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Isolation and phenotypic characterization of *Chlamydomonas reinhardtii* mutants defective in chloroplast DNA segregation

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Summary. Each wild-type *Chlamydomonas reinhardtii* cell has one large chloroplast containing several nuclei (nucleoids). We used DNA insertional mutagenesis to isolate Chlamydomonas mutants which contain a single, large chloroplast (cp) nucleus and which we named *moc* (monokaryotic chloroplast). DAPI-fluorescence microscopy and microphotometry observations revealed that *moc* mutant cells only contain one cp-nucleus throughout the cell division cycle, and that unequal segregation of cpDNA occurred during cell division in the *moc* mutant. One cell with a large amount of cpDNA and another with a small amount of cpDNA were produced after the first cell division. Unequal segregation also occurred in the second cell division, producing one cell with a large amount (about 70 copies) of cpDNA and three other cells with a small amount (only 2–8 copies) of cpDNA. However, most individual *moc* cells contained several dozen cpDNA copies 12 h after the completion of cell division, suggesting that cpDNA synthesis was activated immediately after chloroplast division. In contrast to the cpDNA, the mitochondrial (mt) DNA of the *moc* mutants was observed as tiny granules scattered throughout the entire cell. These segregated to each daughter cell equally during cell division. Electron-microscopic observation of the ultrastructure of *moc* mutants showed that a low-electron-density area, which was identified as the cp-nucleus by immunoelectron microscopy with anti-DNA antibody, existed near the pyrenoid. However, there were no other structural differences between the chloroplasts of wild-type cells and *moc* mutants. The thylakoid membranes and pyrenoid were identical. Therefore, we propose that the novel *moc* mutants are only defective in the dispersion and segregation of cpDNA. This strain should be useful to elucidate the mechanism for the segregation of cpDNA.

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Keywords: *Chlamydomonas reinhardtii*; Chloroplast DNA; Chloroplast nucleus; Chloroplast DNA segregation; Chloroplast division.

Abbreviations: DAPI 4′,6-diamidino-2-phenylindole; VIMPCS video-intensified microscope photon-counting system.

Introduction

Plastids in plant cells contain multiple copies of the plastid genome, which are organized into DNA-protein complexes called plastid (pt) nuclei (nucleoids). The pt-nucleus is the functional unit of pt-DNA, where ptDNA replication, transcription, and segregation occur (Kuroiwa 1991). The multiplication of a plastid in the cytoplasm requires both segregation of the pt-nuclei and plastid kinesis (the binary division of a preexisting plastid by the plastid-dividing ring) (Kuroiwa et al. 1998). The segregation of pt-nuclei is very important in the transmission of the plastid genome to succeeding generations of plastids. However, little is known of the molecular mechanism that controls pt-nuclear segregation.

In higher plants, dynamic changes in the morphology and number of pt-nuclei during the course of plastid development and differentiation have been studied in various species by electron microscopy and fluorescence microscopy with 4′,6-diamidino-2-phenylindole (DAPI) staining (Hashimoto 1985, Miyamura et al. 1986, Lindbeck and Rose 1987, Sato et al. 1997). These cytological observations suggest that the association of pt-nuclei with a thylakoid or envelope membrane and growth of the membranes might play an important role in the segregation of ptDNA,

since changes in the spatial arrangement of pt-nuclei were consistent with the biogenesis and organization of the membranes. In spinach chloroplasts, the ptDNA is bound to the thylakoid membrane in the inverted-repeat region near the rRNA genes (Liu and Rose 1992). The pt-nucleus consists of ptDNA and various DNA-binding proteins (Hansmann et al. 1985; Nemoto et al. 1989, 1990; Sato et al. 1997). Some plastid DNA-binding proteins may be involved in binding the pt-nuclei to the plastid membrane system to segregate ptDNA. Sato et al. (1993, 1998) discovered a PEND (plastid envelope DNA-binding) protein that anchors the pt-nuclei to the inner plastid envelope in the pea. One probable function of the PEND protein is the segregation of ptDNA.

In the unicellular green alga *Chlamydomonas reinhardtii*, the behavior of the cp-nuclei during the cell division cycle and the course of batch cultures have been investigated intensively (Kuroiwa et al. 1981, Nakamura et al. 1986, Ehara et al. 1990). The division and segregation of cp-nuclei in a culture synchronized in a 12 h each light and dark regimen progresses in tandem with morphological changes in the cp-nuclei. The approximately ten ovoid cp-nuclei, which are dispersed throughout the chloroplast at the beginning of the light period, aggregate around the pyrenoid toward the end of the light period. Then the cp-nuclei are transformed into reticulated threads spread throughout the chloroplast. The cp-nuclei are equally apportioned to daughter chloroplasts and combine to form granular bodies (Ehara et al. 1990). This periodical change in morphology (i.e., the dispersion and aggregation of cp-nuclei) may be related to the segregation of cpDNA.

Recently, the development of transformation methods for nuclear genomes with glass beads has significantly enhanced the utility of *C. reinhardtii* (Kindle et al. 1989, Kindle 1990, Gumpel and Purton 1994). By this procedure, various mutants have been identified with defects in different cellular processes such as photosynthesis, cell motility, phototaxis, salt tolerance, and cell division (for a review, see Stevens and Purton 1997). In several cases, the affected gene has been cloned and shown to restore the wild-type phenotype. In addition, *C. reinhardtii* cells are suitable for the study of chloroplast proliferation including the segregation of cpDNA because they contain only a single chloroplast, whose division cycle is synchronized with the mitotic cycle (Goodenough 1970, Mihara and Hase 1971).

To study chloroplast proliferation, we generated a number of mutants by insertional mutagenesis following the transformation of a *nit1* mutant with a plasmid harboring the *NIT1* gene (Kindle 1990). First, the mutants generated were screened to identify mutants with altered cp-nuclei morphology, because the regular changes in the cp-nuclei morphology may contribute to the equal segregation of cpDNA. Mutants with a defect in cpDNA segregation were found in the mutant lines with altered cp-nuclei morphology and were characterized by fluorescence and electron microscopy. This initial phenotypic characterization is the first step toward elucidating the molecular mechanism for the segregation of cpDNA in the chloroplast.

Material and methods

Strain and cultures

Chlamydomonas reinhardtii nit1/cw15, containing a stable *nit1-305* mutation in the nitrate reductase structural gene and a *cw15* mutation which confers a cell-wall-less phenotype, was the generous gift of R. Kamiya (University of Tokyo) and used for transformation. For growth, this strain requires supplemental ammonium as a source of nitrogen. The Algal Culture Collection of the Institute of Applied Microbiology, University of Tokyo provided strains c-562 (Mt^+) and c-9 (Mt^-) used as the wild-type strain. Chlamydomonas cells were grown in liquid SG II medium (Sager and Granic 1953, Harris 1989) for untransformed *nit1/cw15* strain and wild-type strain, and in SG II-NO_3 medium (Kindle 1990) to select transformants, under a 12 h light (light intensity ca. 5,000 lux) and dark cycle, at 22 °C.

Plasmid DNA for transformation

The plasmid pMN24 was a gift of R. Kamiya (University of Tokyo). pMN24, a plasmid containing the cloned wild-type nitrate reductase gene *NIT1* (Fernandez et al. 1989, Kindle et al. 1989), was linearized with *Eco*RI to transform the *nit1/cw15* strain. After restriction digestion, the plasmid DNA was extracted with phenol-chloroform-isoamyl alcohol (25 : 24 : 1, v/v), precipitated with ethanol, and resuspended in TE (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid [EDTA]).

Transformation

The glass bead method was used for nuclear transformation (Kindle 1990). The *nit1/cw15* cells were grown in liquid SG II medium with aeration under continuous light for 4 days (late logarithmic phase) and concentrated by centrifugation. The cell walls were removed from the cells by incubating them in 3 ml of gamatic autolysin for 30–60 min at 30 °C. The *nit1/cw15* strain is cell-wall-deficient strain, yet high-efficiency transformation seemed to require pretreatment with gametic autolysin to remove the cell walls (Kindle 1990). Cells were harvested from the autolysin by centrifugation, resuspended in SG II medium, and transformed immediately to avoid cell wall regeneration. 1 µg of linearized pMN24 DNA was agitated three times with 1×10^8 (0.4 ml) cells in the presence of 5% polyethylene glycol 8,000 (Sigma) and 0.3 g of acid-washed, sterilized glass beads (0.5 mm in diameter; M & S Instruments, Osaka, Japan) for 15 s at

top speed on a Fisher Vortex Gene II mixer in conical disposable polypropylene centrifugation tubes (volume, 15 ml; Corning) at 30 s intervals. Immediately after agitation, the cells were incubated in 50 ml of SG II medium for 1 h at 25 °C with shaking. The cells were then collected by centrifugation and resuspended in 0.8 ml of SG II-NO_3 medium, and spread on 1.5% SG II-NO_3 agar plates to select the Nit^+ transformants. The plates were allowed to dry and then sealed with Parafilm and incubated in a growth chamber (temperature, 22 °C) under a 12 h each light and dark illumination schedule. Nit^+ transformant colonies were visible after 7–10 days of growth.

Screening the mutants with fluorescence microscopy

Transformant colonies were allowed to grow on plates for 7–10 days and then transferred onto fresh SG II-NO_3 plates with a toothpick. Two days after the transfer, a part of each colony was placed on a glass slide and fixed with 1% glutaraldehyde which had been dissolved in culture medium to give a final concentration of 0.3% for 5 min and then stained with a solution of 1 μg of DAPI per ml of TAN buffer (20 mM Tris-HCl, pH 7.6, 0.5 mM EDTA, 7 mM 2-mercaptethanol, 1.2 mM spermidine, 0.4 mM phenylmethyl sulfonylfluoride). A coverslip was placed over the mixture and lightly pressed down. After 20 min, the slides were examined under a fluorescence microscope (BHS-RFC; Olympus, Tokyo, Japan). The fluorescence due to DAPI was observed under excitation with UV light and the red autofluorescence of chlorophyll *a* was eliminated with a suppression filter. The procedure for mitochondrial DNA staining of living cells was the same as described previously (Nishimura et al. 1998). Photographs were taken on 35 mm black-and-white Fuji PREST 400 film.

Quantification of cpDNA by fluorometry

To determine the DNA content of each chloroplast, a quantitative analysis of the fluorescence image of DAPI-stained cp-nuclei was carried out with VIMPCS (video-intensified microscope photon-counting system C1966-20; Hamamatsu Photonics Ltd., Hamamatsu, Japan) connected to the ocular lens of the fluorescence microscope. The number of photons from the DAPI fluorescence was measured with a video image analyzer. T4 phage was used to convert the fluorescence intensity into an absolute amount of DNA, since the size (170 kb) and G + C content of its DNA are very similar to those of chloroplast DNA (Miyamura et al. 1986). To further convert the results into plastid genome equivalents, we used 196 kb as the plastid genome size of *C. reinhardtii*. In this study, we determined the DNA content of each chloroplast in terms of cp-genome number.

Electron microscopy

For ultrastructural observations, cells were fixed with 2% (v/v) glutaraldehyde in the culture medium for 2 h at 4 °C. The cells were then washed three times with 20 mM sodium cacodylate buffer (pH 7.2) and post-fixed with 1% (w/v) OsO_4 in the same buffer for 2 h at 4 °C. The fixed cells were dehydrated in an ethanol series, infiltrated with propylene oxide, and embedded in Spurr's resin. Sections 80 nm thick were double-stained with uranyl acetate and lead citrate and viewed in an electron microscope (JEM-1200 EXII; JEOL, Tokyo, Japan).

For immunoelectron microscopy, the cells were fixed with 2% (v/v) glutaraldehyde in the culture medium for 2 h at 4 °C. After washing with culture medium three times, they were dehydrated with an ethanol series and embedded in LR white resin (The London Resin Co., Woking, Surrey, U.K.), which was polymerized for 36 h in gelatin capsules in an oven (temperature 50 °C). Serial thin sections, each 80 nm thick, were attached to Formvar-coated nickel grids. Thin sections were treated successively with PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4) containing 0.05% Tween-20 for 15 min, 5% bovine serum albumin (BSA) in PBS for 30 min at room temperature, and then 10 μg of monoclonal antibody to single- and double-stranded DNA (Boehringer Mannheim Biochemica, Mannheim, Federal Republic of Germany; cat. no. 980986) per ml diluted fivefold with PBS-supplemented 5% BSA for 1 h at 37 °C. After washing several times with PBS (pH 7.4) containing 0.05% Tween-20, the sections were treated for 1 h at 37 °C with goat antibodies against anti-mouse IgM conjugated to 10 nm colloidal gold particles (EY Lab. Inc., San Mateo, Calif., U.S.A.), which were diluted eightyfold with PBS-supplemented 5% BSA. The sections were then washed several times in 0.05% Tween-20 in PBS (pH 8.2), once in distilled water, and then stained with uranyl acetate.

Results

Isolation of mutants with altered cp-nucleus morphology

To generate mutants by insertional mutagenesis, plasmid pMN24, which contains the nitrate reductase structural gene (*NIT1*), was digested with *Eco*RI and transformed into *nit1/cw15* cells. Nit^+ transformants were selected by growth on SG II-NO_3 plates in which nitrate is the sole source of nitrogen. Several hundred Nit^+ transformants (colonies) were typically obtained per microgram of plasmid DNA from 1×10^8 cells.

The distribution, number, size, and shape of the cp-nuclei were observed by DAPI-fluorescence microscopy. Of 3,000 transformants screened, 6 independent cell lines were recovered in which the morphology of the cp-nucleus was altered. These mutants were classified into three phenotypic classes. The cp-nuclei in a typical wild-type cell are usually observed as 8–10 small granules scattered throughout the stroma (Fig. 1 a). The first class of mutant recovered from 4 independent cell lines (A84, G60, G33, and I29) had only one large cp-nucleus, which was located in the center of the chloroplast (Fig. 1 b). We called these cell lines *moc*, for monokaryotic chloroplast. In the *moc* mutants, the cp-nucleus fluoresced very brightly with DAPI staining and the cp-nucleus was occasionally as large as the cell nucleus (Fig. 1 b). The second class, from one cell line (H57), had many (>15) tiny cp-nuclei spread throughout the chloroplast and was named *fic* for fine-granular cp-nuclei (Fig. 1 c). The third class, from one cell line (A55), had many large brightly-fluorescent cp-nuclei and was named *malc* for many large cp-nuclei (Fig. 1 d). In this strain, the size and shape of the cp-nuclei were irregular.

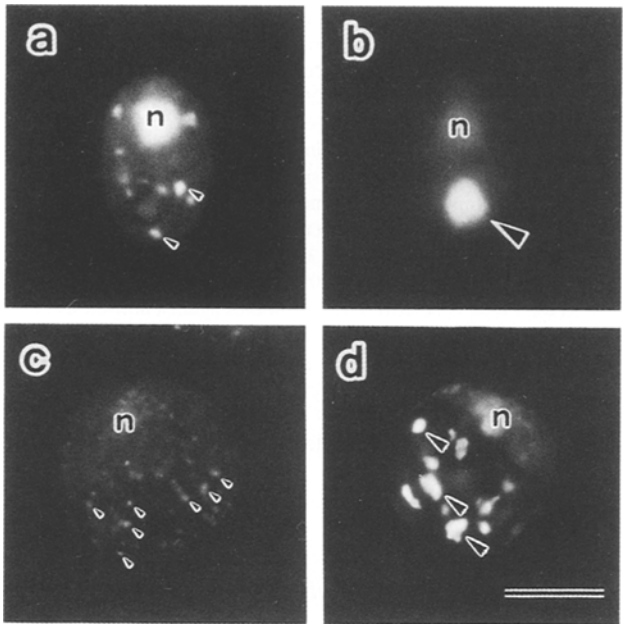

Fig. 1a–d. Fluorescence microscopic profiles of DAPI-stained *C. reinhardtii* cell and chloroplast nuclei. **a** Wild-type strain, **b** *moc* (monokaryotic chloroplast) mutant, **c** *fic* (fine-granular cp-nuclei) mutant, and **d** *malc* (many large cp-nuclei) mutant. *n* Cell nucleus. Arrowheads show cp-nuclei. Bar: 5 μm

Fluorescence microscopic observation of cp-nuclei during the cell division cycle

In order to investigate whether the altered morphology of cp-nuclei was accompanied by defects in the segregation of cpDNA, we observed chloroplasts from each of the three phenotypic classes and their cp-nuclei during the cell division cycle. Under a 12 h each light and dark regimen, most of the wild-type and mutant cells and chloroplasts divided stepwise into two and then four daughter cells during the subsequent 12 h dark period. In wild-type cells, the cp-nuclei were dispersed throughout the chloroplast as 8–10 small granules in nondividing cells at the sixth hour of the light period (Fig. 2a, b). After the first chloroplast division, both of the daughter chloroplasts contained equal numbers of tiny cp-nuclei (Fig. 2c, d). After the second chloroplast division, each of the four daughter chloroplasts also contained equal numbers of cp-nuclei (Fig. 2e, f). The cpDNA of *fic* and *malc* mutant cells also segregated equally during cell division (data not shown). In contrast, the cpDNA of *moc* mutant

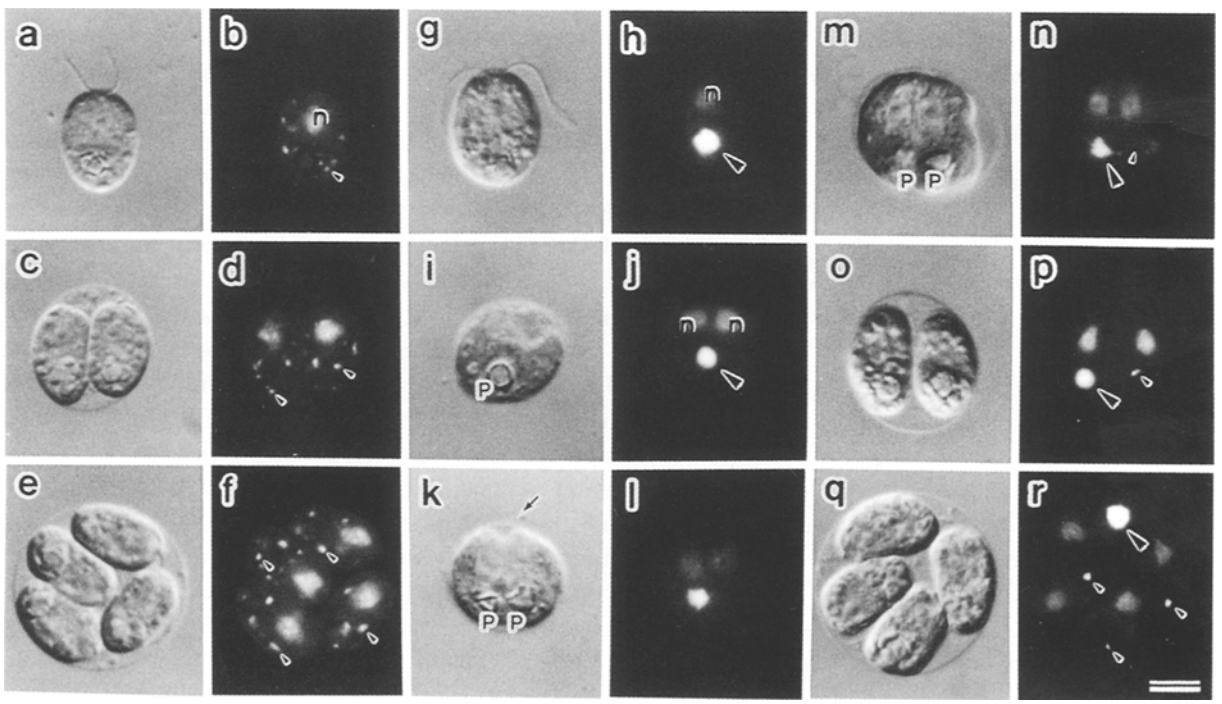

Fig. 2. Differential interference (a, c, e, g, i, k, m, o, and q) and fluorescence (b, d, f, h, j, l, n, p, and r) images with DAPI-stained wild-type (**a–f**) and *moc* mutant (**g–r**) cells of *C. reinhardtii* during the cell division cycle in SG II-NO_3 liquid culture. Photographs of different cells are arranged in sequence. **a**, **b**, **g**, and **h** Single-cell stage in the sixth hour of the light period; **c**, **d**, **o**, and **p** two cells after the first cell division in the second hour of the dark period; **e**, **f**, **q**, and **r** four cells after the second cell division in the sixth hour of the dark period. **i** and **j** After karyokinesis the cell contains a single cp-nucleus (arrowhead in **j**) in the center of the chloroplast. **k** and **l** At the beginning of cytokinesis, the cell begins to form a furrow (arrow in **k**). The pyrenoid has already divided, while the cp-nucleus is still a single body. **m** and **n** At the end of cell division, the cell contains a dividing cp-nucleus (arrowhead in **n**), which is tangled in the pyrenoid. *n* Cell nucleus, *p* pyrenoid. Large arrowheads show cp-nuclei. Bar: 5 μm

cells segregated unequally (Fig. 2g–r). The *moc* mutant cells contained one cp-nucleus in the center of the chloroplast throughout the single-cell phase (Fig. 2g, h). The cp-nucleus was located in the central region on the cleavage plane of the chloroplast at the beginning of chloroplast division following nuclear division (Fig. 2i, j). The cleavage furrow of the chloroplast progressed toward the posterior end of the chloroplast, while the pyrenoid was already divided at this time (Fig. 2k). The structure of the cp-nucleus began to relax at this stage (Fig. 2l) and then the cp-nucleus located on the cleavage plane was pulled apart during chloroplast division. The loose cp-nucleus generally became entangled in the pyrenoid structure (Fig. 2n). Extremely unequal segregation of the cpDNA was observed during this first chloroplast division (Fig. 2o, p). Although each daughter chloroplast contained one cp-nucleus, one of the daughter chloroplasts received most of the cpDNA and the other only had a very small amount of cpDNA. As a result, after the second chloroplast division, one of the daughter chloroplasts contained most of the cpDNA and the other three only contained a little cpDNA (Fig. 2q, r). In the *moc* mutants, the cp-nucleus never dispersed during the cell division cycle, suggesting that equal segregation of cpDNA requires dispersion of the cp-nuclei. Therefore, the *moc* mutants were chosen for further investigation.

Determining the cpDNA content of each daughter chloroplast

To quantify the cpDNA content of each chloroplast, the intensity of DAPI fluorescence from each cp-nucleus was measured with VIMPCS. The intensity of fluorescence corresponding to one copy of *C. reinhardtii* cpDNA (196 kb) was estimated by the mean intensity of fluorescence of T4 phage (170 kb) as a reference. Figure 3A and B shows the frequency of cells containing two or four daughters in a synchronized culture under a 12 h each light and dark cycle in liquid SG II medium without aeration. In wild-type cells, at the end of the light period each chloroplast of an undivided cell contained about 85 cpDNA copies (Fig. 3Ca). In the second hour of the dark period, each daughter chloroplast contained about 40 cpDNA copies (Fig. 3Cc). After the second chloroplast division, the cpDNA also segregated equally and each of the four daughter chloroplasts contained about 20 copies of the cp-genome (Fig. 3Ce). At the middle of the next light period, individual cells contained about 55 copies of the cp-genome (Fig. 3Cg). These results suggest that in the wild-type cells the cpDNA was divided equally between the daughter chloroplasts in both the first and second chloroplast divisions. In the *moc* mutant cells, the chloroplast of an undivided cell contained about 68 cp-genome copies at the end of the light period (Fig. 3Cb). The ratios of cp-genome copy numbers segregated to each daughter chloroplast after the first and second chloroplast divisions in the *moc* mutant cells were about 60 : 2.5 copies (Fig. 3Cd) and about 67.5 : 3.7 : 3.0 : 1.8 copies on average (Fig. 3Cf), respectively. In terms of the certainty of transmission of the cp-genome, it is noteworthy that in the *moc* mutant cells each daughter chloroplast received at least a few cpDNA copies without fail in every chloroplast division, in spite of the extremely unequal segregation. This result suggests that there is some other mechanism concerned with the transmission and maintenance of a minimal cp-genome, as is the case with the chrysophyte alga *Ochromonas danica* (Maguire et al. 1995). Surprisingly, in the middle of the next light period every daughter cell contained several dozen cp-genomes, and cells with a small amount of cpDNA were rarely observed (Fig. 3Ch). This result indicates that immediately after the second chloroplast division cpDNA synthesis was activated in the cells which had only received a small amount of cpDNA. There is some mechanism to maintain a certain copy number of the cp-genome in the *moc* mutant cells.

Mitochondrial nuclei in the moc mutant cells

To investigate the morphology and distribution of mitochondrial (mt) nuclei in the *moc* mutants, the mutant cells were stained with SYBR Green I (Nishimura et al. 1998) and observed under blue-light excitation. Mt-nuclei were spread throughout the cell as many tiny granules (Fig. 4a). During the cell division cycle, the mt-nuclei segregated to each daughter cell equally and were never concentrated in a distinct area (Fig. 4b, c), suggesting that the morphogenesis of mitochondrial and chloroplast nuclei is regulated independently.

Electron-microscopic observation

We investigated whether there was a relationship between changes in the distribution of cp-nuclei and the membrane system or other chloroplast structures in the *moc* mutants. Figure 5a and b shows electron

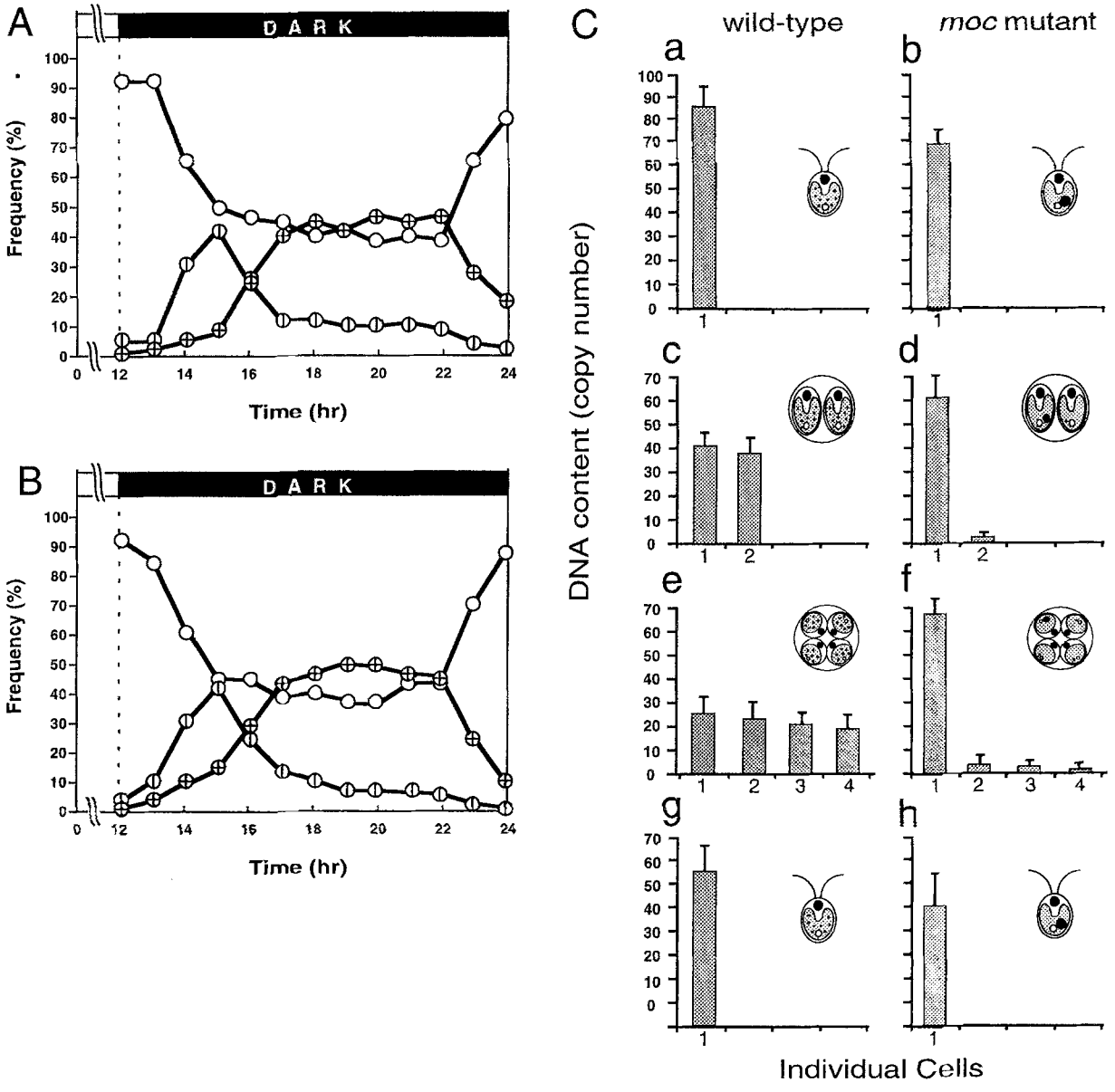

Fig. 3. Process of cell division during the dark period in a synchronized culture of wild-type (**A**) and *moc* mutant (**B**) cells. The frequency of each cell type was estimated by observing more than 200 cells taken from a culture at 1 h intervals. Changes in the frequency of cell types are shown. **C** Quantification of the DNA content of individual chloroplasts at each cell stage. VIMPCS-measured number of photons emitted by DAPI-stained cp-nuclei, which was used to calculate the cp-genome copy number (see Material and methods). On the fourth day of the culture, 20 cells at each stage were sampled at random and the fluorescence intensity of the cp-nuclei was measured. The DNA content of the single-cell stage at the twelfth hour of the light period was measured in wild-type (**a**) and *moc* mutant (**b**) chloroplasts. Twenty individual cells at the two- (**c** and **d**) and four-daughter-cell (**e** and **f**) stages are arranged in order of the fluorescence intensity of the cp-nuclei and the mean value is shown. (The cells were sampled after the first and the second chloroplast division at hours 2 and 6 of the dark period, respectively.) The number of cp-nuclei in a single cell at hour 6 of the light period following the four-daughter-cell stage was also measured (**g** and **h**). The typical appearance of the cell is illustrated in each panel. ○ Undivided cell, ⊕ divided into two daughters, ⊕ divided into four daughters

micrographs of thin sections of a typical chloroplast from a wild-type and a *moc* mutant cell. The chloroplast contains a single pyrenoid, which is surrounded by starch grains, and the thylakoid membranes are arranged in stacks of two to ten discs. The chloroplast regions free of thylakoids and stroma contained chloroplast ribosomes. An area with a lower electron density was recognized near the pyrenoid starch in typical *moc* mutant cells (Fig. 5b). This electron-transparent area where no chloroplast ribosomes were

observed seemed to be the cp-nucleus region, although no fibrillar structures were observed. In wild-type cells, identification of the electron-transparent area in the chloroplast was difficult. No differences in the chloroplast structures of wild-type and *moc* mutant cells were found, except for the electron-transparent area probably corresponding to the cp-nucleus.

To localize the cpDNA in the chloroplast more accurately, we detected cpDNA at the ultrastructural level using an immunogold electron microscopy technique. Figure 5 d shows serial section of a portion of a *moc* mutant cell. In the chloroplast, deposits of gold particles were specifically located near the pyrenoid, indicating that the electron-transparent area observed in Fig. 5 b was indeed the cp-nucleus. In wild-type cells, deposits of gold particles could be seen throughout the chloroplast as small granules (Fig. 5 c), as was observed with DAPI staining.

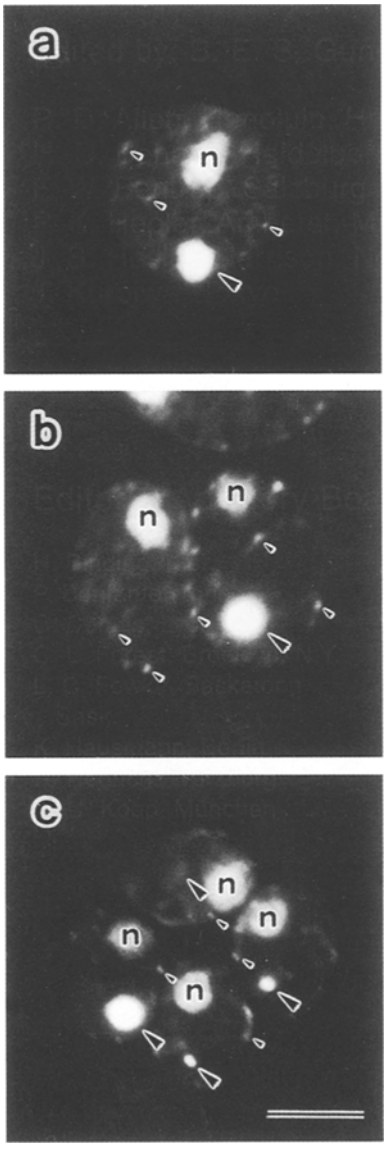

Fig. 4 a–c. Fluorescence images of *moc* mutant cells of *C. reinhardtii* during the cell division cycle with SYBR Green I staining. **a** Single-cell stage at hour 6 of the light period, **b** two cells after the first cell division at hour 2 of the dark period, and **c** four cells after the second cell division at hour 6 of the dark period. Large arrowheads show cp-nuclei and small arrowheads show mt-nuclei. *n* Cell nucleus. Bar: 5 μm

Discussion

This study is summarized in Table 1. In this study, Chlamydomonas mutants with an undispersed cp-nucleus and a defect in the segregation of cpDNA were isolated by insertional mutagenesis, and their phenotype was characterized by DAPI-fluorescence microscopy and electron microscopy. The cp-nuclei of *C. reinhardtii* change their appearance drastically during the cell division cycle (Kuroiwa et al. 1981, Ehara et al. 1990). Dynamic changes in the morphology of cp-nuclei have also been documented repeatedly in higher plants, including wheat (Hashimoto 1985, Miyamura et al. 1986, Marrison and Leech 1992), bean (Lindbeck and Rose 1987), rice (Sodmergen et al. 1991), and pea (Sato et al. 1993, 1997). In spite of differences in the localization of the cp-nuclei between plant species, the cpDNA is normally divided equally among the daughter chloroplasts during chloroplast division (Kuroiwa et al. 1981). However, the molecular mechanisms for separating cp-nuclei remain to be elucidated.

Table 1. Results of cytological studies of wild-type and *moc* mutant cells of *C. reinhardtii*

	Wild type	*moc*
Cell nucleus		
number	1	1
segregation	equal	equal
Chloroplast		
number of nuclei	7–12	1
DNA content (copy number)	80–90	65–75
DNA segregation	equal	unequal
thylakoid membrane stacks (no.)	2–12	2–10
pyrenoid diameter (μm)	1–2	1–2
pyrenoid segregation	equal	equal
DNA replication	active	active
Mitochondria		
number of nuclei	15–50	15–50
DNA segregation	equal	equal

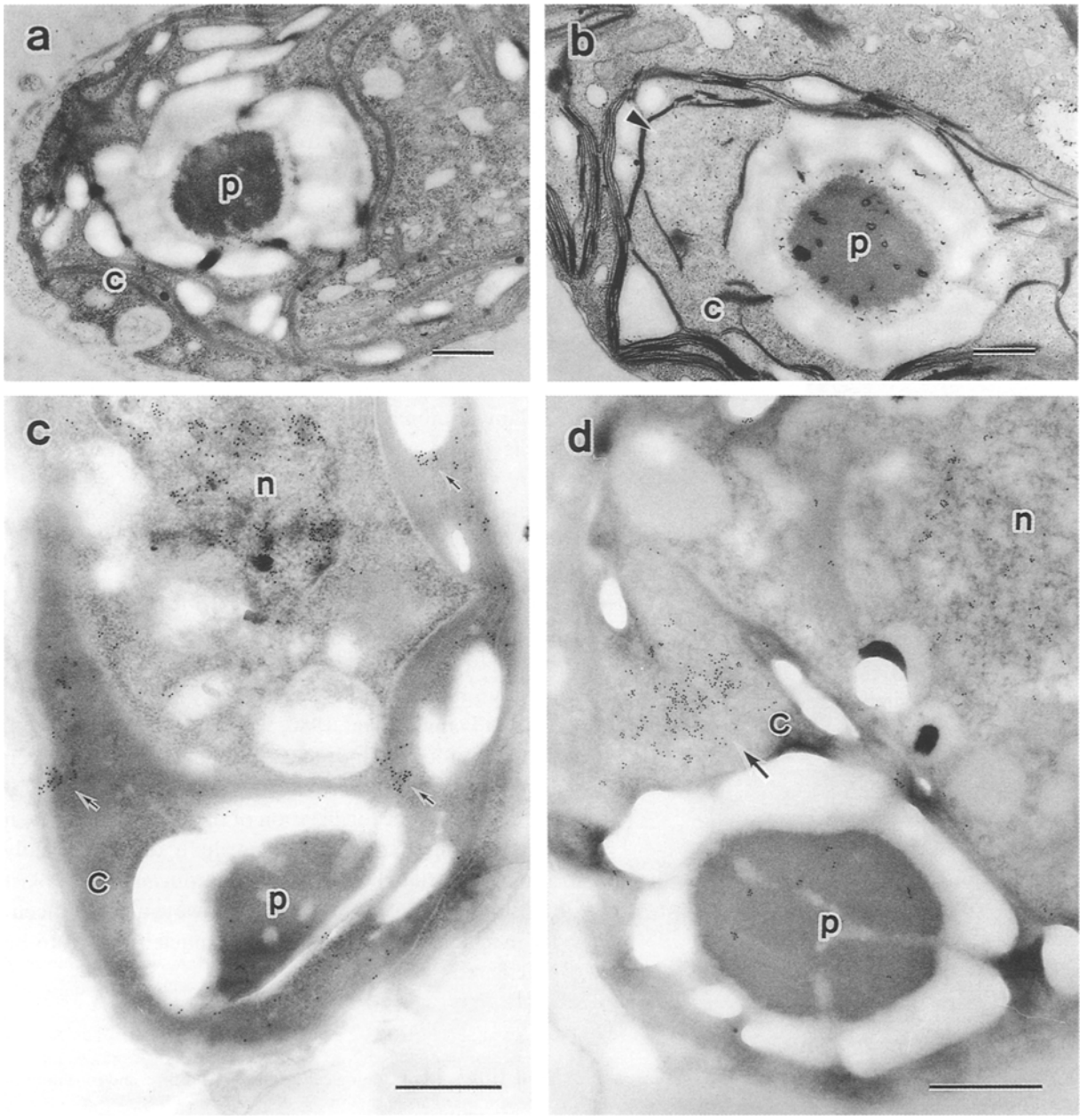

Fig. 5. **a** and **b** Transmission electron micrographs of wild-type (**a**) and *moc* mutant cells (**b**) showing a chloroplast with a pyrenoid. An electron-transparent area (arrowhead in **b**) is seen near the pyrenoid in the *moc* mutant cell. **c** and **d** Deposits of gold particles in the wild-type cell and *moc* mutant cell; the arrows show cp-nuclei. *c* Chloroplast, *n* cell nucleus, *p* pyrenoid. Bar: 0.5 μm

Several investigators have revealed by electron-microscopic observation a close correlation between the changes in the spatial arrangement of cp-nuclei and the development of the membrane systems (Hashimoto 1985, Lindbeck and Rose 1987). In particular, it has been suggested that the growth and formation of the thylakoid membrane may play a crucial role in the movement of cp-nuclei (Rose 1988). However, there is no direct evidence of a specific association at the molecular level with the movement of cp-nuclei. The phenotype of the undispersed cp-nucleus observed in the *moc* mutant cells is probably not caused by an alteration of the membrane system, because there were no significant differences in the arrangement and organization of the thylakoid membrane between wild-type and *moc* mutant cells (Fig. 5 a, b).

The movement of the cp-nuclei not only requires membrane growth, but also attachment to the membrane system. In addition, the morphology of the cp-

nuclei depends largely on the proteins associated with the cpDNA. Ehara et al. (1990) found that the profiles of DAPI-stained cp-nuclei were differently modified in cells fixed in different concentrations of glutaraldehyde or treated with protease before DAPI staining. Therefore, condensation of the cpDNA in vivo may result from the altered configuration of protein components that play a role in membrane binding for dispersion in the *moc* mutant cells, without affecting the membrane structures.

The involvement of DNA gyrase (type II topoisomerase) in cp-nucleus division has been suggested in the primitive red alga *Cyanidioschyzon merolae* (Itoh et al. 1997). *Cyanidioschyzon merolae* contains a single or centrally located cp-nucleus (Kuroiwa et al. 1981). Nalidixic acid, an inhibitor of DNA gyrase, inhibits replication of cpDNA and mtDNA and induces unequal division of the cp-nucleus. Approximately 50–70% of cp-nuclei divided unequally in *C. merolae* cells treated with nalidixic acid. Two different effects of nalidixic acid on cp-nuclei division are postulated. One is the dissociation of cp-nuclei from a protein that anchors DNA to the membrane (assuming that the topoisomerase mediates the association of cp-nuclei with a membrane system); the other is that DNA gyrase inhibits a decatenatic reaction to physically segregate duplicated circular cpDNA. Marrison and Leech (1992) also showed the involvement of DNA topoisomerase II in the replication of cpDNA and division of cp-nuclei in wheat in immunocytochemical experiments.

In *C. reinhardtii*, the replication of cpDNA is also inhibited soon after the addition of the DNA gyrase inhibitor novobiocin, which suggests that DNA gyrase activity is required for cpDNA synthesis (Woelfle et al. 1993). Hence, there is a possibility that DNA gyrase plays an important role in cp-nuclei division in *C. reinhardtii* as well as *C. merolae*.

Insertional mutagenesis has the advantage that it is possible to clone the disrupted gene with exogenous DNA as a clue (Tam and Lefebvre 1993). This approach is suited for studing chloroplast proliferation which is controlled by the cell nucleus. Cloning the affected gene together with a detailed characterization of the gene products should reveal the molecular mechanism for the segregation of cpDNA.

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