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Nuclear rDNA in *Euglena gracilis:* paucity of chromosomal units and replication of extrachromosomal units

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ABSTRACT

Copy number of chromosomal rDNA units was investigated in two Euglena gracilis wild-type strains. It was established by dot blot analysis that these strains possess about four integrated units per haploid genome. This is the first example of a photosynthetic cell with only a few chromosomal ribosomal genes. In addition to these units, Euglena has 800 to 4000 extrachromosomal rDNA units. Electron microscopy revealed that these free rDNA circles bear a replication origin, and intermediates of replication show a D-loop structure.

INTRODUCTION

Nuclear ribosomal genes in eukaryotes are generally clustered and tandemly repeated tens to thousands of times on chromosomes (see Ref 1, 2 for reviews). Well documented exceptions are those of Tetrahymena pyriformis which possesses only one copy in its micronucleus ("genetic" nucleus) (1, 2, 3), and Plasmodium species which have four to eight non similar copies scattered through the genome (4). It is probable that only one or few units exist in the micronucleus of the Ciliates Glaucoma (3) and Tetrahymena pigmentosa (5), and in the slime molds Physarum and Dictyostelium (1,2).

Besides chromosomal rDNA genes, some cells possess extrachromosomal rDNA units. These exist at a low copy number in several yeasts (6). In contrast, they are amplified in oocytes of some insects and amphibians, during magnification in the testes of the Drosophila bobbed mutant (1, 2), in the macronucleus ("metabolic" nucleus) of several protozoans, and in slime molds (1, 2, 3). Depending on the organism, extrachromosomal rDNA can occur in various forms. Single and tandemly repeated units are circular in yeast, amphibians and insects. Free rDNA units are palindromic molecules each containing two transcription units in slime molds and Tetrahymena. Paramecium has nonpalindromic repeated units on linear and circular molecules. Other Ciliates have only linear single copy molecules. In some cases, it has been demonstrated that free rDNA is self-replicating. The majority of

extrachromosomal circular rDNA of Xenopus replicates with rolling circle intermediates (1,2). Linear and palindromic rDNA of Tetrahymena and Physarum replicate from origins located in the non-transcribed spacer of the rDNA unit (1-3, 7, 8).

I report here that very few rDNA units exist in chromosomal DNA of the unicellular green alga Euglena gracilis. Thus, extrachromosomal circular rDNA previously described in this cell (9) represents the majority of cellular rDNA. It was also observed that free rDNA in Euglena is self-replicating.

MATERIALS AND METHODS

Cell cultures, DNA extraction and DNA analyses

Euglena cells were grown in Euglena Broth medium (Difco), and total DNA was extracted as described (9). Electrophoresis was performed with agarose (Sigma) in 10 mM Tris-HCl, 20 mM Na-acetate, 2 mM Na_2EDTA, pH 7.8, 0.5 µg/ml EtBr. Following electrophoresis, DNA was transferred onto nitrocellulose according to Southern (10). Filters were hybridized with an equimolar mixture of labeled plasmids pRH57 and pRH59 which cover the entire Euglena rDNA unit (9, 11), or with rDNA inserts excised from these plasmids. Probes were nick-translated with ^{32}P nucleotides, to specific activities of 0.6 to 2.4 10^7 cpm/µg. Hybridization were performed in 3 X SSC, 1 X Denhardt, 0.1% SDS, 200 µg/ml salmon sperm, at 63°C for 12 to 36 hrs. A final wash was performed with 0.2 X SSC, 0.1% SDS at 63°C. Autoradiography was performed at -80°C with an intensifying screen.

Dot Blots

DNAs were linearized or shortened by digestion with Eco RI (Euglena DNA) or Hind III (plasmids), or by sonication (salmon sperm DNA). DNA in 180 µl was denatured with the addition of 20 µl 2 N NaOH and by incubating for 30-120 min at 37°C. DNA was then placed on ice, neutralized with the addition of 1/20th vol. of 2 M Tris, pH 7.9, and 1/10th vol. of 2N HCl, and 230 µl of 20 X SSC were added. DNAs were fixed on nitrocellulose pre-soaked in 20 X SSC by filtration in an Hybridot (BRL) apparatus. Filters were rinsed in 4 X SSC, activated 2 hrs at 70°C, and hybridized. After autoradiography, radioactive spots were excised and counted for 20 min in 10 ml of scintillation liquid. Counts bound to blank filters were substracted from counts bound to DNA containing filters. Standard curves were linear up to 250 pg rDNA.

Electron microscopy

Total DNA was phenol extracted (9), dialysed against 10 mM Tris, 1 mM EDTA, pH 7.5, and/or digested with 50 µg/ml RNAse at 37°C for 1 hr. Before

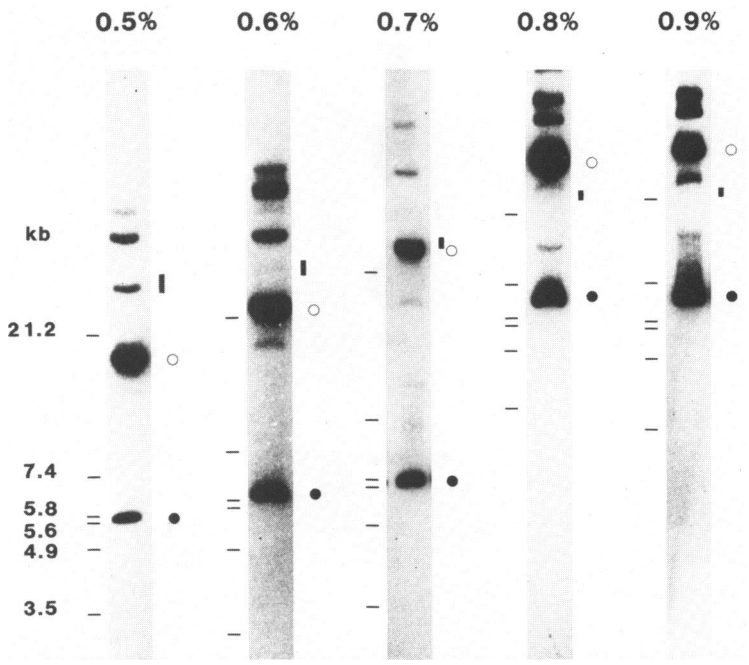

Figure 1
Position of rDNA extrachromosomal forms relatively to chromosomal DNA is dependent on agarose concentration.
Total DNA of Euglena Z 1224 5/25 (about 1.5 µg) was loaded in agarose gels at the specified concentration. Hybridizations were performed with plasmids pRH57 and pRH59 which cover the entire rDNA unit (9, 11). Autoradiographic exposure was for 30 min to a few hours, depending on the probe activity. The position of labelled EcoRI fragments from λ phage is shown on the left of each pattern (9). Vertical bar on the right of each pattern marks the position of chromosomal DNA observed under UV light in the gel stained with EtBr. ○ monomer rDNA, open circle, ● monomer rDNA, supercoiled circle.

spreading, DNA was passed through a CL 2B Sepharose column equilibrated with 10 mM Tris, 1 mM EDTA, pH 7.5. DNA was spread from a hyperphase which is 100 mM Tris, 1 mM EDTA, pH 8.5, 30% formamide, 200 µg/ml cytochrome c, onto a hypophase which is 10 mM Tris, 0.1 mM EDTA, pH 8.9, 10% formamide. It was contrasted with uranyl acetate and shadowed with Pt-Pd (80:20). The microscope was calibrated with a grating replica (2160 lines/mm, Agar Aids).

RESULTS AND DISCUSSION

rDNA genes dosage in chromosomal DNA

We have previously evidenced the existence of circular extrachromosomal rDNA in several wild-type and mutated strains of Euglena gracilis, and

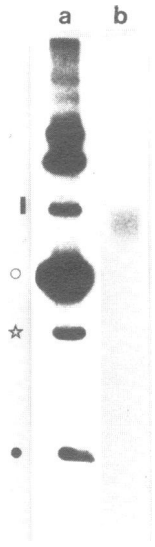

Figure 2
Control of the purity of chromosomal DNA used for rDNA gene dosage (strain Z 1224 5/25).
A) Total DNA (about 1.5 µg), 2 days exposure.
B) Chromosomal DNA (0.5 µg), purified by electrophoresis in 0.6% agarose, and then 0.9% (see text), 3 days exposure.
Electrophoresis in 0.5% agarose. Hybridization with plasmids pRH57 and pRH59.
Star : linear DNA, 11.5 kb (broken monomer circles). Other symbols are the same as in Fig. 1.

postulated that this form represents the majority of the cellular rDNA (9). To investigate this possibility, the number of integrated units in chromosomal DNA of two wild-strains of E. gracilis, strain 1224 5/25 and var. bacillaris, was determined.

Electrophoretic patterns of undigested total DNA hybridized with nuclear rDNA probes usually show two major bands and several minor ones. We previously recognized the former as supercoiled and open rDNA circles of 3.8 µm (11.5 kb, Ref 9, 11, 12), and interpreted the latter as oligomers of these circles. The diversity of the rDNA forms (monomer and oligomer, supertwist and open circle) renders impossible the purification of chromosomal DNA by a single step electrophoresis, because there is always extrachromosomal rDNA at the level or close to the long DNA whatever the agarose concentration used for migration (Fig. 1). Figure 1 shows that migration of the various forms of circular rDNA relative to chromosomal DNA depends on the concentration of

agarose used for electrophoresis. This effect is especially pronounced with the nicked circular monomer. Thus, chromosomal DNA from both wild-type strains was purified by two successive electrophoreses as follows. The first electrophoresis was performed in 0.6% agarose, and chromosomal DNA was electroeluted. In this way, we eliminated the majority of extrachromosomal rDNA. If the faint hybridization appearing at the level of chromosomal DNA in 0.6 % agarose in Figure 1 is due to oligomeric circular rDNA, one may assume from previous results that this form will run at another level far from chromosomal DNA in a very different agarose concentration. Thus, the second electrophoresis was performed in 0.9%. Chromosomal DNA eluted from this gel was checked for purity in a third concentration of agarose, 0.5%. Figure 2 shows that chromosomal DNA thus purified was slightly broken but appeared to be essentially devoid of circular extrachromosomal rDNA.

Purified chromosomal DNA was analysed for rDNA gene dosage by dot blot hybridization. The probe used covers the entire rDNA unit (9, 11). rDNA inserts were excised and purified from the vector. Results show that 0.5 µg of Z strain chromosomal DNA contains about 25 pg rDNA (Fig. 3), indicating that each haploid genome (1.36×10^6 kb, Ref 13, 14) would contain about four 11.5 kb integrated units for Z strain (range between 1 and 6 units) and for bacillaris (range between 2 and 9 units, results not shown). This determination may be an overestimate since it is likely that broken oligomers (larger than dimer, $\geqslant$ 34.5 kb) may accompany chromosomal DNA during the purification.

Among the 800-4000 rDNA units of an Euglena cell (12, 14, 15), at most ten units are located in chromosomal DNA, assuming that the nucleus is diploid (13). Thus, the organization of rDNA units in Euglena is unique in two features. First, the cell has extrachromosomal genes. Some evidence exists for free rDNA units in Chlamydomonas, in which changes in cellular content of rDNA genes may be interpreted as differential synthesis and degradation of extrachromosomal rDNA during cell cycle (16). Secondly, Euglena has few chromosomal units. Other examples of cells with few rDNA units in chromosomes are only found in non-photosynthetic eukaryotic cells such as the Ciliate Tetrahymena pyriformis which has only a single copy in its micronucleus, and Plasmodium which has four to eight copies, depending on the species (4). The Ciliate Glaucoma (3) and slime molds Physarum and Dictyostelium (1, 2) likely possess one or few integrated units. Algae have at least 150 rDNA units per haploid genome, and Embryophytes possess 600-4000 units (1).

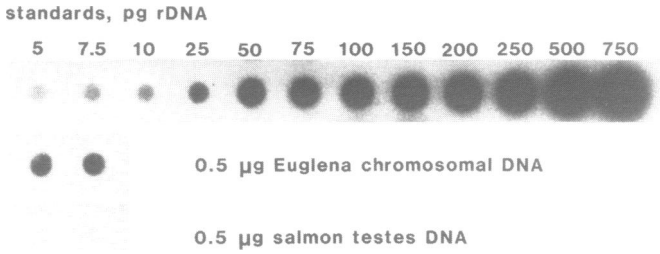

Figure 3
rDNA gene dosage in purified chromosomal DNA (strain Z 1224 5/25). Purified DNA from Figure 2 was used. Standard curve is a mixture of 0.5 µg salmon sperm DNA and equimolar amounts of plasmids pRH57 and pRH59. Specified quantities refer to rDNA sequences only. Hybridization was with rDNA inserts excised and purified from plasmids pRH57 and pRH59. Exposure, 2 days.

Replication of extrachromosomal rDNA units

While the presence of extrachromosomal rDNA was clearly demonstrated, it remained to be determined if they are replicated as copies of the chromosomal units or if they are self-replicating. To address this question, the replication of these units was examined.

To determine if there exists a definite phase of the cycle during which rDNA replication takes place, cells were synchronized by starvation for several days in the dark. At the end of this phase, the cells were transferred into a rich medium and incubated in the presence of light. rDNA was quantitated during the lag phase which precedes the onset of division, and at the beginning of the growth phase. Slight fluctuations in the rates of rDNA synthesis were observed, without correlation to any stages of the synchronized cultures. Cycloheximide (3 or 10 µg/ml) had no effect on the level of extrachromosomal DNA replication. This finding is contrary to that which was observed in animal cell cultures (17).

Cells were sampled from non synchronized cultures during the exponential growth phase, and total DNA was examined by electron microscopy. From a sample of 650 circular rDNA molecules (size between 3.6 to 3.8 µm), twelve molecules showed replication bubbles distinctly composed of a single-stranded branch and a double-stranded one (Fig 4 A, B). These replicating regions represent on the average 5.0 + 1.3% (n = 12) of the molecules (extremes are 2 and 10%), i.e. 575 bp of a 11.5 kb unit. Other molecules showed shorter bubbles (< 2% of the molecule length). Because branches were too small to accurately determine if they were single-stranded or double-stranded, it cannot be excluded with certainty they are denaturation bubbles. However,

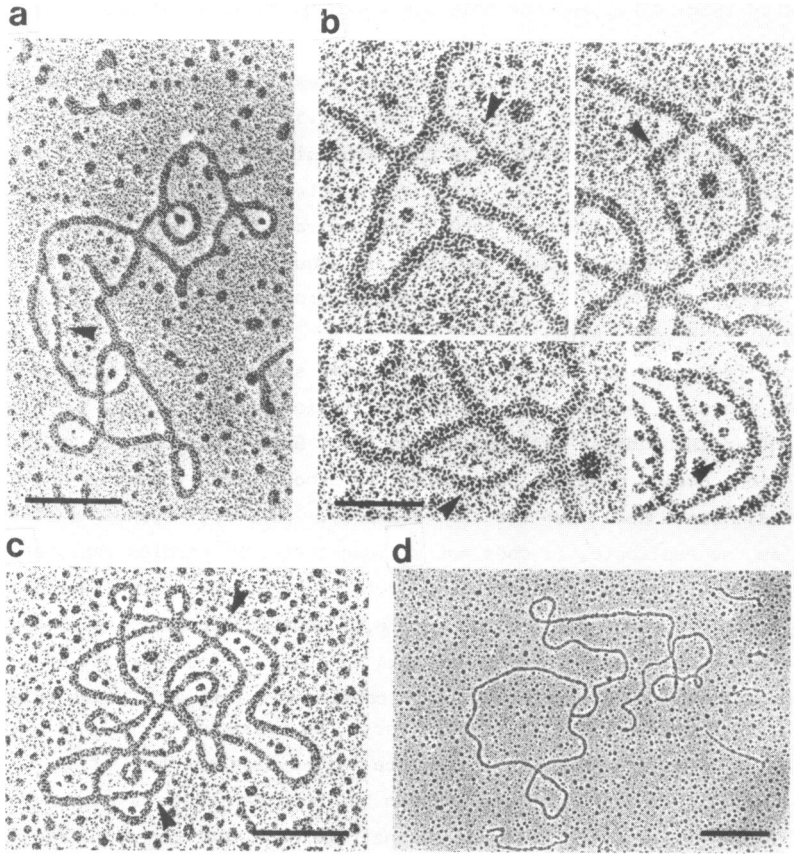

Figure 4
Extrachromosomal rDNA circles replication.
A) 3.5 μm circle, 5.7% replicated. Arrow points to the single-stranded branch of the bubble. Bar, 0.2 μm.
B) Detail of replicated regions showing the D-loop structure (a single-stranded branch, a double-stranded branch) of the bubble. Arrows point to the single-stranded branch. Bar, 0.1 μm
C) Replicated circle with two double-stranded branches. Arrows point to the forks. Bar, 0.2 μm.
D) σ structure (rolling circle). Circle is 3.8 μm, tail is 5.8 μm. Bar, 0.5 μm.

considering that such bubbles were never observed in these preparations on long chromosomal DNA or on rDNA circles extracted by denaturation-renaturation and spread under the same conditions (9), I believe that the smallest bubbles observed represent replicating DNA. I also observed one tangled molecule with two forks which could be a rDNA circle 30%

replicated (Fig. 4 C), and one molecule which could be a rolling circle (Fig. 4 D).

If we consider only the molecules with clear replication bubbles (larger than 2% of the molecule length), about 2.5% of molecules appeared to be replicating. This low number is not surprising since there is no definite stage for extrachromosomal rDNA replication between two mitoses, as described for Physarum (7). Assuming a fork migration rate of 1 kb/min in plant cells (18), a 11.5 kb circle would replicate in about 6 min. At each instant of a cycle (about 12 hours during exponential growth phase), roughly 1% of the circles will be replicating.

The frequency of molecules at the same stage, the structure of D-loop (19) and its size, suggest a pause of replication at this step and evokes the onset of mitochondrial DNA replication (19). Such a structure suggests that replication could continue according to the Cairns model (θ structure molecules) (see Ref. 20 for an example). However, even if part of the molecules so replicate, it does not exclude that rDNA circles replicate also according to the rolling circle model (21, 22). Having observed only one Cairns molecule and one rolling circle, I cannot determine the predominant replication type of extrachromosomal rDNA in Euglena. The majority of Xenopus extrachromosomal rDNA replicate with rolling circles, and Cairns molecules are rarely observed (1, 2, 23). Very few replicating rDNA circles were observed in insects and yeasts : they are tailed circles (1, 2, 6).

To date, no origin of replication has been mapped to a definite region in a green eukaryotic cell. We demonstrate here that Euglena rDNA circles bear an origin of replication, as do extrachromosomal rDNA of Xenopus, Physarum and Tetrahymena (1, 2, 3, 7, 8). A replication origin seems present in chromosomal units of Xenopus (24) and sea urchin (25), and is well evidenced and localized for Drosophila (26) and yeast (27). When precisely mapped inside the rDNA unit, the origin is always in the non-transcribed spacer. One may also notice that the replication origin is close to ribosomal genes on circular mitochondrial DNA of Drosophila and vertebrates (19), on chloroplast DNA (28-30) and on Bacillus subtilis chromosome (31).

The most well characterized origins working with the nuclear enzymatic machinery are viral and yeast 2 μm plasmid origins (32, 33). Though origins are well mapped in the rDNA units of animal and protozoan cells, no structural and functional description of an eukaryotic origin is done to date. Our observation renders possible such a study for a photosynthetic eukaryotic cell. The Euglena replication origin could also be used to build an extrachromosomal transformation vector for plant cells.

Is there filiation between extrachromosomal and chromosomal units ? Since Euglena reproduces only by mitosis and rDNA circles are capable of replication, it is possible that amplification from chromosomal units is not required at any stage of the cycle. If there is no filiation, both kinds of units might independently evolve and, in the extreme, chromosomal units might disappear. It was recently reported that the soil amoeba Naegleria is a cell without chromosomal rDNA (34).

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