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MITOCHONDRIAL GENOME CONFORMATION AMONG CW-GROUP CHLOROPHYCEAN ALGAE[1]

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Most green algal taxa have circular-mapping mitochondrial genomes, whereas some have linear genome- or subgenomic-sized mitochondrial DNAs (mtDNA). It is not clear, however, if the circular-mapping genomes represent genome-sized circular molecules, if such circular molecules and the linear forms are the predominant *in vivo* mtDNA structures, or if the linear forms arose only once or multiple times among extant green algal lineages. We therefore examined the DNA components detected with homologous mtDNA probes after pulsed-field gel electrophoresis of total cellular DNA from the chlorophycean basal bodies displaced clockwise(CW)-group taxa *Chlamydomonas reinhardtii* and *Chlamydomonas moewusii*. For *C. reinhardtii*, the 15.8-kb linear mtDNA was the only DNA component detected, and there was no evidence of circular or large linear precursors of this DNA. In the case of *C. moewusii*, which is known to have a circular-mapping 22.9-kb mitochondrial genome, three DNA components were detected; these appeared to be circular (relaxed and supercoiled) and genome-sized linear DNA molecules, the latter of which likely resulted from random double-strand breaks in the circular forms during DNA isolation. In further studies, DNA from additional CW-group taxa was examined using conventional gel electrophoresis and DNA-filter blot analysis with *C. reinhardtii* and *C. moewusii* mtDNA probes. We conclude that all taxa from the "*Volvox* clade" (*sensu* Nakayama et al. 1996) of the CW-group have genome- or subgenomic-sized linear mtDNAs as their predominant mtDNA form and that these arose from a genome-sized circular form in an ancestor that existed near the base of this clade.

Key index words: Chlamydomonas; **Chlorophyceae; gel electrophoresis; mitochondrial DNA conformation; phylogeny**

Abbreviations: **CW, basal bodies displaced clockwise; mt, mitochondrial; PFGE, pulsed field gel electrophoresis**

Mitochondrial genomes vary in size and structure both among and within kingdoms of the Eukarya (Gray 1999, Gray et al. 1999, Lang et al. 1999b). In terms of size, at one extreme are the 6-kb mitochondrial genomes of the apicomplexa that encode five genes (Feagin 1995), whereas at the other extreme are 200 kb and larger land plant counterparts that encode 50–70 genes (Ward et al. 1981, Lang et al. 1999b) and the 100-kb mitochondrial genome of the jakobid protozoan *Reclinomonas americana* that encodes 91 genes (Lang et al. 1999a). The conformation of mitochondrial DNA (mtDNA) can be a genome-sized circular molecule as in most animals, although genome-sized linear forms and subgenomic-sized circular or linear forms have been reported in some animal taxa (reviewed in Moritz et al. 1987, Bridge et al. 1992, Armstrong et al. 2000). Linear genome-sized mtDNAs with distinct telomeres have been identified in diverse taxa, most notably in some yeasts (reviewed in Nosek et al. 1998) and ciliates (Burger et al. 2000). In the apicomplexa (Wilson and Williamson 1997) and many fungi (Maleszka et al. 1991, Maleszka and Clark-Walker 1992, Maleszka 1993), most mtDNAs exist as larger than genome-sized linear molecules, with a small fraction of genome-sized circular molecules that are thought to give rise to the linear forms by a rolling-circle replication mechanism. Finally, land plant mtDNAs, the most structurally complex of these DNAs, exist primarily as linear molecules or "rosette-like" structures that can be larger than the genome size, and it has been proposed that these use a recombination-dependent DNA replication mechanism similar to that of phage T4 (Backert et al. 1997, Oldenburg and Bendich 2001).

Information pertinent to mitochondrial genome size and conformation in green algae (Chlorophyta, *sensu* Sluiman 1985) is summarized in Table 1. Those mitochondrial genomes that have been sequenced to completion vary in size from 16 to 55 kb, encode 12 to 60 genes, and are most commonly circular-mapping, although linear genome structures are found in some taxa. The smallest mitochondrial genomes in the green algae are found in the CW-group (Lewis et al. 1992, Friedl 1997) of the Chlorophyceae (*sensu* Mattox and Stewart 1984); the CW-group is characterized by the clockwise displacement of basal bodies in flagellated cells and includes all species of the artificial taxon *Chlamydomonas* and related genera (see Pröschold et al. 2001, and references therein). Essentially the same 12 genes are recognized in the mitochondrial genomes of this group, and the size variation of these genomes (Table 1) is largely a result of optional intron content (reviewed by Nedelcu and Lee 1998a). The almost completely sequenced mitochondrial genome of the CW-group taxon *Polytomella parva* exists in at least two subgenomic-sized linear pieces of 13.5

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and 3.5 kb, and so far 10 genes have been identified in this genome. All the linear mitochondrial genomes identified in the Chlorophyta are associated with the chlorophycean "*Volvox* clade" of Nakayama et al. (1996). The existence of this clade is supported by phylogenetic analyses of nucleus-encoded small and large subunit rRNA (Buchheim et al. 2001) and chloroplast large subunit rRNA sequence data (Turmel et al. 1993, Buchheim et al. 1996). Although all green algal mitochondrial genomes characterized outside this clade are circular-mapping, little is known about the *in vivo* structure of these mtDNAs. For example, although restriction fragment analysis reveals a circular-mapping mitochondrial genome for two *Chlamydomonas moewusii* strains (Lee et al. 1991, Denovan-Wright and Lee 1992), Bendich (1993) commented that these mtDNAs may exist as larger than genome-sized linear molecules. In addition, although the existence of a linear genome-sized mtDNA in *Chlamydomonas reinhardtii* is indisputable (Ryan et al. 1978, Boer et al. 1985, Vahrenholz et al. 1993), the possibility that this mtDNA is produced by the site-specific cleavage of a genome-sized circular (Ryan et al. 1978, Grant and Chiang 1980) or concatemeric mtDNA (Bendich 1993) has not been eliminated.

Because of the importance of mitochondrial genome conformation to our understanding of the function and evolution of this genome, we studied mtDNA structure in the CW-group of the Chlorophyceae. We used a variety of gel electrophoretic techniques and obtained direct physical evidence regarding the *in vivo* conformation of the mtDNA in taxa from this group. We addressed several questions. Does the mtDNA of *C. reinhardtii* exist in conformations other than the 15.8-kb linear form? Is the major component mtDNA molecule in *C. moewusii* a circular genome-sized molecule, or does this mtDNA exist mostly as larger than genome-sized linear molecules? Is linear genome- or subgenomic-sized mtDNA a feature among green algae that is characteristic of and restricted to the *Volvox* clade of the CW-group?

MATERIALS AND METHODS

Strains, culture conditions, and cell harvesting. The algal strains used in this study and their sources are listed in Table 2. All photosynthetic strains were grown as synchronized cultures under the conditions described by Whiteway and Lee (1977); in some instances, identified in Results, cells were cultured under exponential growth conditions (continuous light at 250 μmol photons·m^{-2}·s^{-1} PAR). *Polytomella parva* was grown in the medium of Sheeler et al. (1968) and bubbled with air. When cell numbers reached about 4×10^6 cells·mL^{-1}, cultures were cooled on ice and the cells were harvested by centrifugation (6000*g*) at 4° C.

Preparation of total DNA, gel electrophoresis, and hybridization analysis. Total DNA was prepared as in Lemieux et al. (1980), except that after incubation with RNase A, ammonium acetate

Table 1. Apparent mtDNA conformation among green algal taxa.

Class/group	Strain and source[a]	Size (kb)	Conformation	References
Chlorophyceae				
CW group				
Volvox clade	*Chlamydomonas reinhardtii* Dangeard–UTEX 2244	15.8	Linear[b,c]	Michaelis et al. 1990, Boer and Gray 1991, Vahrenholz et al. 1993
	Chlamydomonas reinhardtii Dangeard–UTEX 1062	16.9	Linear[b]	Boynton et al. 1987
	Pandorina morum Müller, syngen 1–UTEX 854	20[d]	Linear[b]	Moore and Coleman 1989
	Polytomella parva, Pringsheim–UTEX L193	13.5, 3.5	Linear[e]	Fan and Lee 2002
Dunaliella clade	*Chlorogonium capillatum* Dangeard–SAG 12-2e	22.7	Circular[b]	Kroymann and Zetsche 1998
Tetracystis clade	*Chlamydomonas moewusii* Gerloff–UTEX 9	22.9	Circular[b]	Denovan Wright et al. 1998;
	Chlamydomonas moewusii Gerloff–UTEX 97	22	Circular[b]	Denovan Wright and Lee 1992
	Chlamydomonas pitschmannii Ettl–SAG 1473	16.5	Circular[b]	Boudreau and Turmel 1995
DO group	*Scenedesmus obliquus* (Turpin) Kützing–UTEX 78	42.9	Circular[b]	Nedelcu et al. 2000
	Scenedesmus obliquus (Turpin) Kützing–KS3/2	42.7	Circular[b]	Kück et al. 2000
Trebouxiophyceae[f]	*Prototheca wickerhamii* Soneda & Tubaki–SAG 263-11	55.3	Circular[b]	Wolff et al. 1994
	Chlorella N1a Beijerinck	75	Circular[b,c]	Waddle et al. 1990
Pedinophyceae[g]	*Pedinomonas minor* Korshikov–UTEX LB1350	25.1	Circular[b]	Turmel et al. 1999
Prasinophyceae[h]	*Platymonas subcordiformis* (Willie) Hazen	43	Circular[b]	Kessler and Zetsche 1995
	Nephroselmis olivacea Stein–NIES 484	45.2	Circular[b]	Turmel et al. 1999
	Mesostigma viride Lauterborn–NIES-296	42.4	Circular[b]	Turmel et al. 2002

DO group, taxa with basal bodies directly opposed. The *Volvox* clade, *Dunaliella* clade, and the *Tetracystis* clade are based on Nakayama et al. (1996); support for the inclusion of additional taxa in these clades is provided by the results of Buchheim and Chapman (1992), Turmel et al. (1993), Buchheim et al. (1996), and Hepperle et al. (1998). Nuclear (Buchheim et al. 2001), plastid (Buchheim et al. 1996), and mitochondrial (Nedelcu et al. 2000) sequence data support the view that the *Dunaliella* clade and the *Tetracystis* clade are sisters to the exclusion of the *Volvox* clade.

[a] Strains labeled UTEX derive from the University of Texas Algal Collection; SAG, the Sammlung von Algenkulturen; and NIES, the National Institute for Enviromental Forum (Japan).

[b] DNA conformation inferred by restriction fragment mapping analysis and/or complete DNA sequence data.

[c] DNA conformation observed by EM.

[d] Other syngens were analyzed and have mtDNA sizes ranging from 20 to 38 kb.

[e] Two linear pieces of the mitochondrial genome corresponding to 13.5 kb and 3.5 kb have been described.

[f] *sensu* Friedl (1995).

[g] *sensu* Moestrup (1991).

[h] *sensu* Steinkötter et al. (1994).

TABLE 2. Taxa used in this study.

Taxon	Source/comments
Chlamydomonas sphaeroides–Gerloff (V)	SAG 25.72, (formerly *C. iyengarii*) identical to UTEX 221
Sphaerellopsis aulata–(Pasher) Gerloff (V)	SAG 69.72 (formerly *Chlamydomonas gelitanosa*)
Chlamydomonas reinhardtii–Dangeard (V)	UTEX 2244, identical to SAG 34.89
Chlamydomonas reinhardtii–Dangeard (V)	UTEX 2337, (AKA CW15) identical to SAG 83.81
Chlamydomonas asymmetrica–Korshikov (V)	SAG70.72 (formerly *C. peterfii*)
Polytomella parva–Pringsheim (V)	UTEX L193 (formerly *P. agilis*)
Chlorogonium capillatum–Dangeard (D)	SAG 12-2e (formerly *C. elongatum*)
Chlamydomonas pitschmannii–Ettl (T)	SAG 14.73
Chlamydomonas moewusii–Gerloff (T)	UTEX 9, (formerly *C. eugametos*) identical to SAG 20.90
Chlorococcum echinozygotum–Starr (T)	UTEX 118, identical to SAG 213-5

V, D, and T identify taxa in the *Volvox* clade, the *Dunaliella* clade, and the *Tetracystis* clade, respectively, See Table 1 for references and abbreviations.

was added to a final concentration of 2.5 M and precipitable material was removed before ethanol precipitation of the DNA. In the case of CW15, the cell wall–deficient mutant of *C. reinhardtii*, cells were lysed using an in-gel technique, as described by Carle and Olsen (1987), and agarose plugs were used directly for gel electrophoresis.

Total DNA (normally 1–3 μg) was fractionated using either pulsed-field gel electrophoresis (PFGE) with the CHEF-DRII system (Biorad, Hercules, CA, USA) or conventional gel electrophoresis, and fractionated DNA was transferred to nylon membranes (Hybond N^+, Amersham Biosciences, Piscataway, NY, USA) using standard methods (Sambrook et al. 1989). The probes used for hybridization analysis were constructed in the following manner. The five largest *Hin*dIII fragments and the 1.2-kb *Pvu*II fragment, which constitute the entire previously cloned mtDNA of *C. moewusii* (= *C. eugametos*) as reported by Denovan-Wright and Lee (1992), were cut out of their vectors and purified by gel electrophoresis before nonradioactive labeling using the AlkPhos Direct Labelling and Detection System (Amersham Biosciences). These labeled DNA fragments were mixed in stoichiometric proportions and termed the "*C. moewusii* mtDNA probe." Two DNA fragments (E1 and H1), which constitute approximately 80% of the mitochondrial genome of *C. reinhardtii* (Boer et al. 1985), were labeled and mixed as above and termed the "*C. reinhardtii* mtDNA probe." Membranes were hybridized with either of these probes at 60° C for 18 to 24 h in the hybridization buffer supplied and were washed according to the directions of the labeling and detection system before exposure of the film.

RESULTS

Search for additional mitochondrial DNA conformations in Chlamydomonas reinhardtii. We wished to determine if *C. reinhardtii* contains conformations of mtDNA other than the well-characterized 15.8-kb linear form. In these experiments, cells were grown under exponential culture conditions so that all vegetative cell cycle stages would be represented. Total cellular DNA was fractionated by PFGE at a 15-s switch time after in-liquid cell lysis and DNA purification, in the case of the wild-type strain, and after in-gel lysis, in the case of the cell wall–deficient strain, CW15. Only one hybridizing component, which migrated as a 16-kb linear molecule, was detected in both samples after DNA filter blot analysis with the *C. reinhardtii* mtDNA probe, even after overexposure of the films (Fig. 1A). Similar results were obtained after PFGE at a 75-s switch time (data not shown), which makes it unlikely that a non-linear mtDNA form was comigrating by chance with the linear one under both conditions of PFGE.

Conformation of Chlamydomonas moewusii *mitochondrial DNA*. We were interested in determining the conformation of the mtDNA in *C. moewusii*. Because there is no known cell wall–deficient strain of this species and we were unsuccessful in using the in-gel lysis procedure with the walled strains, we used our in-liquid method of cell lysis and total DNA extraction. Three bands hybridizing with the *C. moewusii* mtDNA probe were visible after fractionation of total *C. moewusii* DNA at the 15- and 75-s pulse times (Fig. 1B, lane 1). Based on its mobility relative to linear markers, the fastest migrating component under both gel

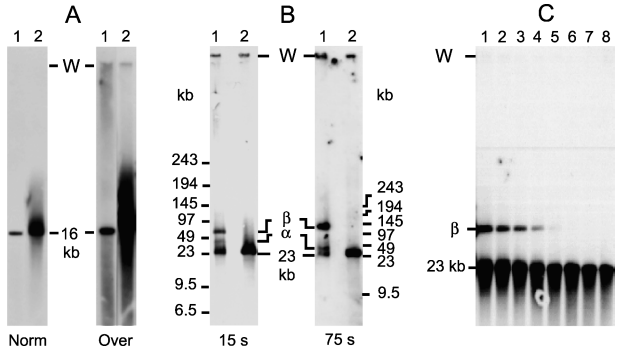

FIG. 1. Analysis of *Chlamydomonas reinhardtii* and *Chlamydomonas moewusii* mtDNA fractionated by PFGE. DNA was run through 1% agarose in 1× TAE (40 mM Tris-acetate; 1 mM EDTA, pH 8.0) at 6 $V \cdot cm^{-1}$ for 13.5 h. "W" indicates the wells in all panels. The DNA size markers used were the Low Range PFG Markers (New England Biolabs). (A) Total undigested DNA from *C. reinhardtii* fractionated by PFGE at a 15-s switch interval and hybridization with the *C. reinhardtii* mtDNA probe: Norm, normal exposure (15 min); Over, overexposure (60 min). Lanes: 1, *C. reinhardtii* (wild-type) DNA; 2, *C. reinhardtii* (CW15) DNA. (B) Total DNA from *C. moewusii* fractionated by PFGE using either 15- or 75-s switch intervals as indicated, and hybridization with the *C. moewusii* mtDNA probe. Lanes: 1, undigested DNA; 2, DNA digested with *Sal* I. (C) Total DNA from *C. moewusii* fractionated by PFGE at a 15-s switch interval and hybridization with the *C. moewusii* mtDNA probe. DNA was digested for 30 min with increasing amounts of *Sal* I. Lanes: 1, DNA undigested; 2–8, DNA digested with 0.01, 0.05, 0.1, 0.25, 0.5, 1, and 2 units of *Sal* I, respectively.

conditions appeared to be linear 23-kb DNA, the size of the *C. moewusii* mitochondrial genome. The slowest migrating component, designated β, was likely circular DNA because it migrated as would linear DNA of approximately 55 kb at the 15-s pulse time and as would linear DNA of approximately 135 kb at the 75-s pulse time. The third DNA component detected by the mtDNA probe, designated α, showed less hybridization than the other two components and migrated as would a linear DNA of about 35 and 40 kb at the 15- and 75-s pulse times, respectively.

After digestion with *Sal* I, a restriction enzyme having a single recognition site in the circular physical map of this taxon's mtDNA (Denovan-Wright et al. 1998), only one hybridizing component was visible in the electrophoretically fractionated total cellular DNA of *C. moewusii*; this DNA component comigrated with the 23-kb linear marker at both pulse times (Fig. 1B, lane 2) and therefore was likely linear DNA. The disappearance of components α and β and the increased amount of the 23-kb linear DNA after *Sal* I digestion suggest that these components were circular molecules, likely supercoiled and relaxed circles, respectively, that were converted into the linear 23-kb DNA. Similar results were obtained after digestion with *Xba* I or *Eag* I (data not shown); these restriction enzymes also have only one recognition site in the *C. moewusii* mtDNA physical map, and each cuts at a different region of this map.

We wished to test the hypothesis that the proposed circular forms of *C. moewusii* mtDNA identified in Figure 1B were genome-sized molecules. To this end, total cellular DNA from *C. moewusii* was digested with a range of *Sal* I concentrations such that the lower concentrations produced only partial digestion and the higher concentrations produced complete digestion. In contrast to the undigested samples shown in Figure 1B, the control DNA sample in Figure 1C had a lower proportion of component β and no detectable component α. This could have resulted from the complete and partial degradation of the α and of the β components, respectively, as a result of the 37° C incubation in *Sal* I buffer. From the results shown in Figure 1C it is apparent that the gradient of loss of component β, in relation to the increased opportunity for *Sal* I digestion, was not associated with the appearance of any linear DNAs with sizes that were multiples of 23 kb, as one might expect from partial digestion of circular multimers. Rather these data suggest that the β component was converted directly into genome-sized linear DNA. It is noteworthy that the 23-kb linear DNA in this experiment, in contrast to the sample from Figure 1B, appeared to decrease rather than increase after *Sal* I digestion. This can be explained by the greater proportion, before digestion, of linear mtDNA in the sample from Figure 1C compared with the samples from Figure 1B, and the likelihood that the linear mtDNAs in the undigested samples are derived from circular monomers that were broken randomly relative to the single *Sal* I site.

Distribution of CW-group taxa with linear mitochondrial genomes. We performed a limited survey of mtDNA conformation, using gel electrophoresis, among selected CW-group taxa with the goal of testing the hypothesis that linear mitochondrial genomes are a feature characteristic of and restricted to the *Volvox* clade of this group. In these experiments, conventional gel electrophoresis was employed using both 0.6% and 1.0% agarose. Total cellular DNA prepared from all taxa from the *Volvox* clade revealed only one band after hybridization with the *C. reinhardtii* mtDNA probe (Fig. 2, A and B). In each case, these bands comigrated with the linear size markers and correspond to sizes of about 14 kb in *P. parva* and *Chlamydomonas asymmetrica* and approximately 25 kb or more in *Chlamydomonas sphaeroides* and *Sphaerellopsis aulata*: the latter two mtDNAs are at the limit of the linear size range for these gels.

The results of DNA filter blot analysis of total cellular DNA from taxa outside the *Volvox* clade, specifically, within the "*Dunaliella* clade" and the "*Tetracystis* clade" of Nakayama et al. (1996), following standard gel electrophoresis and using the *C. moewusii* mtDNA probe are shown in Figure 2, C and D. All these samples, including the one from *C. moewusii*, revealed two or more hybridizing components. The lower most band, in each case, comigrated with linear size markers in both the 0.6% and 1.0% gels; these are assumed to have resulted from randomly broken circles with the smallest one being about 16 kb in *Chlamydomonas pitschmannii* and the largest ones being about 23 kb in *C. moewusii* and *Chlorogonium capillatum*, the size of the mitochondrial genome maps for each of these taxa (Table 1). DNA from all taxa, with the exception of *C.*

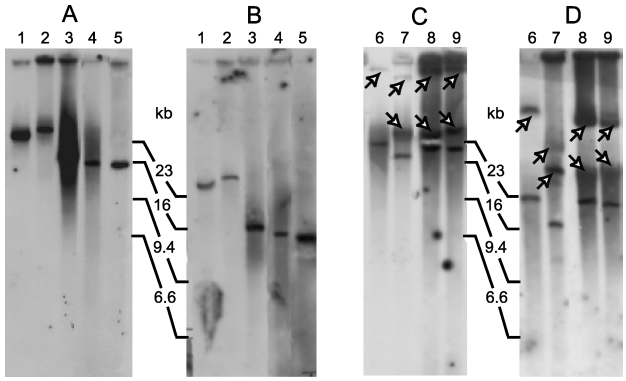

FIG. 2. Analysis of mtDNA from various CW-group taxa fractionated by conventional gel electrophoresis. DNA was run through 0.6% (A and C) or 1.0% agarose (B and D). Gels (20 cm) were run in 1× TAE at 0.5 V·cm^{-1} for 24 h; hybridization analysis was done with either the *Chlamydomonas reinhardtii* mtDNA probe (A and B) or the *Chlamydomonas moewusii* mtDNA probe (C and D). The 23-, 9.4-, and 6.6-kb pointers correspond to the position of the three largest λ-*Hin*dIII fragments, whereas the 16-kb pointer corresponds to the position of the 15.8-kb linear DNA of *C. reinhardtii*. Lanes: 1, *Chlamydomonas sphaeroides*; 2, *Sphaerellopsis aulata*; 3, *C. reinhardtii*; 4, *Chlamydomonas asymmetrica*; 5, *Polytomella parva*; 6, *Chlorogonium capillatum*; 7, *Chlamydomonas pitchsmannii*; 8, *C. moewusii*; 9, *Chlorococcum echinozygotum*. Arrowheads indicate the location of the proposed nonlinear mtDNA forms in C and D.

capillatum, showed two bands above the compression zone of linear DNA in these gels; these bands likely represent relaxed and supercoiled circular DNA. The *C. capillatum* mtDNA showed only one DNA component above the linear DNA band and this probably represented relaxed circular DNA.

DISCUSSION

Mitochondrial DNA conformation in Chlamydomonas reinhardtii *and* Chlamydomonas moewusii. Our analyses suggest that the only mtDNA conformation in vegetative cultures of *C. reinhardtii* is its well-characterized 15.8-kb linear DNA molecule. Ryan et al. (1978), using electron microscopy, detected rare (<1%) circular DNA molecules in mitochondrial-enriched DNA fractions of *C. reinhardtii* similar in density and contour length to that of the linear mtDNA. If such molecules represent circular forms of the *C. reinhardtii* mtDNA, as proposed by Ryan et al. (1978) and Grant and Chiang (1980), and they represent at least 0.1% of the mtDNA, we should have been able to detect them during our DNA filter blot analyses, especially with the overexposed films. On the other hand, if the fraction of circular mtDNA represents less than 0.1% of the mtDNA and given estimates of mtDNA copy number in *C. reinhardtii* at about 50 molecules per cell (Ryan et al. 1978), such circular molecules would occur, assuming random distribution among cells, in fewer than one of 20 cells. Although Ma et al. (1992) and Duby et al. (2001) obtained a 0.8-kb PCR product that seemingly bridges the ends of the *C. reinhardtii* mtDNA, this product is missing part of the right terminus and may have resulted from a naturally occurring partly circular mtDNA structure, such as proposed in the recombination-mediated replication model of Vahrenholz et al. (1993).

Our data do not support the possibility that the mtDNA in vegetative cultures of *C. reinhardtii* also exists as large linear molecules composed of a multimeric array of unit genomes as detected in other systems (Maleszka 1993, Bendich 1996). Such molecules, if present in at least one copy per cell, should have been detected after our DNA filter blot analyses. The faint hybridization signals corresponding to the position of the wells and only visible after overexposure of the film represent much less than 1% of the total hybridization signal.

Restriction fragment mapping (Lee et al. 1991) and DNA sequencing (Denovan-Wright et al. 1998) have shown that two strains of *C. moewusii*, UTEX 97 and UTEX 9, have circular-mapping genomes. Bendich (1993) pointed out, however, that these data are consistent not only with genome-sized circular mtDNAs, but also with a linear concatemeric array of unit genomes. The *C. moewusii* DNA samples show hybridization signals in the well regions of the gel corresponding to about 10% to 20% of the total signal, therefore leaving open the possibility that large multimeric mtDNA molecules are being retained in the wells. We note, however, that after *Sal* I digestion, such molecules should have been converted into genome-sized molecules, and we detected no reduction in the amount of well-bound hybridization when such a digestion was performed. Also, in another, albeit more degraded, DNA preparation, no label was detected in the well region and there were no signs of larger than genome-sized degradation products, therefore arguing against the possibility of large multimeric mtDNA molecules in *C. moewusii*.

The *C. moewusii* mtDNA seems to exist as a mixture of genome-sized circular and linear DNA molecules. Of the two proposed circular conformations detected (α and β), we favor the view that component β is a relaxed circular form and component α is a supercoiled circular form. In support of this possibility, we observed that DNA isolated from CsCl gradients, which involves much physical manipulation, or DNA that has been stored for extended periods of time shows a higher proportion of the linear form and little or no component α (data not shown). In addition, it seems that both forms α and β can be directly converted into linear genome-sized molecules by the action of restriction endonucleases having only one restriction site in the physical map of the *C. moewusii* mtDNA. We favor the hypothesis that the 23-kb linear mtDNA component observed in the undigested DNA samples is the result of random double-strand breaks in components α and β, which occur during DNA isolation, rather than by the site-specific cleavage of an unknown endonuclease *in vivo*. In the latter case, one would expect that digestion of this linear DNA fraction with restriction enzymes having only one recognition site in the circular mitochondrial genome map would produce two distinct DNA components with a combined size of 23 kb; this was not observed when the DNA was digested individually with three such restriction enzymes that cut in different regions of the mitochondrial genome map.

Conformation of CW-group mtDNAs. We were interested in testing the hypothesis that genome- or subgenomic-sized linear mtDNAs are characteristic of the *Volvox* clade and that other CW-group taxa have circular genome-sized mtDNAs. In support of this hypothesis, DNA from all *Volvox* clade taxa tested revealed only single hybridizing bands after DNA filter blot analysis with the mtDNA probe. It is probable that the linear hybridizing components reflect unit mitochondrial genomes, although in the case of *P. parva* Fan and Lee (2002) showed at least one additional small subgenic mtDNA encoding *nad6*, which is not detected with the *C. reinhardtii* mtDNA probe used in this study. Although we did not include any colonial CW-group algae in the present study, it has been demonstrated that the mitochondrial genome from one such taxon, *Pandorina morum*, is linear (Moore and Coleman 1989). In addition, it has been shown that this taxon, *Volvox carteri* and a variety of other colonial algae are closely related to one another and that as a group they form a lineage with *C. reinhardtii* to the exclusion of taxa from the *Dunaliella* clade and the *Tetracystis* clade

(Coleman 1999). Hence, it is likely that all these taxa have linear mitochondrial genomes.

In contrast, all taxa examined from the *Dunaliella* clade and the *Tetracystis* clade, with the exception of *C. capillatum*, had three DNA components detected by the *C. moewusii* mtDNA probe. These bands likely correspond to open- and closed-circular mtDNA molecules, along with linear genome-sized mtDNA molecules that were derived from the circular forms during DNA isolation, as suggested for *C. moewusii*. It is expected that under the electrophoretic conditions used here, the faster migrating (lower) nonlinear DNA is a supercoiled circular DNA and the slower migrating (upper) non linear DNA is a relaxed circular DNA. Consistent with this possibility, we note that the faster migrating nonlinear form of the 16-kb mtDNA of *C. pitchsmannii* migrated to approximately the same position as the faster migrating nonlinear form of the 23-kb mtDNA of *C. moewusii*, an observation that could be explained by the unpredictable migration of supercoiled molecules through agarose gels that depends not only on the size of the molecules but also on their superhelical density (Johnson and Grossman 1977). The single nonlinear mtDNA form of *C. capillatum* is probably the open circular form, with the closed circular form being absent as a result of DNA degradation. We note also that although the physical map of the *C. capillatum* mtDNA is smaller than that of *C. moewusii*, the *C. capillatum* linear mtDNA migrated slower than the linear mtDNA of *C. moewusii*. This could be explained by a migration retardation of the *C. capillatum* DNA due to the intentional overloading of this sample to obtain an appropriate hybridization signal.

Concluding comments. Available data are consistent with the hypothesis that the linear genome- or subgenomic-sized mtDNA conformation in green algae is limited to the *Volvox* clade of the chlorophycean CW-group and that this linear DNA was derived from an ancestral circular form, which appears to have been maintained in all other CW-group taxa examined and possibly all other green algae. In an attempt to determine if the origin of the linear mtDNA form occurred at the base of the *Volvox* clade or earlier in the evolution of the CW-group, it will be necessary to examine mtDNA conformation in other extant clades that are closely related to the *Volvox* clade, such as the "*Oogamochlamys* clade" of Pröschold et al. (2001). Although this clade has been resolved by molecular phylogenetic analysis of chloroplast-encoded large subunit (Turmel et al. 1993, Buchheim et al. 1996) and nucleus-encoded small and large subunit rRNA sequences (Pröschold et al. 2001, Buchheim et al. 2001), only the chloroplast data provide strong statistical support for it being a sister clade of the *Volvox* clade; the other data, however, are not inconsistent with this possibility.

It has been suggested (Nedelcu and Lee 1998b) that linearization of mtDNA within the chlorophycean class may have been the result of incidental recombination between a circular genome-sized mtDNA and a linear episome with terminal inverted repeat sequences, such as described in some strains of maize (Schardl et al. 1984). The apparently rare occurrence of extant clades with linear mitochondrial genomes in green algae is in contrast to the situation in yeast where taxa with linear- or circular-mapping mtDNAs are interspersed on phylogenetic trees (Fukuhara et al. 1993, Nosek et al. 1998), therefore suggesting a different mechanism or mechanisms for the origin of the linear mtDNA forms in yeast.

Aside from the need for a special mechanism to replicate the 5'ends of any linear DNA, the long-term biological significance of having a linear mitochondrial genome in the green algae or any other group of organisms is not clear. Based on available data, there seems to have been no effect of mtDNA linearization in the CW-group on the size or gene content of this DNA (Nedelcu and Lee 1998a, Fan and Lee 2002). As more linear and circular mitochondrial genomes from this group are sequenced, it will be of interest to determine if there is any correlation between mtDNA conformation and the rate and pattern of its evolution. For example, it has been suggested that in yeast, gene rearrangements occur much less frequently among mitochondrial genomes with a particular type of linear structure compared with those that are circular (Drissi et al. 1994, Nosek et al. 1998). Current information from CW-group taxa, however, reveals coding regions in the mtDNA to be highly rearranged both among and between taxa with linear or circular mitochondrial genomes (Nedelcu and Lee 1998a, Fan and Lee 2002).

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