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Isolation and characterization of mitochondrial DNA from *Chlamydomonas reinhardtii*

(sedimentation/melting/reassociation/organelle/evolution)

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ABSTRACT **Mitochondrial DNA (mtDNA) has been isolated from a mitochondrial pellet of *Chlamydomonas reinhardtii*. The mtDNA has a buoyant density of 1.706 g/ml in CsCl, a melting temperature of 87.9° in standard saline citrate, and a nucleoside composition of 47.5% deoxyguanidine plus deoxycytidine with no odd nucleosides. Thermal denaturation and renaturation studies have shown that (*i*) mtDNA contains no extensive intramolecular heterogeneity nor significant base bias between the complementary polynucleotide chains and (*ii*) mtDNA renatures as a single homogeneous class with a kinetic complexity of 9.78×10^6 daltons. Although rare (≤1%), both open and supercoiled circular mtDNA molecules have been observed in the electron microscope. Contour lengths of linear and open and closed circular molecules are all within the range of 4.0–5.4 μm with a mean of 4.67 ± 0.30 μm. This size is similar to that of animal mtDNA but approximately ⅛ that of the higher plant mtDNAs. The magnitude of mtDNA reiteration in *C. reinhardtii* is estimated to be of the same order as that of chloroplast DNA.**

Primarily due to a number of advantageous biological characteristics, *Chlamydomonas reinhardtii*, a unicellular green alga, has been increasingly exploited for fruitful investigations of the cooperation between chloroplast and nuclear genomes in nucleic acid metabolism (1–4) and protein synthesis (5, 6). Genetic studies with a few non-Mendelian mutants have also suggested that there is probably another level of cooperation between the chloroplast and mitochondria genomes in organelle protein synthesis (7–9). Dissection of this postulated interorganelle cooperation at the molecular level should prove to be feasible and of interest, because *C. reinhardtii* is a facultative photosynthetic organism with an abundance of well-characterized nonphotosynthetic mutants (10). The unusually fragile structure of the single large chloroplast in *C. reinhardtii*, however, has hampered repeated attempts in several laboratories to isolate contamination-free mitochondria for biochemical characterization.

We report in this paper the successful isolation of a DNase-impermeable mitochondrial preparation from a cell-wall-less mutant of *C. reinhardtii* and the characterization of the mitochondrial DNA (mtDNA). We document here the basic physicochemical properties of this mtDNA and its complexity. The unexpected findings of our present study are that (*i*) the complexity of *C. reinhardtii* mtDNA is strikingly small in comparison with other plant mtDNAs but very close to that of animal mtDNA, and (*ii*) the reiterations of mtDNA and chloroplast DNA (cpDNA) molecules are comparable in the vegetative cells of *C. reinhardtii*.

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MATERIALS AND METHODS

Strain and Culture Conditions. A mating type + subclone of the cell-wall-less mutant CW-15 (11) was used for all studies. Cells were grown in minimal medium supplemented with 0.2% sodium acetate (4). Twelve- or 18-liter cultures were illuminated with six circular 40-W cool-white fluorescent lamps and aerated with 3% CO_2 in air.

Mitochondria Isolation Procedures. *Procedure A.* Cells were collected at early stationary phase, washed once in buffer 1 (0.25 M sucrose/0.1 mM EDTA/10 mM Tris·HCl, pH 9), once in buffer 2 (0.25 M sucrose/0.1 mM EDTA/10 mM Tris·HCl, pH 7.4), and suspended in 10 ml of buffer 2 per liter of culture volume (12). This cell suspension was passed once through a chilled French pressure cell at 500 psi (34.5×10^2 kPa), diluted with an equal volume of buffer 2, and, after standing 5 min, centrifuged at 2000 × *g* for 10 min. The pellet was suspended in buffer 2 at 5 ml/liter of culture and centrifuged for 10 min at 2000 × *g*. This and the previous supernatant were combined and centrifuged sequentially three times for 5 min each at 2000 × *g* to remove sedimentable material. The final supernatant was centrifuged for 20 min at 10,000 × *g*. The resulting pellet was washed twice in buffer 2 and suspended in NET buffer (0.15 M NaCl/0.1 M EDTA/10 mM Tris·HCl, pH 8.0) for DNA isolation. All steps were carried out at 0°–4°.

Procedure B. Cells were collected at early stationary phase, washed twice in buffer A (0.3 M mannitol/3 mM EDTA/0.1% bovine serum albumin/1 mM 2-mercaptoethanol, 50 mM Tris·HCl, pH 8) and suspended in buffer A at 10 ml/liter of culture volume (13). This cell suspension was passed once through a chilled French pressure cell at 500 psi, diluted with an equal volume of buffer A, and, after standing 5 min, centrifuged at 1000 × *g* for 15 min. The pellet was suspended in buffer A at 5 ml/liter of culture and centrifuged for 15 min at 1000 × *g*. The supernatants were combined and centrifuged for 20 min at 10,000 × *g*. The resulting pellet was suspended in buffer A at 2.5 ml/liter of culture and digested with DNase I (50 μg/ml, Worthington) in the presence of 10 mM $MgCl_2$. After incubation at 0° for 60–90 min, 4 vol of buffer B (0.15 M NaCl/0.1 M EDTA, pH 8) was added. This mixture was centrifuged for 20 min at 10,000 × *g*. The resulting pellet was suspended in NET buffer for DNA isolation. All steps were carried out at 0°–4°.

Procedure C. Same as *Procedure B* except that the two 1000 × *g* and the first 10,000 × *g* centrifugation steps were omitted.

DNA Isolation. Mitochondrial pellets were lysed in 2%

Abbreviations: mtDNA, mitochondrial DNA; cpDNA, chloroplast DNA; NET buffer, 0.15 m NaCl/0.1 M EDTA/10 mM Tris·HCl, pH 8.0; SSC, 0.15 M NaCl/0.015 M Na_3 citrate, pH 7.0; t_m, melting temperature.

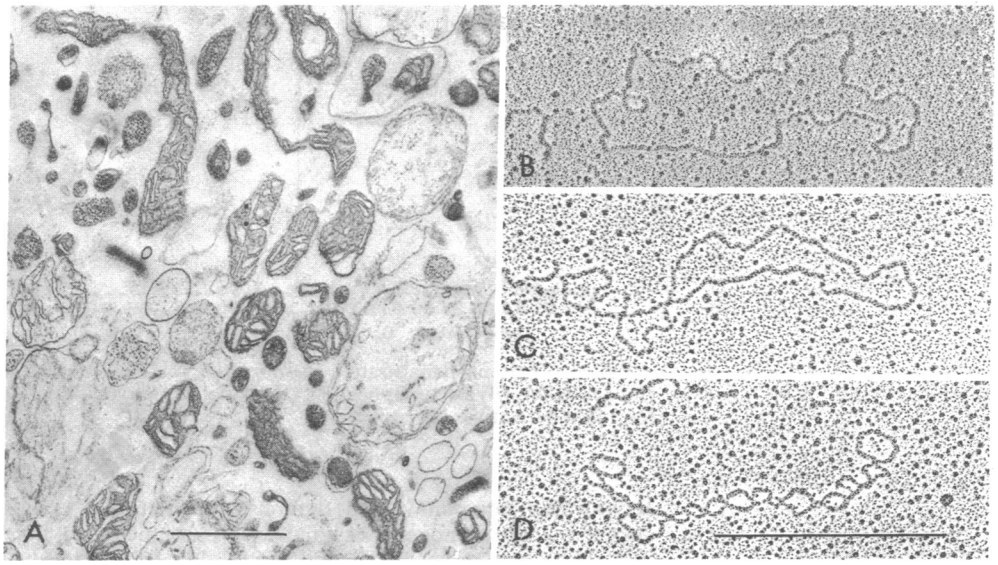

FIG. 1. Electron micrographs. (*A*) Mitochondrial pellet prepared by *Procedure A*. (*B–D*) Circular mtDNA molecules. Bars represent 1 μm. (*B*, Open circle, 4.14 μm; *C*, open circle, 4.53 μm; *D*, supercoiled circle, 4.55 μm.)

Sarkosyl/1% sodium dodecyl sulfate/NET buffer with 1 mg of heat-treated Pronase (80° for 10 min, then 37° for 2 hr) per ml and incubated for 16 hr at 4° with gentle mixing. This lysate was sequentially extracted with NET buffer-saturated phenol and chloroform/isoamyl alcohol, 24:1 (vol/vol), and the final aqueous phase precipitated at −20° with 95% ethanol. The precipitate was collected by centrifugation, dissolved in 0.15 M NaCl/0.015 M Na_3 citrate, pH 7.0 (SSC), and digested at 37° for 60 min with 0.05 unit of heat-treated (80° for 10 min) RNase T1 and 50 μg of heat-treated (80° for 10 min) RNase A per ml. After an extraction with chloroform/isoamyl alcohol, the aqueous phase was ethanol-precipitated twice. The second precipitate was used for further purification on preparative CsCl gradients.

Ultracentrifugation. The analytical CsCl equilibrium sedimentation procedures have been described (1). Alkaline CsCl centrifugation was carried out by adjusting the sample to pH 12.3 with K_3PO_4. Neutral CsCl centrifugation of denatured DNA was accomplished by making the sample 0.2 M in NaOH for 15 min at 37° followed by 5 min in the presence of 3.6% previously neutralized formaldehyde. The sample was then adjusted to pH 7 with KH_2PO_4. Preparative CsCl gradients of 4.5 ml with a refractive index of 1.4010 were centrifuged in a Beckman L2-65 using a type 65 rotor for 42 hr at 40,000 rpm at 25°. Preparative CsCl/EtBr gradients of 6 ml with a refractive index of 1.3380 containing 3 mg of EtBr were sedimented at 35,000 rpm at 20° in a Beckman type 65 rotor for 66 hr. After centrifugation, the gradients were viewed under UV light and the upper DNA band and lower region of the gradient were removed separately. These fractions were dialyzed against 0.02 M Na_2EDTA/0.2 M Tris·HCl, pH 8, for further analysis.

Melting and Renaturation Analysis. Purified mtDNA in SSC was melted under a linear temperature increase rate of 18°/hr. Bacteriophage T4 DNA in SSC was melted in a separate cuvette of the same run as a control. For renaturation analysis, purified mtDNA or bacteriophage T4 DNA was sheared to an average size of 350 nucleotides as determined by analytical ultracentrifugation in alkaline solution by three consecutive passes through a French pressure cell at 20,000 psi (14). Renaturation was carried out at [melting temperature (t_m) −25]° in SSC.

Nucleoside Composition Analysis. The high-pressure liquid chromatography procedure of Singhal (15) was used.

Electron Microscopy. Mitochondrial pellets were prepared and examined by using the methods of Martin and Goodenough (16). mtDNA was prepared for microscopy by using the formamide technique of Davis *et al.* (17). DNA contour lengths were determined on micrographs with a magnification of ×51,000 by the use of a map measurer. A grating replica calibration grid of 58,400 lines per in. (23,000 lines per cm) was used as a calibrating standard.

RESULTS

Isolation and Identification of the Mitochondrial DNA. An electron micrograph of a typical mitochondrial fraction prepared by *Procedure A* is shown in Fig. 1. A high field density of mitochondria with very little chloroplast membrane contamination was evident.

In addition to approximately 10% nuclear DNA (with a peak density, ρ, of 1.720 g/ml) (1) contamination, the major DNA species isolated from this pellet with a density of 1.706 g/ml was identified as mtDNA. cpDNA (ρ = 1.695 g/ml) (1) was undetectable (Fig. 2*B*). Unless there is intentional heavy overloading of the analytical centrifuge cell (see Fig. 6), the mt DNA, due to its minute content, does not usually appear as a distinct band in neutral CsCl gradients of whole cell DNA. The origin of the mtDNA was confirmed by its resistance, in the mitochondrial pellet as well as in the whole cell homogenate (prepared by *Procedure C*), to DNase digestion. As shown in Figure 2*E*, when isolated mitochondria were treated with DNase I, all of the cpDNA and more than 95% of the nuclear DNA was removed but the amount of mtDNA was unchanged. Similar resistance to DNase I has been reported with other mtDNAs (18).

Biophysical Characterization of mtDNA. After denaturation, the mtDNA exhibited a density increase of 0.014 g/ml in neutral CsCl gradient (Fig. 2*C*) as would be expected for double-stranded DNA (19). Only one broad, symmetrical mtDNA peak was observed in alkaline CsCl gradient (data not shown), indicating that little or no bias exists with respect to the G + T content of the complementary DNA strands (20).

The hyperchromic transition of the mtDNA in SSC was highly cooperative with a narrow t_m range between 83° and 91° (Fig. 3*A*). The first derivative of the melting curve revealed that, although a small amount of intramolecular heterogeneity may exist in the very early melting region, mtDNA is devoid of extensive intramolecular heterogeneity and is at least as intramolecularly homogeneous as T4 DNA (Fig. 3*B*).

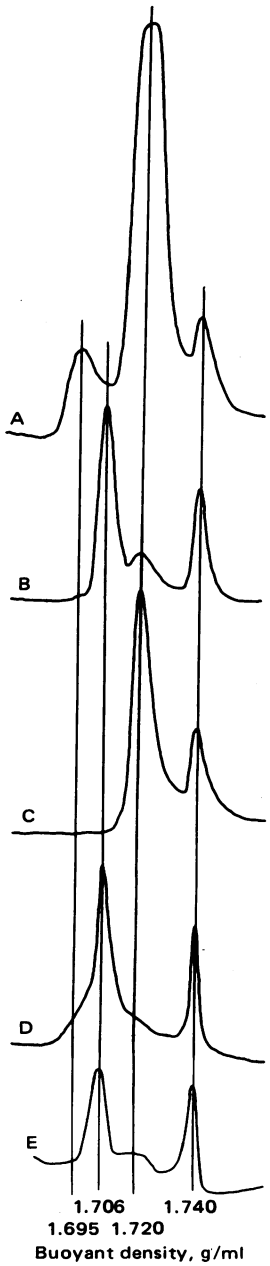

FIG. 2. Densitometer traces of DNA samples after CsCl equilibrium sedimentation in an analytical centrifuge. (*A*) Whole cell DNA from *C. reinhardtii*; (*B*) DNA isolated from the mitochondrial pellet as shown in Fig. 1; (*C*) purified mtDNA after denaturation; (*D*) purified mtDNA denatured and allowed to renature at $(t_m - 25)°$ to a C_0t value of 0.6; (*E*) DNA isolated from DNase I-treated mitochondrial pellet.

When the mtDNA was heat denatured, allowed to renature to a C_0t of 0.6, and remelted, the resulting profile was superimposable on the original melting profile to within 0.1°. This result and the fact that the renatured mtDNA returned to within 0.001 g/ml of its original density in a neutral CsCl gradient (Fig. 2*D*) suggest that the mtDNA has a simple genomic complexity.

The G + C content of the mtDNA measured by nucleoside analysis was 47.5% deoxyadenosine, 26.1 mol %; deoxythymidine, 26.4; deoxycytidine, 24.5; deoxyguanosine, 23.0. This value is in good agreement with the G + C content of 45.4% and 46.9% as calculated from the t_m (i.e., 87.9°) (20) and the buoyant density in CsCl (i.e., 1.706), (21) indicating that odd base(s) was not present in significant amounts. Odd nucleoside(s) was also not detected (<0.1%) in the high-pressure liquid chromatograms of deoxyribonucleosides.

To analyze the genetic information content of the mtDNA, renaturation kinetic studies were carried out. The second-order

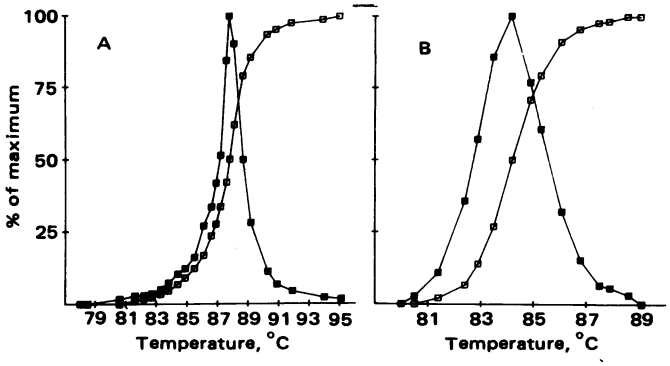

FIG. 3. Thermal denaturation profiles of DNA in SSC. (*A*) Purified mtDNA. (*B*) Bacteriophage T4 DNA. Melting curves (■) and their first derivatives (□) are shown.

renaturation rate plots (Fig. 4) demonstrate that mtDNA consists of a single homogeneous kinetic class with no detectable redundant sequences. On the basis of the kinetic complexity of T4 (Fig. 4*B*) being 110×10^6 daltons (22), the kinetic complexity of the mtDNA was calculated, according to Wetmer and Davidson's procedure (23), to be 9.78×10^6 daltons. Because G + C content of this DNA is close to 50%, a significant base content effect on the observed rate of renaturation is unlikely.

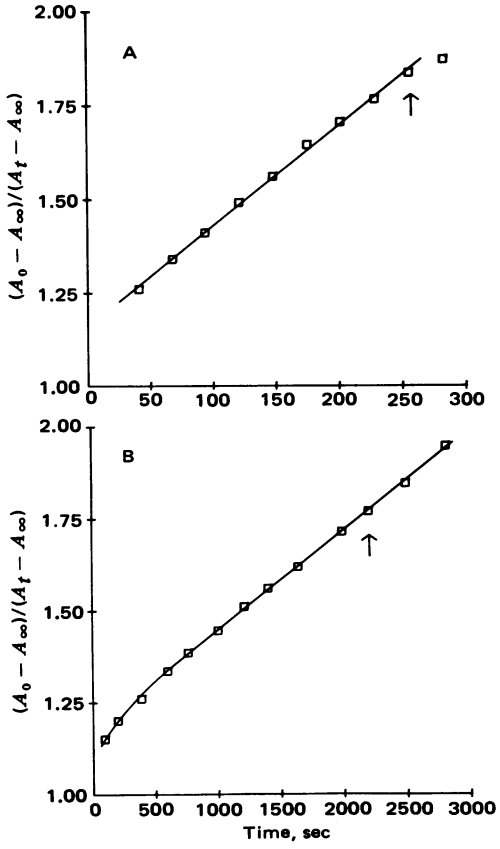

FIG. 4. Renaturation kinetics of DNA in SSC at $(t_m - 25)°$. (*A*) Purified mtDNA. The arrow marks 46.5% completion of the renaturation reaction. The k_2 was calculated to be 68.6 liters mol^{-1} sec^{-1}. (*B*) Bacteriophage T4 DNA. The arrow marks 43.5% completion of the renaturation reaction. The k_2 was calculated to be 6.47 liters mol^{-1} sec^{-1}.

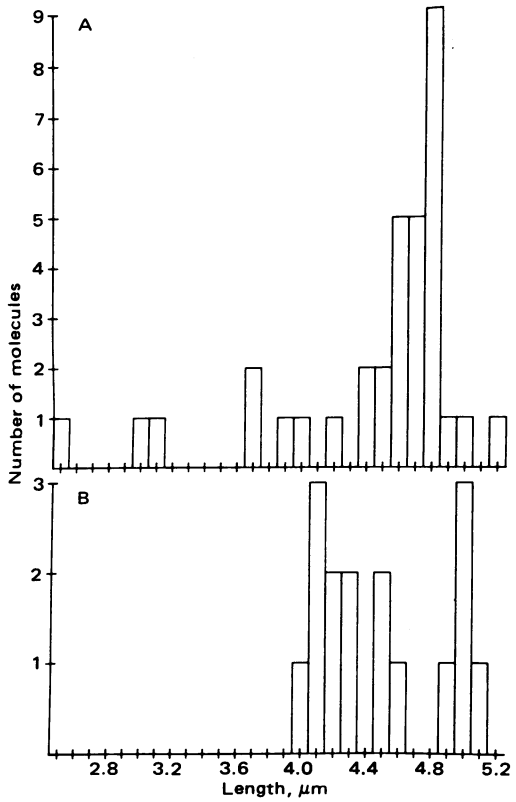

FIG. 5. Length distribution of linear (*A*) and circular (*B*) mtDNA molecules.

Molecular Size and Conformation of mtDNA. mtDNA to be examined by electron microscopy was isolated from mitochondrial pellets obtained by *Procedure B*. The mitochondrial pellet was lysed with 2% Sarkosyl and digested with Pronase (1 mg/ml) at 4° for 16 hr. A trace amount of radioactively labeled *Escherichia coli* DNA (ρ = 1.710 g/ml) was added, and the lysate was centrifuged to equilibrium in a neutral CsCl gradient. The region of the gradient starting from one fraction less dense than the *E. coli* marker was pooled for further analysis. Only DNA with a buoyant density attributed to mtDNA was detected in this pooled fraction by analytical CsCl centrifugation. Both linear and circular molecules were observed in the electron microscope. Fig. 1 *B* and *C* shows two of the circular molecules seen in a typical DNase-treated mitochondrial fraction. Circular molecules seen in this preparation ranged in size from 4.0 to 5.2 μm with a mean ($\pm$ SD) of 4.60 $\pm$ 0.42 μm (Fig. 5*B*). The frequency of observed circular molecules was less than 1% of the total DNA molecules scanned. Linear molecules in this particular preparation were in the range 0.3–5.2 μm, with greater than 50% of the molecules in the range 4.0–5.2 μm. In more recent preparations obtained with minimal mechanical shear, molecules less than 4.0 μm were not observed. For this reason, only those molecules greater than 4.0 μm were shown in Fig. 5*A*. The mean of these linear molecules was 4.72 $\pm$ 0.25 μm.

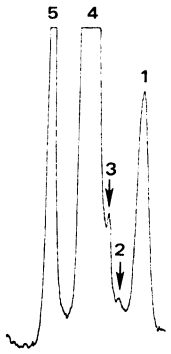

FIG. 6. Densitometer trace of an intentionally overloaded whole cell DNA preparation after CsCl equilibrium sedimentation in an analytical ultracentrifuge. 1, cpDNA; 2, mtDNA; 3, the γ satellite (cytosol ribosomal RNA cistron); 4, nuclear DNA; 5, bacteriophage SP01 DNA (density taken as 1.742 g/ml).

Because no supercoiled molecules were seen in a DNase-digested mitochondrial fraction, the DNA isolated from a mitochondrial preparation in the absence of a DNase treatment was analyzed on a CsCl/ethidium bromide gradient. The region of the gradient denser than the linear/open circular DNA band was pooled. Analytical ultracentrifugation, in CsCl, of DNA from this fraction showed only mtDNA to be present. Supercoiled molecules were observed in the electron microscope although they represented only a small fraction of the mtDNA molecules observed. Nine such molecules were found, of which one was a circular dimer and four were too tightly collapsed to be measured accurately. The contour lengths of the four measurable molecules were 4.0, 4.17, 4.30, and 4.55 μm with a mean of 4.27 $\pm$ 0.21 μm. Another with a contour length of 8.30 μm was thought to be a dimer. One of the supercoiled molecules is shown in Fig. 1*D*. The contour lengths of linear, open, and closed circular molecules were within the range 4.0–5.2 μm and showed a mean of 4.67 $\pm$ 0.30 μm. Hence, the size of the mtDNA as determined by electron microscopy is approximately 9.3×10^6 daltons (24). This value is in good agreement with the kinetic complexity of the mtDNA—i.e., 9.78×10^6.

DISCUSSION

From a mitochondria-enriched fraction of *C. reinhardtii*, we have isolated a DNA species with a buoyant density of 1.706 g/ml in CsCl. Observations of a satellite DNA component with the same or similar density in CsCl have been reported (25, 26). The identification of this component as the mtDNA is based on the facts that (*i*) it is the major species of DNA isolated from the mitochondrial fraction, and (*ii*) treatment of either a whole cell homogenate or a mitochondrial fraction with DNase I removes all contaminating chloroplast and nuclear DNA species while leaving the mtDNA unaffected (Fig. 1*C*). At least two other small circular DNA species of suggested chloroplast origin have been reported: a 3.13-μm molecule from *Euglena* (27) and a 4.2-μm molecule from *Acetabularia* (28).

Characterization by renaturation kinetics and electron microscopy has shown the *C. reinhardtii* mtDNA to be an approximately 9.54×10^6 dalton molecule with no pronounced intra- or intermolecular heterogeneity. Although rarely seen, a minor fraction (i.e., $\leq$1%) of the mtDNA has been observed to exist as a supercoiled, closed circular molecule in DNA isolated from mitochondria-enriched fractions. Because virtually all the linear molecules have unique ends (29, 30), the circular-to-linear transition is apparently due to a site-specific break rather than to random mechanical shear. Whether a greater proportion of the mtDNA exists *in vivo* as supercoiled, closed circular molecules at a given stage in the cell cycle is not known at present.

It is not clear at present why the lengths of the rarely observed circular mtDNA molecules did not fall into a very narrow range as circular DNA molecules usually do and whether this nonclustered distribution of circular DNA molecules possesses any significant (or trivial) explanation. Satisfactory answers will not be forthcoming until the circular molecules can be successfully isolated in some quantity for detailed biophysical-chemical characterizations.

Due to certain technical difficulties, the precise content of mtDNA per cell has yet to be determined. A rough estimate of

the relative content of mtDNA per cell, however, can be made from heavily overloaded whole cell DNA preparations sedimenting in analytical ultracentrifuge. An example is shown in Fig. 6. mtDNA probably represents 5% of the cpDNA and thus roughly 0.6% of the total cellular DNA. Because *C. reinhardtii* contains about 14% of cpDNA or 1.23×10^{-7} μg (7.4×10^{10} daltons) of DNA per cell (1), it appears that the mtDNA is present at about 46 copies per cell. This reiteration is in the same order of magnitude as that of the cpDNA in the same cell: 52 copies of cpDNA were estimated to be present in *C. reinhardtii* (31–33). It would be of considerable interest to ascertain whether these comparable reiterations in mtDNA and cpDNA molecules in these two organelles in *Chlamydomonas* possess any biological significance.

Among different organisms, the mtDNA sizes are highly variable, ranging from the 5-μm circles of animals to the 30-μm circles found in pea leaves (13, 34–42). However, this large variance in size does not necessarily imply that comparable differences in information content exist among these mtDNAs. Irrespective of speculation as to whether or not the larger mitochondrial genomes contain greater untranscribed regions (42–44), the nature of this variability of the mitochondrial genome size remains unclear. Our findings indicate that the *Chlamydomonas* mitochondria contain a genome more animal-like in size than the nearly 8-fold larger mtDNAs of higher plants (45), which incidentally have the same buoyant density as the *C. reinhardtii* mtDNA, although these similarities may be entirely fortuitous. In contrast to this large variation in the mtDNA size, the size of cpDNA from a great diversity of plants including *Chlamydomonas* and the colorless alga *Polytoma* has been shown to be relatively more constant (i.e., approximately $100 \pm 20 \times 10^6$ daltons) (46–48). The same is also true for their buoyant densities in CsCl (46, 47). This apparent difference in the relative constancy of the genome complexities between mtDNA and cpDNA may relate to differences in evolutionary constraints upon two quite different organelle systems.

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