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[Original]

Immunogold Localization of Mitochondrial DNA in Synchronized Cells of *Euglena gracilis* Z

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Euglena nuclear, mitochondrial and chloroplast DNA was visualized by immunoelectron microscopy. Normal sized reticulate mitochondria contain discrete separate DNA molecules. At specific cell cycle stages giant mitochondria are formed by fusion. Mitochondrial DNA molecules are homogenously distributed throughout the enlarged matrix of giant mitochondria suggesting that direct interactions among otherwise separately existing mitochondrial DNA molecules occurs in the enlarged matrix. If mitochondria differ in their genetic composition, temporary formation of giant mitochondria through mitochondrial fusion would provide an opportunity during the cell cycle for gene exchange between individual mitochondrial DNA molecules and for mitochondrial DNA replication prior to mitochondrial division.

Key words: *Euglena gracilis* strain Z, Freeze-substitution method, Immuno-electron microscopy, Synchronous culture, Mitochondrial DNA

Introduction

Osafune *et al.*[1,2] examined the morphology of mitochondria in cell populations of *Chlamydomonas reinhardtii* Dangeard and *Euglena gracilis* strain Z in synchronous culture by high resolution fluorescence microscopy, serial ultrathin sectioning-electron microscopy, and computer graphics analysis, and found that mitochondria undergo dynamic morphological changes such as fusion, fission, and branching in the cell cycle. Furthermore, we found for the first time such phenomena in chloroplasts, and we clarified their arrangement in the cell cycle of *Euglena*[2]. Consequently, direct analysis of the relationship between morphological data and physiochemical findings of mitochondria became possible. Mitochondria in *Euglena* and *Chlamydomonas* become giant form by fusion, and cause temporary reduction of respiratory function, suggesting that giant mitochondria play roles in the cell cycle other than the primary respiratory function[3-6]. Clarification of such phenomena is very important for our understanding of life processes.

In this study, we attempted to elucidate the importance of the temporary change in intracellular organelles that become giant form with changes in biological functions, and examined the localization of mitochondrial DNA using *Euglena gracilis* strain Z cells in synchronous culture[7,8].

Materials and Methods

Synchronized culture

Euglena gracilis strain Z was obtained from the Algal Culture Collection in the University of Tokyo (IAM Z-6), and has been maintained in Department of Life Science, Nippon Sport Science University. Cells of *Euglena* were synchronized under a 14 hour light : 10 hour dark regimen at 26℃ under photoautotrophic culture

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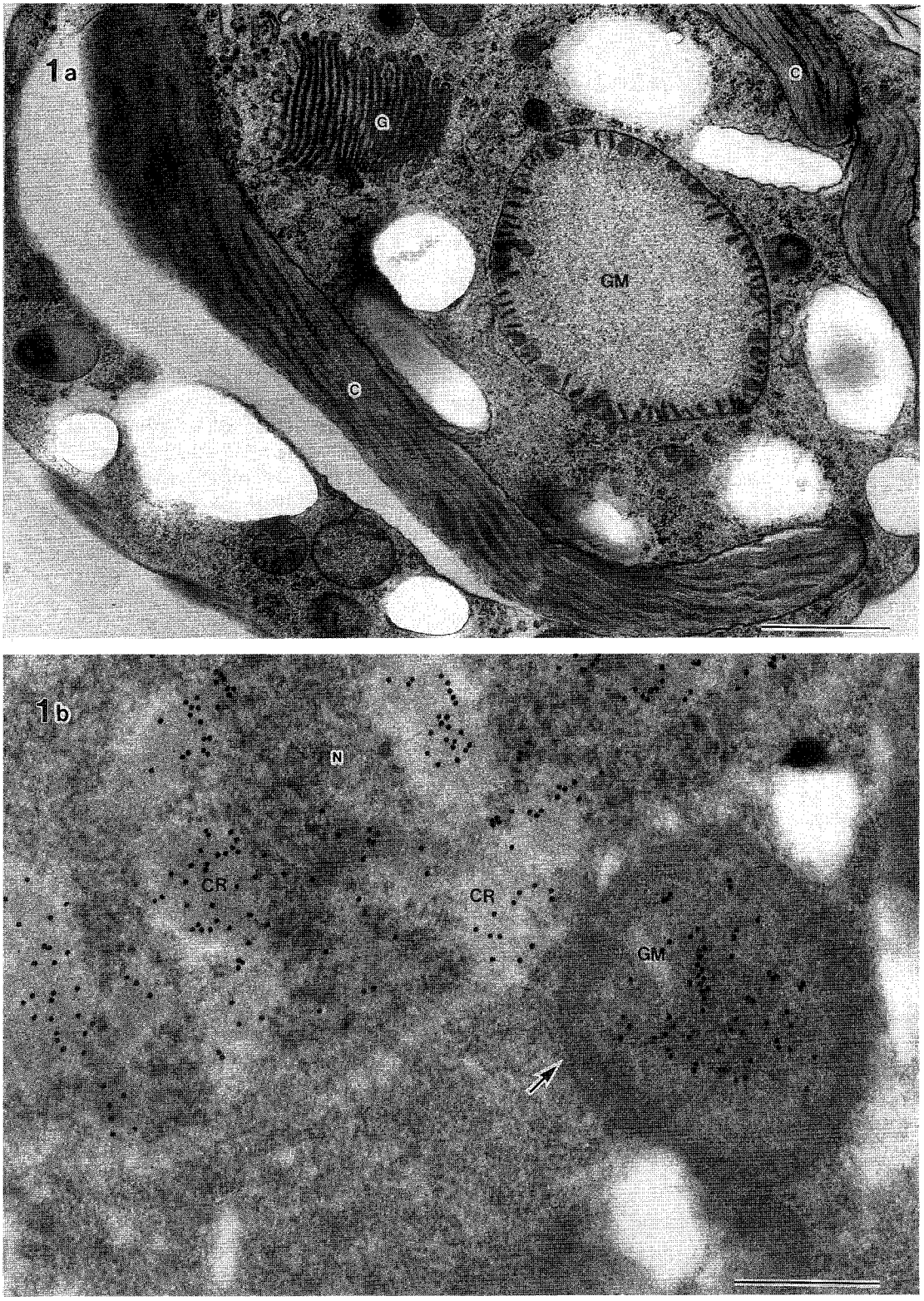


Fig. 1-a, 1-b.

conditions. The cell suspension was aerated with 1.5% CO_2 in air. Details of the synchronized culture have been described previously[7]. The light intensity was 6000 lux at the surface of the culture container in this work. In the experiments, the synchronized cells were placed under a 14 hour light : 10 hour dark cycle or in continuous light[9].

Freeze-substitution method

Cells of *Euglena gracilis* strain Z were placed in liquid propane cooled with liquid nitrogen (−180℃). The cell samples were in cooled acetone (−85℃) containing 1% glutaraldehyde, for 18 hours to replace ice in cells[10]. The temperature of the acetone was raise to −20℃ for 2 hours, and then allowed to rise gradually to room temperature over a period of 2 hours. The cells were washed with acetone and embedded in Epon 812[11].

Immunoelectron microscopy

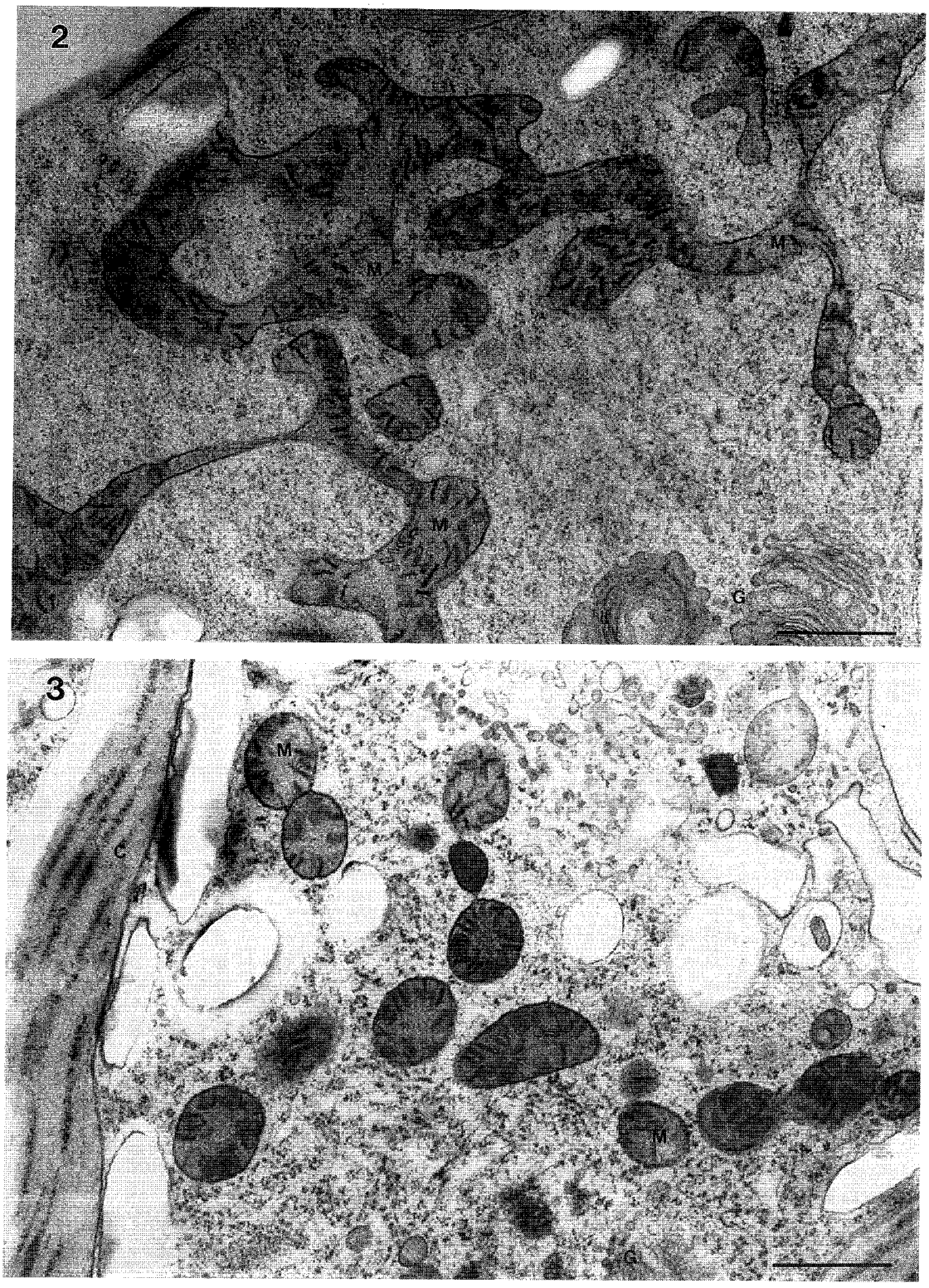
Cells of *Euglena gracilis* strain Z were fixed with 1% (v/v) glutaraldehyde for 45 min in an ice bath. They were then washed with phosphate buffer (pH 7.4) and embedded in 2% agarose which was cut into small cubes. The cubes were dehydrated with an ethanol series, placed in acetone, and embedded in L. R. White resin, medium grade (London Resin Co., Ltd., Hampshire, England). Thin sections cut on a Sorvall MT-1 micro-tome were placed on nickel slit grids which were floated section side down on drops of 0.01 M potassium phosphate buffer (pH 7.4) containing 0.85% (w/v) sodium chloride (phosphate-buffered saline, PBS) and 0.5–1.0% (w/v) bovine serum albumin for 30–60 min at room temperature. The grids were then floated on PBS containing mouse anti-DNA monoclonal MAB-030 antibodies (Chemicon, CA, USA) at 20–40 fold dilution overnight at 4℃. The grids were washed twice in PBS plus 0.05% (w/v) Tween 20 (Sigma), and floated on PBS containing a 20 fold dilution of goat anti-mouse IgG 15 nm gold conjugate (Zymed, San Francisco, CA, USA) for 20 min. They were subsequently floated on 3% (w/v) uranyl acetate for 30 min, and after drying viewed with a JEOL 100 CX electron microscope at 80 kV[12–14]. When cell sections were treated with 0.5 mg/ml DNase I (Sigma) before application of primary antibodies in the normal procedure, no labeling of cellular structures with gold was seen[15].

Results

As shown previously, giant mitochondria appear in cells at 9 hours of synchronous culture in *Euglena gracilis* strain Z[7,16]. The cells were

Fig. 1-a. Electron microscopic profile of giant mitochondrion in a *Euglena*.
The cells taken from a synchronous culture 9 hour after onset of the light period. Cells of *Euglena gracilis* Z were collected by centrifugation, and then put into liquid propane that had been cooled with liquid nitorogen (−198℃) and rapidly frozen using Leica EM-cryofixation-equipment. The frozen cells were transferred to OsO_4 dissolved in acetone at −80℃ and incubated for 48 hours, then the temperature of the samples was gradually increased (Fig. 1-a). The mitorix of giant mitochondrion (GM) was very large in volume and no cresitae were found to extend into the middle of the matrix.

Fig. 1-b. Immunoelectron microscopic profile of giant mitochondrion in a *Euglena*.
The cells of *Euglena gracilis* Z taken from a synchronous culture 9 hour after onset of the light period. The arrow shows giant mitochondrion (GM). Using a monoclonal antibody to DNA from mouse together with gold-conjugated anti-mouse IgG antibody (Au-Anti IgG) it has been possible to visualize the DNA of the nucleus, chloroplast and mitochondrion of *Euglena gracilis* Z with the electron microscope. Immuno-electron microscopic localization of mitochondrial DNA in *E. gracilis* Z shown that DNA molecules gather in the large matrix of the giant mitochondrion (GM).
Abbreviations used here and on subsequent pictures: C, chloroplast; CR, chromosome; GM, giant mitochondrion; G, Golgi apparatus; M, mitochondrion; N, nucleus.



Figs. 2 and 3.

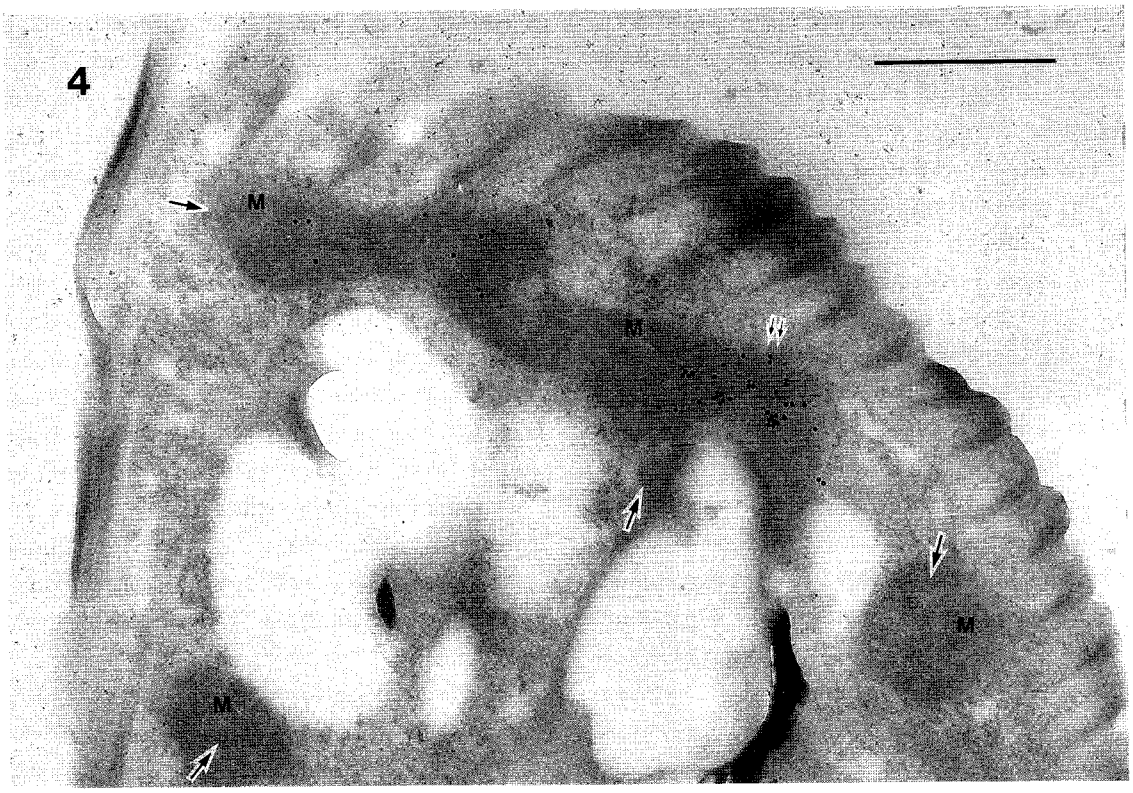


Fig. 4. Immuno-electron microscopic profile of mitochondria in a *Euglena*.
The cells of *Euglena gracilis* Z taken from a synchronous culture 14 hour after onset of the light period. The single arrow shows mitochondria (M). The double arrows indicate gold particles (mitochondrial DNA) in the small mitochondria (M). Immunoelectron microscopic localization of mitochondrial DNA in *E. gracilis* Z shown that DNA molecules are separately localized in the reticulate form of mitochondria (M).

fixed with rapid freezing and substitution, and the cross section of giant mitochondria was observed by electron microscopy (Fig. 1-a). The matrix of the giant mitochondria were wide, while the cristae were characteristically localized in the vicinity of the inner membrane (Fig. 1-a). The giant mitochondria observed in the cells of *Euglena* obtained at 9 hours of synchronous culture showed temporary reduction of respiratory function.

Figures 2 and 3 show part of a cell of *Euglena* obtained at 14 hours of synchronous culture. The cross section of the mitochondria was circular or oval with a diameter of about 1 mm (Figs. 2 and 3). In the cells in this stage reconstructed by computer graphics analysis, the morphology of mitochondria differed from what had been expected based on their cross sections (data not shown). Figure 2 shows reticular mitochondria in cells at 14 hours of synchronous culture.

Figures 1-b and 4 show the localization of

Figs. 2. and 3. Electron microscopic profile of small mitochondria in a *Euglena*.
The cells of *Euglena gracilis* Z taken from a synchronous culture 14 hour after onset of the light period (Figs. 2 and 3). Cells were fixed by the rapid freeze substitution method as same as Fig. 1-a. The mitochondrial profiles existing separately on the section in Fig. 3 are parts of a complicated ramified structure as shown in Fig. 2.

Euglena DNA visualized by immuno-electron microscopy after treatment with the anti-double stranded DNA monoclonal antibody, indicating that colloidal gold particles were localized specifically in 3 intracellular organelles (Fig. 4), *i.e.*, nuclear chromosomes, mitochondria, and chloroplasts. Immuno-electron microscopic observation of these samples demonstrated for the first time that colloidal gold particles were homogeneously localized in the matrix of giant mitochondria (Fig. 1-b). In the nuclei, colloidal gold particles were localized specifically in chromosomes (Figs. 1-b, 4).

Figure 4 shows the cells observed by immunoelectron microscopy, which were obtained by synchronous culture for 14 hours and stained with the anti-double stranded DNA monoclonal antibody. The arrows indicate cross sections of mitochondria, and the double arrows indicate the distribution of mitochondrial DNA as identified by colloidal gold particles (Fig. 4). On the cross section of some mitochondria, only a few colloidal gold particles were distributed in specific regions of the matrix, while in others, DNA molecules were not observed (Fig. 4). In the stage when mitochondria are changed to the reticular morphology, it was found that DNA was characteristically distributed in the mitochondrial matrix.

Discussion

The morphology, size, mobility, and number of mitochondria can be observed to change in the cytoplasm of living cells by light microscopy[17] The morphology of mitochondria also varies depending on physiological conditions. Osafune *et al.*[1] discovered in 1972 that mitochondria in *Chlamydomonas* show dynamic changes in the cell cycle, and arranged them in the cell cycle for the first time. Calvayrac *et al.*[18] in France reported on these phenomena in the cell cycle of *Euglena* in synchronous culture at almost the same time as us. Arnold *et al.*[4] constructed three-dimensional images of mitochondria by serial ultra-thin sectioning and electron microscopy, and confirmed the occurrence of complicated changes in the morphology of mitochondria. Ehara *et al.*[5,6] examined mitochondria in cell populations of *Chlamydomonas* in synchronous culture by DASPMI staining and fluorescence microscopy, classified the morphology of mitochondria, statistically analyzed, and confirmed the phenomena discovered by Osafune[1,2]. Ehara *et al.*[6] found that giant mitochondria are formed twice in the early cell cycle and before cell division. Based on data such as the number, external morphology, volume and surface area of mitochondria obtained by DASPMI fluorescence microscopy and three-dimensional computer graphics analysis, it was confirmed that giant mitochondria were formed by fusion. As shown on the electron microscopic image of *Euglena* cells fixed by rapid freezing and substitution (Fig. 1-a), temporary formation of giant mitochondria has been reported with *Polytoma* and *Candida*, suggesting that it is biologically common[19,20].

Mitochondria proliferate only by fission within cells[1,5,7,8]. Therefore, it is considered that mitochondria are not made *de novo*. It is speculated that mitochondria are doubled by fission while maintaining their DNA, and mitochondrial DNA is subjected to free recombination when they fuse. Much remains to be solved about the importance of their showing dynamic changes during the cell cycle and temporarily becoming giant forms.

In this study, *Euglena* mitochondrial DNA was examined in the time course by fixation with rapid freezing and substitution, staining with the anti-DNA monoclonal antibody, by immunoelectron microscopy[11,13]. Circular or oval reticulate mitochondria with a diameter of about 1 mm are observed in cross section by freeze substitution electron microscopy (M: mitochondrion, C: chloroplast, G: Golgi apparatus).

Cristae are seen protruding from the inner membrane into the matrix. Figure 1-a and b shows a giant mitochondrion (GM) in a cell of *Euglena gracilis* Z sampled from a synchronous culture 9 hours after the onset of light. The mitochondrial matrix is enlarged significantly while the cristae have not increased proportionately in size appearing as short protrusions of the inner membrane (Fig. 1-a). DNA was localized in the 9 hour cells by immuno-electron microscopy after treatment with a mouse monoclonal antibody to double stranded DNA together with gold-conjugated anti-mouse IgG antibody. Colloidal gold particles localized DNA to the nucleus (Fig. 1-b; CR), and to mitochondria (M). In nuclei, colloidal gold particles were localized specifically to chromosomes (CR). Cross sections of normal size reticulate mitochondria in cells sampled 14 hours after the onset of light showed only a few colloidal gold particles confined to specific regions of the matrix (Fig. 4; double arrows) while in other mitochondria (M), DNA molecules were not observed (Fig. 4). Individual mitochondrial DNA molecules appear to be localized to discrete spatially separated regions of the reticulate mitochondrial matrix. Serial ultra-thin sectioning and immuno-electron microscopy found colloidal gold particles homogeneously distributed throughout the matrix of giant mitochondria (GM).

The results of staining with the anti-DNA monoclonal antibody and immuno-electron microscopy suggested that the temporary formation of giant mitochondria by reduction of respiratory function occurs for the purpose of recombination, regulation, and information exchange between mitochondrial DNA by fusion of reticular mitochondria and several independent small-sized mitochondria. This study suggested that the temporary formation of giant mitochondria provides such an occasion in the cell cycle. This demonstrates for the first time the distribution of mitochondrial DNA throughout the matrix of giant mitochondria of *Euglena gracilis* strain Z suggesting that individual DNA molecules are not spatially separated as found in reticulate mitochondria. If mitochondria differ in their genetic composition, the temporary formation of giant mitochondrion through mitochondrial fusion would provide an opportunity during the cell cycle for gene exchange between individual mitochondrial DNA molecules and for mitochondrial DNA replication prior to mitochondrial division.

Conclusion

Using a monoclonal antibody to DNA from mouse together with gold-conjugated anti-mouse IgG antibody (Au-Anti IgG) it has been possible to visualize the DNA of the nucleus, chloroplast and mitochondrion of *Euglena gracilis* strain Z in the electron microscope[13]. In this work, immunoelectron microscopic localization of mitochondrial DNA in *Euglena* shown that DNA molecules gather in the large matrix of the giant mitochondrion, while they are separately localized in the reticulate form, suggesting that direct interactions among otherwise separately existing mitochondrial DNA molecules occur in the large matrix. If each mitochondrion is somewhat different in its genetic makeup, mitochondrial gene exchange might also occur during the fusion of different mitochondria.

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