The shape of mitochondria and the number of mitochondrial nucleoids during the cell cycle of *Euglena gracilis*

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**Summary**

The shape of mitochondria and the number of mitochondrial nucleoids in *Euglena* cells were examined throughout the cell cycle by fluorescence microscopy. Both photoheterotrophic and heterotrophic cells contained a network of mitochondria that did not divide into fragments at any stage of the cell cycle. Mitochondrial nucleoids could be clearly detected in the mitochondria by staining with ethidium bromide and with DAPI. Half of the mitochondrial nucleoids entered each daughter cell during cytokinesis. Nucleoids in the newly produced daughter cells increased in number as the cells increased in size. The number of nucleoids reached double the initial value in cells at the stage just prior to mitosis. The total length of the mitochondrial net was proportional to the cell volume.

Key words: cell cycle, *Euglena*, fluorescence microscopy, mitochondria, mitochondrial nucleoid.

**Introduction**

Fluorescence microscopy can provide valuable information about the shape and patterns of distribution of mitochondria in the cell (Johnson *et al.* 1980, 1981; Goldstein & Korczack, 1981; Kuroiwa *et al.* 1981a; Terasaki *et al.* 1986). It is a much more convenient technique than electron microscopy for investigation of such aspects of the morphology of mitochondria. It is not necessary to make serial sections or to construct three-dimensional figures as in the case of electron microscopy. Using fluorescence microscopy, one can accurately assess the morphology of mitochondria in cells by the thousands if an appropriate fluorescent dye, which specifically combines with the mitochondria, is employed.

The fluorescent dye DiOC6(3) (3,3'-dihexyloxocarbocyanine iodide) was chosen for detection of mitochondria by Johnson *et al.* (1981). Hatano & Ueda (1988) and Noguchi & Ueda (1989) compared the fluorescent structure in a solution of DiOC6(3) with the mitochondria examined by electron microscopy, and they proved that the fluorescent structure corresponds to the mitochondria in algal cells.

Nucleoids in mitochondria and in chloroplasts can also be visualized by fluorescence microscopy (Kuroiwa *et al.* 1981a,b; Hashimoto, 1985; Coleman, 1979; Hatano & Ueda, 1987; Possingham *et al.* 1983). Mitochondrial nucleoids in a slime mold, *Physarum polycephalum*, were well studied using the dye DAPI (4',6-diamidino-2-phenylindole) and their division cycle was observed in detail (Kuroiwa, 1982). Fusion of mitochondrial nucleoids has also been traced in a yeast, *Saccharomyces cerevisiae*, by fluorescence microscopy (Miyakawa *et al.* 1984). However, reports on analysis of the morphology of mitochondria and mitochondrial nucleoids by fluorescence microscopy are very few in number, because this method has been only recently developed. Little is known about the number of nucleoids in the large reticulated mitochondrion that is frequently found in algal cells, or about changes in the numbers of nucleoids during the cell cycle. This report describes the shape of mitochondria and the changes in the numbers of mitochondrial nucleoids during the cell cycle of *Euglena*.

**Materials and methods**

Green and leuco strains of *Euglena gracilis* Z were used in this investigation. The leuco strain was a mutant induced by ultraviolet (u.v.) irradiation in our laboratory. Cells were cultured at 25°C in a culture medium that contained 0.05% KH2PO4, 0.1% citric acid, 0.1% sodium citrate, 0.02% MgSO4, 0.5% glucose, 0.5% polypeptone, and 10 µgml⁻¹ vitamin B12. Cells were illuminated under a fluorescent light with a photon flux density of 50 µmol m⁻² s⁻¹ for 14 h per day.

For visualization of mitochondria, the dye DiOC6(3) (3,3'-dihexyloxocarbocyanine iodide) (Eastman Kodak Co., NY, USA) was used. It was dissolved in ethanol at a concentration of 1%, and then diluted in culture medium to a concentration of 5 µg ml⁻¹. Cells at various stages were treated with this solution of dye for 5 min at room temperature. After a brief
rinse with culture medium, cells were mounted in culture medium and examined under a fluorescence microscope (Olympus Kogaku Co., BHS-RFA type) with a blue excitation filter. Photographs were taken on Kodak Tri-X film.

For double staining of mitochondria and mitochondrial nucleoids, cells stained with DiOC6(3) were further treated for 5 min with 0.2% ethidium bromide, washed with culture medium, and mounted in culture medium. Photographs were taken on Fuji DX-400 film under the same conditions as used for single staining with DiOC6(3).

For counting the number of mitochondrial nucleoids, photographs were taken through many different optical focal planes in the cells, which were treated with DAPI (4',6-diamidino-2-phenylindole) (Sigma St Louis, USA) in a 0.05% aqueous solution. Each nucleoid shown in the photographs was carefully checked to ascertain whether it was visible in other photographs taken at the upper or lower adjacent focal planes, to avoid counting the same nucleoids more than once.

For digestion experiments of mitochondrial nucleoids, cells were partially broken by a homogenizer in 1/15 M-phosphate buffer (pH 6.5) containing 5 mM-MgCl₂ and 0.5 M-mannitol, and were treated for 30 min at 30°C with deoxyribonuclease I (Sigma, St Louis, USA) at a concentration of 1500 Kunitz units ml⁻¹. The mitochondrial nucleoids in the broken cells were detected with ethidium bromide before and after digestion with the nuclease.

The measurements of the total lengths of mitochondria and of the cell volumes were carried out on the photographic figures. As the cells were considerably pressed between coverslips and slides, the cells assumed a discoid shape with uniform thickness, so that the cell volume was represented by the surface area of the cell. The total lengths of the mitochondria were also represented by twice the length of the mitochondria that were photographed near the upper surface of the cell, because near the opposite surface of the cell the distribution of a similar quantity of mitochondria was observed.

Results

Mitochondria during the cell cycle

A network of mitochondria was clearly visualized under the fluorescence microscope after staining with the dye DiOC6(3). The network was apparent in the leuco Euglena (Fig. 1A–D) as well as in the green Euglena (Fig. 2A–D). The network of mitochondria was situated close to the cell surface, surrounding other organelles in the cell. There was only one network of mitochondria in each cell. The distribution density of the mitochondria in the network was usually low in the region above the nucleus; one or two thinner strands of mitochondria were frequently seen in this region (see arrow in Fig. 1A). The network of mitochondria was also less dense in the region of the reservoir (see r in Fig. 1A).

The volume of the cell gradually increased during the growing phase and reached a maximum at the initial stage of mitosis. Fig. 1B shows the network of mitochondria at the mitotic telophase, by which time two nuclei have been produced. In Fig. 1B, the mitochondrial network was absent over the nuclei and the reservoir.

A cleavage by constriction proceeded from the anterior part to the posterior part of the cell after mitosis (Fig. 1C). The networks of mitochondria were maintained during the cleavage of the cells. The networks had more rod-shaped terminals than those found at previous stages, which may result from the partial cutting of the networks. The cleavage of the cells by constriction inevitably resulted in cutting of the mitochondrial networks, so that the number of terminals in the networks increased. Small cells, which were assumed to be those at stages soon after cytokinesis, also contained a network of mitochondria (Fig. 1D).

The green Euglena emitted primary fluorescence from the chloroplasts. However, strong fluorescence from the mitochondria, which were treated with DiOC6(3), enabled us to trace the mitochondrial network accurately, provided that the primary fluorescence from chlorophylls

Fig. 1. Mitochondria in leuco Euglena. A. Growing cell. An arrow points to the thin strand of mitochondria at the nuclear region. B. Cell with two daughter nuclei (n) formed by nuclear division. C. Dividing cell. D. Young cell soon after cytokinesis. r, reservoir. ×1400.
Mitochondria in green *Euglena*. A. Fully grown cell. in mitosis. C. Cell with two daughter nuclei after D. Dividing cell. n, nucleus. ×1400.

Cell double-stained with DiOC6(3) and ethidium z. The network of mitochondria is colored green, point to the mitochondrial nucleoids. n, nucleus.
in the chloroplasts was blocked with a green filter. The behavior and the appearance of mitochondria in the green *Euglena* throughout the cell cycle were the same as those in the leuco *Euglena* (Fig. 2A–D).

**Mitochondrial nucleoids**

Mitochondrial nucleoids could be visualized after the treatment of cells with DAPI or ethidium bromide, under a fluorescence microscope. Fig. 3 is a fluorescence micrograph of a leuco *Euglena* double-stained with DiOC6(3) and ethidium bromide. The nucleus strongly emitted a vermilion fluorescence (see n in Fig. 3). Mitochondria emitted a green fluorescence. There were many small granules in the network of mitochondria (see arrows in Fig. 3). These granules appeared to be yellow, probably as a result of the combination of their original vermilion and mitochondrial green. They lost their fluorescence after treatment with DNase, indicating that they contain DNA. Accordingly, the granules were identified as the mitochondrial nucleoids. As shown in Fig. 3, small fluorescent granules were not detected outside the mitochondrial network. This may imply that the leuco strain used in this investigation contains no detectable plastid nucleoids.

The leuco *Euglena* was used to examine the numerical changes in the mitochondrial nucleoids during the cell cycle, in order to avoid misidentification of chloroplast nucleoids as mitochondrial nucleoids. For calculation of the number of nucleoids, DAPI was used for staining, because the intensity of fluorescence from DAPI is stronger than that from ethidium bromide. An interphase cell at an optical focal plane is shown in Fig. 4A. Besides the nucleus (n) and the assimilation products (a), many small spherical mitochondrial nucleoids can be seen. About 75% of the nucleoids were uniform in size, 0-3 μm in diameter, and the remaining ones were larger in size, 0-6 μm in diameter. Elongated nucleoids (Fig. 4B) and pairs of nucleoids situated close to one another (Fig. 4C,D) were always observed in the non-dividing growing cells, though they were fewer in number. They may be the dividing and separating nucleoids. Spherical assimilation products, 2 μm in diameter, fluoresced weakly (see a in Fig. 4A). This fluorescence was due to the non-specific adsorption of the dye DAPI to the assimilation products, because treatment with DNase did not diminish the fluorescence. Fig. 5A is a representation of all the mitochondrial nucleoids detected in the cell in Fig. 4A. They were 278 in number.

In cells that had completed nuclear division, mitochondrial nucleoids were similar in size and in fluorescence intensity as those in the interphase cells (Fig. 4E). All mitochondrial nucleoids in the cell in Fig. 4E have been plotted in Fig. 5B and 322 nucleoids can be counted. About half the mitochondrial nucleoids were distributed to each of the two daughter cells during cytokinesis (Fig. 4F). Mitochondrial nucleoids did not change in size or in appearance during cytokinesis. The distribution of nucleoids in the two daughter cells in Fig. 4F is reconstructed in Fig. 5C where 352 nucleoids can be counted. The daughter cell, soon after cell division, contained mitochondrial nucleoids without any visible change in their size (Fig. 4G). All the nucleoids in the daughter cell are shown in Fig. 5D and 139 nucleoids can be counted.

The total length of the mitochondrial net has been plotted against the cell volume in Fig. 6. The total length appears to be nearly proportional to the cell volume. Small cells, which probably were cells soon after cell division, contained about 800 μm of mitochondria, and the large cells contained about 1600 μm. The number of mitochondrial nucleoids was also proportional to the cell volume, but not as strictly proportional as the mitochondrial length. Small cells contained about 150 nucleoids, and large ones about 350.

**Discussion**

Early studies demonstrated that *Euglena* contained many ovoid or rod-shaped mitochondria, by the examination of single random sections of the cell under the electron microscope (Gibbs, 1960; Malkoff & Buetow, 1964). Reticular mitochondria were observed by Leedale & Buetow (1970) in living *Euglena gracilis*. Next, an interpretation was proposed that the mitochondria are reticular in non-dividing cells and are rod-shaped in dividing cells. The rod-shaped mitochondria grow and fuse with each other to form a mitochondrial network after cell division (Calvayrac et al. 1972, 1974; Osafune et al. 1975a,b). Later, Pellegrini (1980a,b) reconstructed the shape of mitochondria three-dimensionally from serial sections of *E. gracilis* Z, and emphasized that the mitochondria are reticular in shape throughout the cell cycle. The present investigation confirmed the Pellegrini's conclusion after examination of thousands of cells, both non-dividing and dividing, in which a clear, fluorescent reticulum was visible.

The increase in the diameter of mitochondria in cells grown in darkness or under heterotropic conditions has been reported (Siegesmund et al. 1962; Lefort, 1964; Pellegrini, 1980b). Pellegrini (1980b) described mitochondria that were three times larger under heterotropic conditions than under phototrophic conditions. These mitochondria appeared as networks with narrow meshes. Our results showed that there were no visible differences between the mitochondria in phototrophic and heterotropic cells of *Euglena* under our culture conditions. As shown in Figs 1 and 2, the diameter of mitochondria and the size of the networks of the mitochondrial reticulum were similar in both strains. It should be pointed out that the thickness of mitochondria after vital staining was much smaller than that of mitochondria reconstructed by electron microscopy. The size and the form of mitochondria observed by the present method probably reflected their real size and form.

The division of mitochondrial nucleoids and the succeeding division of mitochondria have been described in some organisms (Kuroiwa, 1982; Nishibayashi & Kuroiwa, 1985). In most cases, spherical nucleoids become slightly elongated, then become dumbbell-shaped and are finally pinched off at the middle to form two spherical daughter nucleoids of similar size. Mitochondrial nucleoids shown in Fig. 4B–D in this investigation.
Fig. 4. Mitochondrial nucleoids stained with DAPI. A. Growing cell. B–D. Three chosen photos suggesting the division of nucleoids. B. Elongated nucleoids. C. Pair of nucleoids positioned close to one another. D. Pair of nucleoids. E. Cell with two daughter nuclei. F. Dividing cell. G. Young cell soon after cytokinesis. a, assimilation product; n, nucleus. A, E–G, x1900. B–D, x7700.
are very similar in appearance to the dividing nucleoids described to date. They probably are the dividing nucleoids. Nishibayashi & Kuroiwa (1985) showed that dumbbell-shaped nucleoids in *Paramecium caudatum* and *Allium cepa* contained about twice the amount of DNA found in spherical nucleoids. One of the characteristics of the mitochondria in *E. gracilis* is that mitochondrial division does not immediately follow the division of nucleoids. The mitochondrial network increases in volume in proportion to, and according to, the number of nucleoids and then it divides in two at cytokinesis.

There have been several reports on the timing of synthesis of mitochondrial DNA during the cell cycles of various organisms. The synthesis of mitochondrial DNA in *P. polycephalum* occurs at a different time to that of nuclear DNA synthesis (Kuroiwa et al. 1978). Mitochondria in *Tetrahymena pyriformis* have been reported to incorporate thymidine at all stages of the cell cycle independently of nuclear DNA synthesis (Parsons & Rustad, 1968). By contrast, both the mitochondrial and nuclear DNA in *Euglena* appeared to be synthesized at the beginning of and during the first phase of cell division from an examination of incorporation of tritiated adenine (Calvayrac et al. 1972). In the present investigation, we did not find any specific figures of mitochondrial nucleoids that suggested the synthesis of mitochondrial DNA at the beginning of nuclear division in *Euglena*. As shown in Fig. 6, the number of mitochondrial nucleoids increased as the cell increased in volume after cytokinesis. If the amount of mitochondrial DNA is proportional to the number of nucleoids, then the mitochondrial DNA is probably synthesized continuously during the non-dividing, growing phase.

**References**


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