exponential growth phase (lanes 1), at the beginning (lanes 2) or after six days (lanes 3) of the stationary growth phase. Autoradiography of the Southern blots for 1 hour at -80°C .

B. Southern blots of undigested total DNA from wild-type and mutant *Euglena* strains (autoradiography for two hours at -80°C) and from *Petunia* and *spinach*

(autoradiography for 24 hours at -80°C). Y3BUD sample was the same as in lane 1 of A, but after storage for six weeks at 4°C . HMW: High Molecular Weight.

Southern blots were probed with a mixture of plasmids pRH57 and pRH59 (see Figure 2B).

(Difco). Initial density was 0.19×10^6 cells/ml. For Figure 1A, three aliquots of Y3BUD cells were collected when density reached 0.75×10^6 cells/ml (lanes 1), 2.95×10^6 cells/ml (lanes 2), and after 6 days of stationary phase (3.0×10^6 cell/ml, lanes 3). For Figure 1B, cells were collected before the cultures reached the beginning of the stationary phase. Cells were washed twice with 10 mM Tris-HCl, pH 7.6, 50 mM Na_2EDTA (buffer A) and frozen. *Petunia hybrida* and spinach leaves were washed with distilled water and ground with a waring blender in the presence of buffer A. The slurry was filtered through four layers of cheese cloth. Thawed *Euglena* cells resuspended in buffer A or leaf homogenates were treated for 15 min. at room temperature with 1% SDS and 1% sarkosyl. NaCl, 0.3 M final concentration, was added and total DNA extracted with phenol-chloroform. DNA was precipitated with ethanol, centrifuged and then resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM Na_2EDTA (buffer B).

Purification of extrachromosomal rDNA

Total DNA from Y3BUD extracted as above was enriched in DNA circles using the denaturation-renaturation technique (9). Nucleic acids were recovered by ethanol precipitation. The pellet was resuspended in buffer B containing 2.5 M NaCl. After overnight incubation at 0°C , the precipitated RNA were removed by centrifugation and the supernatant dialysed against buffer B. DNA (70-160 μg , estimated as before) were loaded on an 0.7% agarose gel. DNA 1 and DNA 2, clearly visible under U.V. light after ethidium bromide staining, were recovered by electroelution. Purified DNA from several electrophoreses was recovered by ethanol precipitation and ultracentrifugation (10), resuspended in buffer B, extracted by phenol-chloroform and ethanol precipitated. After ultracentrifugation, DNA was resuspended in buffer B.

Analyses of DNA

DNA concentration was determined by the specific fluorometric assay using DABA (11). Total DNA, 1.5 μg , was electrophoresed in 0.7% agarose (Sigma) gels. DNAs 1 and 2 were analysed in 0.9% agarose gels. Electrophoreses were at 30 volts, overnight, with ethidium bromide 1 $\mu\text{g}/\text{ml}$. DNA was transferred onto nitrocellulose filters according to Southern (12), and hybridized with an equimolar mixture of nick-translated pRH57 and pRH59 plasmids (Figure 2B), specific activity was 2×10^7 cpm/ μg . Autoradiographies were performed with an intensifying screen. Purified DNA 1 and 2 were digested in buffers recommended by the supplier (Boehringer) for 4 hours in the presence of 10 enzyme units/ μg .

λ DNA digested with Eco RI or Hind III was used as a standard. Hind III fragments of λ DNA were labelled by filling in with the Klenow fragment of DNA polymerase I and radioactive nucleotides.

Electron microscopy

R-loop formation and spreading of DNA were carried out as before (13). Hybridization temperature was 53°C, and DNAs were spread from an hyperphase 40% in formamide onto an hypophase 10% in formamide. In a few cases, DNA was spread after addition of pBR322 as an internal standard.

RESULTS AND DISCUSSION

The Euglena nuclear rDNA unit is 11.5 kb long and contains genes for cytoplasmic rRNAs, 20S, 25S, 5.8S and 5S (Figure 2B) (14). Bacillaris and Z type strains have similar rDNA units except for two additional Sal I sites in bacillaris in a non coding region (unpublished results). Total DNA has been extracted from several Euglena strains and electrophoresed without digestion, denatured, transferred to nitrocellulose filters and hybridized with a probe formed by an equimolar mixture of the two Hind III fragments (Figure 2B) cloned in pBR322 (pRH57 and pRH59). The pattern obtained with Y3BUD, a mutant derived from bacillaris (15, 16) shows several radioactive bands (Figure 1A). A faint one corresponds to high molecular weight DNA (chromosomal) visible under U.V. light after ethidium bromide staining. Two heavy ones correspond to a region of the gel where DNA is present in low amounts : generally, no band is visible under U.V. light; in some preparations however, one or two faint bands which correspond to the two hybridizable DNAs are visible. On 0.7% agarose gel these bands migrate with an apparent size for a linear fragment of about 20 kb and 5.7 kb. To simplify matters, these two bands will be called DNA 1 and DNA 2, respectively. These DNAs are present in all the growth stages although they appear less abundant after six days in the stationary phase (Figure 1A). After storage of DNA for six weeks at 4°C, we found that DNA 2 had disappeared while a band at 11.5 kb had become clearly visible (Figure 1B). DNA 1 has been found in three wild-type strains, Z 1224 5/25, bacillaris and ATCC 10616 (17, 18), and in three other mutants impaired in photosynthesis derived either from bacillaris (Y1BXD and W3BUL) (15, 16) or from Z (W34ZUD) (19) (Figure 1B). DNA 2 is generally less abundant than DNA 1. As for Y3BUD, one or two faint bands are sometimes visible at the position of DNAs 1 and 2 after ethidium bromide staining.

To further analyse these DNAs, we have enriched Y3BUD DNA extract using the denaturation-renaturation technique described for preparation of bacterial

Fig. 2 Organization of *Euglena* extrachromosomal rDNA.

A. Restriction patterns obtained with DNA 2. Purified DNA 2 from Y3BUD (see Materials and Methods) was digested, electrophoresed, transferred and hybridized with plasmids pRH57 and pRH59. No fragment less than 0.6 kb was observed, even on overexposed autoradiography. < point to bands faintly visible on the autoradiograms. Autoradiography was for 8 hours at -80°C (20 min. for the λ lane).

B. Map of the rDNA unit, adapted from Curtis and Rawson (14) and our analyses of plasmids pRH57 and pRH59 by restriction enzymes and R-loop mapping (Neyret et al., unpublished results), is given for easy identification of rDNA fragments and localization of probes. ★, Sal I sites specific of *bacillaris* rDNA. Restriction fragments (in kb) obtained for *bacillaris*: Bam H I, 4.8, 3.5, 1.9, 0.7, 0.7; Eco R I, 11.5; Xho I, 6.6, 3.4, 1.5; Sal I, 7.1, 3.5, 0.6, 0.4; Hind III, 5.9, 5.7.

plasmids (9). This eliminates high molecular weight DNA while DNA 1 and DNA 2 are clearly visible under U.V. light after ethidium bromide staining, and can be further purified by preparative electrophoreses. These purified DNAs have then been analysed with restriction enzymes and electron microscopy.

Restriction patterns obtained for DNA 2 after hybridization with pRH57 and pRH59 (Figure 2A) are similar to those obtained for an *Euglena* nuclear rDNA unit from *bacillaris* (Figure 2B). Identical patterns were observed with DNA 1

Fig. 3 R-loops formed between DNA 1 or DNA 2 and 20S RNA. Arrowheads point to the R-loop. Scale bar, 0.5 μ m.

(not shown). Electron microscopic observations of DNA 1 and DNA 2 reveal the presence of circular molecules (20 to 50% of the molecules). Circular DNA from both bands are homogeneous with a length $3.82 \pm 0.08 \mu$ m ($n=16$) which corresponds to a size of 10.8 kb close to the sum (11.2 kb) of the hybridized fragments observed Figure 2A. We have further hybridized DNA 1 and DNA 2 with 20S cytoplasmic rRNA under conditions of R-loop formation. Figures observed (Figure 3) clearly show that DNA circles contained sequences for 20S rRNA. The size of the R-loop is 2.07 ± 0.09 kb ($n=14$), the same as that measured on pRH59 (unpublished results). This size is close to the presumed length of the 20S gene (2.3 kb). The presence of circular DNAs with the same size in bands 1 and 2, together with the modification of the hybridization pattern after conservation at 4°C, lead us to assume that DNA 1 and DNA 2 correspond respectively to open and supertwist circles, while the bands at 11.5 kb in Figure 1 correspond to linearized circles.

All the data shown above clearly demonstrate that nuclear rDNA in *Euglena* is present as circular DNA molecules of 3.8 μ m. The presence of the 25S rRNA gene was not directly evidenced, but we demonstrate that the size and the restriction pattern of the circle are those of the complete rDNA unit (14). Faint hybridization bands observed sometimes on autoradiograms, particularly above the DNA 1 band, may represent dimers or trimers of the rDNA unit : few 8 μ m circles were observed before (20). In contrast to amphibian and insect oocytes where most of the extrachromosomal rDNA are oligomers, in *Euglena* most of them appear as monomer circles. These molecules are probably similar to the

3.6 μm (in formamide) DNA circles described by Nass and Ben-Shaul (21). As in our studies, they observed that these circles are less abundant during the stationary than during the exponential phase of growth. Such a decrease has already been found for Tetrahymena extrachromosomal rDNA (22). It is possible that part of the 3.8 μm DNA circles observed here are not nuclear rDNA. To check that, we have looked for chloroplast origin but all the approaches used, such as comparative denaturation of chloroplast DNA and 3.8 μm circles, or heteroduplex formation between chloroplast DNA and 3.8 μm circles, were unsuccessful. It remains possible that some circular DNA molecules represent various types of DNA sequences cut out by looping due to the presence of direct repeat sequences (23-27).

The ease of detection of these molecules in total undigested DNA suggests that they represent a large portion of the nuclear rDNA. In gels where DNA 1 and DNA 2 are visible under U.V., we estimated the amount of such circular DNAs to be at least 1% of total DNA, which corresponds to about 2,400 units per cell, assuming a DNA content of 3 pg per cell (28). This value is in agreement with the range found in the literature, namely 800, 1,000 and 4,000 rDNA units per Euglena cell (14, 29, 30). This and the fact that low hybridization is observed on undigested chromosomal DNA (Figure 1) suggest that most of the nuclear rDNA, if not all, is extrachromosomal in Euglena. This is probably why, in spite of several attempts, cloning of Mbo I partially digested nuclear DNA in λ EMBL4 has led to the cloning of one rDNA unit at most (Lebrun and Freyssinet, unpublished results); Curtis and Rawson (14) also obtained recombinant phages with only one rDNA unit. One may then ask if the cloned rDNAs analysed (14, pRH57 and pRH59) are not extrachromosomal circular rDNA. This situation would be similar to that observed in Physarum and Dictyostelium which have only a few rDNA units in the chromosomes, and in Tetrahymena where only one rDNA unit is integrated in the chromosome of the micronucleus (1).

In higher plants, the rDNA unit ranges from 7.7 to 12.7 kb (31). If there are large amounts of extrachromosomal circular rDNA units, they should be easily visible. Thus we have looked for them in total DNA from Petunia and spinach. Our probe effectively detects rDNA in chromosomal DNA (Figure 1B), but no hybridization was found in lower molecular weight DNA even after long exposure of the filters. As DNA was extracted from leaves, we cannot rule out that extrachromosomal rDNA might be present in other parts of the plant; it might also appear during a short phase of the development or under the influence of environmental factors.

This work describes for the first time the presence of extrachromosomal

rdNA in a photosynthetic cell. Up to now, amplification has been suggested but never demonstrated in any algae and higher plants (5-8). Work is in progress to determine whether the 3.8 μ m circles replicate. If this is the case, it would constitute a model to study the replication process of nuclear DNA and its regulation in photosynthetic cells.

While this paper was being written, a short report appeared (32) which describes 11.3 kb circles but does not identify them. As in our studies, these authors estimated the number of these circles to be at least 1000/cell.

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*Present address: UNICET, BP 11, 69572 Dardilly, France

+Present address: Rhône-Poulenc Agrochimie, Biologie Moléculaire Végétale, BP 9163, 69263 Lyon Cedex, France

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