A Mating Type-linked Mutation that Disrupts the Uniparental Inheritance of Chloroplast DNA also Disrupts Cell-Size Control in *Chlamydomonas*

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An intriguing feature of early zygote development in *Chlamydomonas reinhardtii* is the active elimination of chloroplast DNA from the mating-type minus parent due presumably to the action of a zygote-specific nuclease. Meiotic progeny thus inherit chloroplast DNA almost exclusively from the mating-type plus parent. The plus-linked nuclear mutation mat3 prevents this selective destruction of minus chloroplast DNA and generates progeny that display a biparental inheritance pattern. Here we show that the mat3 mutation creates additional phenotypes not previously described: the cells are much smaller than wild type and they possess substantially reduced amounts of both mitochondrial and chloroplast DNA. We propose that the primary defect of the mat3 mutation is a disruption of cell-size control and that the inhibition of the uniparental transmission of chloroplast genomes is a secondary consequence of the reduced amount of chloroplast DNA in the mat3 parent.

**INTRODUCTION**

A characteristic feature of eukaryotes is the uniparental transmission of mitochondrial and chloroplast genomes to sexual progeny, an inheritance pattern accomplished by one of two general mechanisms. In unicellular organisms, where gametes are generally isogamous and contribute equal numbers of organelles to the zygote, uniparental inheritance is accomplished by selective destruction of one set of organelles (Braten, 1971, 1973) or organelle genomes in the developing zygote (Harris, 1989; Kuroiwa, 1991; Meland *et al.*, 1991). In multicellular organisms, gametes are generally anisogamous and organelles are transmitted almost exclusively via the egg or, in the case of conifers, via the male gamete (Whatley, 1982). Until recently, this pattern has been attributed to dilution—the small male gamete simply fails to contribute much organelle DNA to the zygote (Kondo *et al.*, 1990; Gyllensten *et al.*, 1991; Hoeh *et al.*, 1991), but it now appears that at least in mammals, both dilution and selective destruction are involved (Yaneda *et al.*, 1995). Despite the nearly universal nature of uniparental inheritance, the driving force for its evolution remains controversial (Cosmides and Tooby, 1981; Hurst and Hamilton, 1992; Charlesworth, 1994; Sears and VanWinkle-Swift, 1994) and underlying molecular mechanisms remain mysterious.

The unicellular haploid alga *Chlamydomonas reinhardtii* has become an important system for analyzing the uniparental inheritance of chloroplast DNA (Gillham, 1994). *C. reinhardtii* generates isogamous gametes of two mating types, *plus* (*mt*⁺) and *minus* (*mt*⁻), that each contain a single cup-shaped chloroplast. The 200-kb chloroplast genome is present in 80–90 copies per cell (Gillham, 1978) and is localized into 8–10 discrete protein/DNA complexes called nucleoids (Ris and Plaut, 1962). Greater than 90% of meiotic progeny inherit their chloroplast genomes from the *mt*⁺ parent only, a phenomenon first described over 40 years ago (Sager, 1954). Both genetic and molecular data indicate that uniparental inheritance results from the selective destruction of *mt*⁻ chloroplast DNA within 1–2 h of zygote formation (reviewed in Gillham, 1994). Two distinct events are believed to underlie the selective process: protection of *mt*⁺ chloroplast DNA, perhaps occurring during gametogenesis, and

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subsequent destruction of unprotected mt⁻ chloroplast DNA in the newly formed zygote.

Protection is the less well understood event. A mutant strain mt11, apparently defective in protector function, has been identified in the homothallic species *Chlamydomonas monica*: when the mt⁺ parent carries mt11, both mt⁺ and mt⁻ chloroplast DNA is destroyed in the zygote, a lethal phenotype (VanWinkle-Swift and Salinger, 1988). No comparable mutation has yet been identified in *C. reinhardtii*. Protection was originally proposed to result from the methylation of cytosine residues in mt⁺ chloroplast DNA (Sager and Lane, 1972). Subsequent studies, however, found that neither hypermethylation (Bolen et al., 1982) nor hypomethylation (Feng and Chiang, 1984) of mt⁺ chloroplast DNA affected inheritance patterns. Thus, the mechanisms underlying protection remain unclear.

More is known about destruction, a process hypothesized to involve a nuclear-encoded (Kuroiwa et al., 1983a,b; Kuroiwa, 1995) zygote-specific nuclease that degrades unprotected mt⁻ chloroplast DNA (Kuroiwa, 1985; Sager and Grabow, 1985; Armburst et al., 1993). In *C. reinhardtii*, destruction can be prevented, and biparental inheritance thereby enhanced, by three different means. In the first, exposure of mt⁺ gametes to low levels of ultraviolet (UV) irradiation just before mating disrupts destruction in the zygote (Sager and Ramanis, 1967; Gillham et al., 1974). We have recently shown that the mt⁻-linked gene ezy1 is expressed within minutes of zygote formation and its gene product localizes to chloroplast nucleoids (Armburst et al., 1993). Importantly, ezy1 expression displays the same sensitivity to UV irradiation as uniparental inheritance, suggesting that ezy1 plays a role in destruction (Armburst et al., 1993).

Second, inheritance patterns are affected by the amount of chloroplast DNA carried by the mt⁺ parent. An increased percentage of zygotes inherit chloroplast markers from the mt⁻ parent if the amount of chloroplast DNA contributed by the mt⁺ parent is reduced, either by mating cells of different ploidy levels (Matagne and Mathieu, 1983) or by treating cells with 5-fluorodeoxyuridine (FdUr), a specific inhibitor of chloroplast DNA replication (Wurtz et al., 1977; Matagne and Beckers, 1983).

Finally, destruction of mt⁻ chloroplast DNA is almost completely inhibited when the mt⁺ gamete carries the mt⁻-linked mutation mat3, an observation that led to the proposal that the mat3 gene encodes a key component of the destruction process (Gillham et al., 1987b; Gillham, 1994; Munaut et al., 1990). Here we show that mat3 cells display two additional mutant phenotypes not previously described: the cells are remarkably small, and they contain very little mitochondrial or chloroplast DNA. We propose that the primary defect induced by the mat3 mutation is a disruption of cell-size control and that the disruption of uniparental inheritance of chloroplast genomes is a secondary consequence of the reduced level of chloroplast DNA in mat3 mt⁺ gametes.

**MATERIALS AND METHODS**

**Strains and Culture Conditions**

All *C. reinhardtii* strains were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC) and were maintained in continuous light on Tris-acetate phosphate (TAP) media (Gorman and Levine, 1965) solidified with 1.5% agar. Liquid TAP cultures were shaken continuously in constant light. The wild-type mt⁺ strain used was CC-620 and the wild-type mt⁻ strains used were CC-124 and CC-621. The mutant mt⁻ strain CC-2660 (nic7 ac29a mt⁻ kr-u-24-2) is grown in TAP + 2 μg/ml nitocanamide, is sensitive to 7.5 μg/ml 3-acytlimidipryidine (AP), and is resistant to 100 μg/ml kanamycin (kan⁶) due to a single C to T change in the chloroplast 16S rRNA gene (Harris et al., 1989). The mat3 strains used were CC-1993 (mat3⁻1 mt⁺ spr-u-1-27-3), CC-1995 (mat3⁻2 mt⁺ spr-u-1-27-3), CC-2646 (mat3⁻3 mt⁺ spr-u-1-27-3), and CC-1996 (mat3⁻3 mt⁺ spr-u-1-27-3). CC-2646 is a subclone of CC-1995. Each mat3 strain is resistant to 100 μg/ml spectinomycin due to a single G to A change in the chloroplast 16S rRNA gene (Harris et al., 1989).

Growth rates were determined for cultures maintained both on TAP plates and in liquid TAP. For growth rates on plates, the entire plate was flooded with 5 ml 2% glutaraldehyde and replicate aliquots were counted using a hemocytometer. For growth rates in liquid, aliquots were fixed in 2% glutaraldehyde and counted directly. At least 200 cells were counted for each sample.

FdUr treatment was essentially as described by Matagne and Beckers (1983). Briefly, cells were grown to stationary phase (approximately 1 wk) on TAP agar plates containing a range of concentrations of FdUr and then transferred to a new TAP + FdUr plate for an additional week of growth.

**Genetic Analysis of Mutant Phenotypes**

Gametes were generated by transferring cells maintained on TAP agar plates for at least 7 days (Martin and Goodenough, 1979) to nitrogen-free high salt minimal media (Sueoka, 1960) for a minimum of 1 h. Genetic analysis of nuclear markers was performed as described by Levine and Ebersold (1960). Tetrad analysis was restricted to complete tetrads or to tetrads in which three of four meiotic products were viable and the genotype of the fourth product could be unambiguously determined.

Chloroplast inheritance patterns were determined essentially as described by Gillham et al. (1987b). Briefly, the mt⁺ parent to be tested was mated to CC-2660 for 1–2 h and plated on TAP + 7.5 μg/ml AP. The presence of AP in the media is lethal to both the mt⁻ parent and the resulting mt⁺ meiotic progeny because the nic7 mutation, which confers sensitivity to AP, is tightly linked to the mt⁻ locus. Zygotes were matured as described by Levine and Ebersold (1960). The zygote plates were transferred to the light, flooded with nitrogen-free high salt minimal media, and aliquots were spread on fresh TAP + AP plates. These plates were exposed to chloroform vapors for 30 s to kill unmated gametes. Depending on the cross, the resulting zygote colonies were replica plated onto TAP + AP plates with or without either spectinomycin or kanamycin. At least 90 zygote colonies were tested for each cross.

**Video Microscopy**

A Leitz Diaplan microscope equipped with a Hamamatsu C2400 SIT camera was used to obtain images of mutant and wild-type cells. The resulting images were processed with an Argus-10 Image Processor (Hamamatsu) and recorded on a Panasonic Optical Disk Storage Unit model TQ-3038F. Size measurements for each sample were performed on 100 random cells using the quick draw feature.
of the image processor. Micrographs were obtained using a Sony Video Graphic Printer model UP-860.

**Electron Microscopy**

Fixation and sectioning for electron microscopy were performed as described by Goodenough et al. (1982).

**Western Analysis**

Western analysis was performed as described by Armbrust et al. (1993).

**Southern Analysis**

Total DNA was isolated essentially as described by Weeks et al. (1986). Restriction enzyme-digested DNA was electrophoresed on 0.8% agarose gels and transferred to nitrocellulose according to the method of Sambrook et al. (1989). 32P-labeled probes were generated using the random primed labeling kit (Boehringer Mannheim, Indianapolis, IN). Plasmids containing chloroplast (p123) and mitochondrional DNA (p85) were obtained from the *Chlamydomonas* Genetics Center (Duke University). p123 is composed of the 6.8-kb BamHI chloroplast DNA fragment (BamHI 13) isolated from *C. reinhardtii* CC-125 (Harris, 1989) and ligated into pBR322; the chloroplast-specific probe was the entire 6.8-kb BamHI fragment. p85 is composed of the 5.6-kb BamHI/SalI fragment (Harris, 1989) isolated from *C. reinhardtii* CC-125 and ligated into pBR322; the mitochondrion-specific probe was the entire 5.6-kb BamHI/SalI fragment. The nuclear-specific cyt1 probe was a 0.75-kb fragment from the 3' end of cyt1 (Armbrust et al., 1993) and the nuclear-specific aryl sulfatase probe was a 1.05-kb BamHI fragment derived from an aryl sulfatase cDNA (de Hostos et al., 1989). The blots were prehybridized and hybridized at 65°C according to Church and Gilbert (1984). Preflashed autoradiograms were scanned with a Computing Densitometer model 300A (Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

**The mat3 Mutation Generates a Cell-size Defect in Addition to the Biparental Inheritance Phenotype**

The biparental inheritance phenotype associated with the mt+–linked mutation mat3 (Gillham et al., 1987b) is readily suppressed (Gillham, personal communication). Indeed, none of the mat3 strains obtained from the *Chlamydomonas* Genetics Center displayed the biparental phenotype and therefore one strain was back-crossed to wild type. Unexpectedly, some of the resulting mat3 mt+ progeny displayed a phenotype not previously described: the vegetative cells and gametes are substantially smaller than wild-type cells (Figure 1). The average cell length of wild-type mt− gametes (CC-621) is 6.6 μm, whereas the average cell length of the small mat3 mt− gametes is 2.8 μm (Figure 1C), a decrease in cell volume of approximately 13-fold. The flagella of the small mat3 mt− cells are also reduced in length from 12.1 μm to 8.7 μm (Figure 1D), but they are still disproportionately long relative to cell size. Nonetheless, the cells display no obvious impairment in their ability to swim or to engage in the mating reaction.

Because the original report of the mat3 mutation did not describe a cell-size defect (Gillham et al., 1987b), experiments were performed to determine whether the new phenotype was due to a second mutation, unrelated to mat3, that had arisen spontaneously during the intervening years. The mat3 mutation has never been observed to recombine with the mt+ locus (Gillham et al., 1987b). Therefore each of the three suppressed mat3 strains was back-crossed to wild type to determine if the cell-size defect was also tightly linked to mt+. The resulting meiotic progeny that displayed the size defect were invariably mt+ (Table 1). Moreover, when small mat3 mt+ progeny were crossed to wild type, the resulting tetrads were always parental ditypes with all the mt− progeny displaying the size defect (Table 1). If instead, a wild-type–sized (suppressed) mat3 mt+ strain was crossed to wild type, equal numbers of parental and nonparental ditypes were recovered: approximately one-half of the mat3 mt+ progeny were large (suppressed) and one-half were small, but again, none of the mt− progeny were small. Therefore the cell-size defect segregates as a single nuclear mutation while suppression of the size defect segregates as a second extragenic nuclear mutation unrelated to either mat3 or the mt+ locus (Table 1). Hundreds of progeny resulting from back-crosses of either suppressed or unsuppressed mat3 strains have been examined and no small mt− progeny have ever been observed, confirming that the size defect, like the biparental inheritance phenotype, is tightly linked to the mt+ locus.

Because the mt+ locus encompasses a large region of suppressed recombination (Ferris and Goodenough, 1994), the mat3 mutation could, in theory, reside almost 850 kb away from a cell-size mutation and the two mutant phenotypes would still co-segregate. However, it is unlikely that a single extragenic suppressor would suppress both the cell-size and the uniparental inheritance defects unless they were pleiotropic defects of a single gene. The relationship between cell size and chloroplast inheritance patterns was therefore examined. Small and wild-type–sized (suppressed) mat3 strains were mated to a mt− strain carrying a chloroplast mutation that conveys resistance to the antibiotic kanamycin (kanR), and the number of zygotes that displayed kanR were scored (Table 2). When wild type–sized (suppressed) mat3 mt+ strains were tested, less than 4% of the resulting zygotes inherited kanR from the mt− parent (Table 2), values similar to those obtained using a wild-type mt+ parent. In contrast, when small mat3 mt− strains were tested, high levels of biparental inheritance were observed. Depending on the small mat3 strain used, anywhere from 33–64% of the resulting zygotes inherit kanR from the mt− parent (Table 2), inheritance patterns consistent with the original description of mat3 (Gillham et al., 1987b). Thus, the biparental inheritance of chloroplast markers and the disruption of cell-size control...
Figure 1. Cell size characteristics of the wild-type mt− strain CC-620 and an unsuppressed mat3 mt+ strain. (A) Wild-type mt− gamete. Magnification, 787.5X. (B) Unsuppressed mat3 mt+ gamete. Magnification, 787.5X. (C) Cell length distribution of 100 unsuppressed mat3 mt+ gametes (thick line) and 100 wild-type mt− gametes (thin line). (D) Flagella length distribution of 100 unsuppressed mat3 mt+ gametes (thick line) and 100 wild-type mt− gametes (thin line).

are both tightly linked to mt+ and are both suppressed by an unlinked extragenic suppressor, indicating that both mutant phenotypes are generated by the mat3 mutation.

**At Least Two Extragenic Mutations Can Suppress the mat3 Phenotypes**

Suppression of two of the mat3 strains was studied in more detail by first crossing a suppressed mat3−1 mt+ strain to wild type. The two mt− progeny from a zygote in which both mat3 mt+ progeny were small (nonparental ditype) were assumed to carry the suppressor mutation, and one of these was designated smt1 mt− (for suppressor of mat3). Because the smt1 mt− strain does not display an obvious mutant phenotype, its genotype was confirmed by crossing it to an unsuppressed mat3−1 mt+ strain. Thirteen of the 21 mt+ progeny examined were wild-type sized (suppressed), confirming that the mt− parent did carry the smt1 mutation (Table 3). The smt1 mt− strain was then crossed to an unsuppressed mat3−2 mt+ strain. Again, one-half of the resulting mt+ progeny were wild-type sized (suppressed), indicating that smt1 can suppress both mat3−1 and mat3−2 (Table 3). To determine whether smt1 was identical to the suppressor carried by mat3−2 (designated smt2), smt1 mt− was crossed to mat3−2 smt2 mt+. If smt1 is the same as smt2, all the
Table 1. Meiotic segregation of cell-size defect and mating type

<table>
<thead>
<tr>
<th>Cross</th>
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<th>Minus progeny</th>
<th>Tetrads</th>
<th>Tetrads</th>
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<td></td>
<td>Large</td>
<td>Small</td>
<td>Large</td>
<td>Small</td>
</tr>
<tr>
<td>mat3-1 smt mt⁺⁺</td>
<td>41</td>
<td>47</td>
<td>87</td>
<td>0</td>
</tr>
<tr>
<td>X CC-124</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mat3-2 smt mt⁺</td>
<td>29</td>
<td>43</td>
<td>91</td>
<td>0</td>
</tr>
<tr>
<td>X CC-124</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>mat3-2 smt mt⁺⁺</td>
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<td>44</td>
<td>81</td>
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<tr>
<td>mat3-3 smt mt⁺</td>
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<td>19</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>X CC-124</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mat3-2 mt⁻</td>
<td>0</td>
<td>41</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>X CC-124</td>
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<td>mat3-2 mt⁻</td>
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<td>28</td>
<td>29</td>
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</tr>
<tr>
<td>X CC-621</td>
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a PD, parental ditype; NPD, nonparental ditype; T, tetratype. Tetrad analysis was restricted to complete tetrads or ones in which three of four meiotic progeny were viable and the genotype of the fourth product could be determined.
b smt indicates the presence of a suppressor of the cell-size defect.
c The mat3-2 strain used for this cross was CC-1995. All other mat3-2 crosses were performed with CC-2646.

resulting mat3 mt⁺ progeny should be wild-type sized because they will all carry the suppressor. If the two suppressors are different, however, independent assortment will generate small mat3 mt⁺ progeny carrying neither suppressor. Approximately 28% of the resulting mt⁺ progeny were small, indicating that at least two different extragenic nuclear mutations can suppress the mat3 phenotypes. Again, no obvious mutant phenotypes were observed in the smt1, smt2, or smt1 smt2 double mutants generated in these crosses.

The mat3 Strains Display Growth Defects Under Nutrient-limited Conditions

The fact that at least two different suppressors arise quickly and spontaneously in a mat3 mt⁺ background

Table 2. Relationship between cell-size defect and biparental inheritance of chloroplast markers

<table>
<thead>
<tr>
<th>mat3 parent</th>
<th>size of mat3 parent</th>
<th>Chloroplast inheritance patterns</th>
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</thead>
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<tr>
<td>mat3-1 smt mt⁺⁺</td>
<td>wild type</td>
<td>%UP⁺</td>
</tr>
<tr>
<td>mat3-2 smt mt⁺⁺</td>
<td>wild type</td>
<td>100</td>
</tr>
<tr>
<td>mat3-3 smt mt⁺⁺</td>
<td>wild type</td>
<td>100</td>
</tr>
<tr>
<td>mat3-2 mt⁺+</td>
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<td>96</td>
</tr>
<tr>
<td>mat3-3 mt⁺</td>
<td>wild type</td>
<td>100</td>
</tr>
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<td>36</td>
</tr>
<tr>
<td></td>
<td>small</td>
<td>66</td>
</tr>
</tbody>
</table>

a All mat3 mt⁺ strains are resistant to 100 μg/ml spectinomycin (spec⁺); the mt⁻ parent is resistant to 100 μg/ml kanamycin (kan⁻).
b UP⁺, all the meiotic progeny within a zygote colony inherit only spec⁺ from the mt⁺ parent; BP, meiotic progeny within a zygote colony inherit spec⁻ and kan⁻. UP⁻, all the meiotic progeny within a zygote colony inherit only kan⁻ from the mt⁻ parent; Exceptionals, BP + UP⁻.
c smt indicates the presence of a suppressor of the cell-size defect.
d The chloroplast inheritance patterns of wild-type-sized and small mat3-2 strains were tested on three separate occasions.
Table 3. Demonstration that at least two mutations can suppress mat3

<table>
<thead>
<tr>
<th>Cross</th>
<th>Plus progeny</th>
<th>Minus progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Large</td>
<td>Small</td>
</tr>
<tr>
<td>mat3-1 mt⁺</td>
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<td>8</td>
</tr>
<tr>
<td>X smt1 mt⁻</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mat3-2 mt⁺</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>X smt1 mt⁻</td>
<td></td>
<td></td>
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<tr>
<td>mat3-2 smt2 mt⁺</td>
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<td>18</td>
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<tr>
<td>X smt1 mt⁻</td>
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</tbody>
</table>

suggests that mat3 cells are under strong selection. To determine whether the selection reflected a reduced growth rate, wild-type-sized (suppressed) and small mat3 strains were maintained in exponential growth on either TAP agar plates or in liquid TAP. Under nutrient replete conditions, suppressed and unsuppressed mat3 strains are indistinguishable: both form either four or eight mitotic progeny per mother cell and both display a mean doubling time of about 6 h on plates and about 10 h in liquid. The strains exhibit very different growth characteristics, however, if they are first grown on TAP plates for 1 wk, a treatment that results in nitrogen starvation (Martin and Goodenough, 1975) and induces cells to enter G0, before they are transferred to liquid TAP. Under these conditions, the mutant cells fail to divide for up to 75 h after the transfer, whereas the suppressed cells resume division within 18 h (Figure 2). This suggests that mat3 cells are defective in their ability to exit from stationary phase and initiate mitotic divisions. Importantly, once cell division is underway in the mat3 culture, the population becomes dominated by wild-type-sized (suppressed) cells, suggesting that under these conditions, suppressed mat3 cells have a growth advantage. Moreover, mat3 cells die more quickly than either suppressed or wild-type cells on agar plates when maintained under conditions of prolonged nitrogen starvation, again suggesting that small mat3 cells have difficulty in stationary phase.

Expression of ezy1 in mat3 Zygotes

The biparental inheritance phenotype associated with mat3 can also be generated by a brief UV irradiation of mt⁺ gametes just before mating (Sager and Ramanis, 1967). This treatment disrupts the zygote-specific transcription of ezy1, a gene postulated to be involved in destruction of mt⁻ chloroplast DNA (Armbrust et al., 1993). To determine whether the mat3 mutation affects expression of ezy1, wild-type or mat3 mt⁺ parents were mated to wild-type mt⁻ parents and total protein was isolated from the resulting zygotes 1 and 3 h later. Western analysis using polyclonal antibodies generated against an ezy1 fusion protein (Armbrust et al., 1993) was used to detect ezy1 expression. The timing and level of expression of ezy1 protein are comparable in wild-type and mat3 zygotes (Figure 3), indicating that UV irradiation and the mat3 mutation disrupt uniparental inheritance through different mechanisms.

Small mat3 Cells Possess a Small Chloroplast and a Reduced Amount of Organelle DNA

Approximately 40% of the Chlamydomonas cell volume is occupied by the chloroplast (Harris, 1989). Perhaps not surprisingly, therefore, small mat3 cells possess a much smaller chloroplast than wild-type cells, although the relative proportions appear similar (Figure 4). All other organelles are also present in the small mat3 cells, again in apparently appropriate proportions (Figure 4).

Figure 2. Growth rates of suppressed (thin line) and unsuppressed (thick line) mat3 cells in liquid TAP after transfer from nutrient-depleted conditions.

Figure 3. Western analysis of total protein isolated from wild-type and mat3 zygotes. Approximately equal amounts of total protein from 1- and 3-h zygotes were separated using SDS/PAGE, transferred to nitrocellulose, and probed with polyclonal antibodies generated against an ezy1 fusion protein.
Figure 4. Transmission electron microscopy of two mat3 gametes (A and B) and a wild-type gamete (C). Magnification, 20,000×.

Southern analysis was used to determine the relative amount of organelle DNA in a number of strains. A suppressed mat3–2 strain was crossed to CC-124 and total DNA was isolated from replicate samples of
a wild-type-sized \( mt^- \) progeny, a wild-type-sized (suppressed) \( mat3^-2 \) smt2 \( mt^- \) progeny, and a small \( mat3^-2 \) \( mt^- \) progeny all from the same tetrad. Chloroplast-specific and mitochondrial-specific probes were used to determine organelle DNA content and two nuclear-specific probes were used to determine nuclear DNA content. Regardless of the nuclear probe used for normalization, the relative amount of chloroplast DNA in the small \( mat3^-2 \) \( mt^- \) strain is about 3.5-fold less than the wild-type \( mt^- \) strain while the relative amount of mitochondrial DNA is about 2.5-fold less (Figure 5), indicating that the \( mat3^-2 \) mutation generates not only a reduced cell size but also a reduced organelle DNA content.

Unexpectedly, the suppressed \( mat3^-2 \) strain contained about 1.6 times as much chloroplast DNA and about 1.3 times as much mitochondrial DNA as the wild-type \( mt^- \) strain from the same tetrad (Figure 5), suggesting that these suppressed cells might be larger than wild-type. Cell size was therefore determined for a number of strains. The mean cell size of suppressed \( mat3^-1 \) and \( mat3^-3 \) is 7.7 \( \mu m \), comparable to the 7.6 \( \mu m \) observed for the wild-type \( mt^- \) strain CC-125 used to generate \( mat3^-2 \) (Gillham et al., 1987b), and slightly larger than the 6.6 \( \mu m \) observed for the wild-type \( mt^- \) strain CC-621. In contrast, the suppressed \( mat3^-2 \) strain is about 9.0 \( \mu m \), an increase in cell volume about 1.7 times that of either wild-type strain and comparable to the observed increase in organelle DNA (Figure 5), again suggesting a coupling between cell size and organelle DNA content.

**Figure 5.** Southern analysis of total DNA isolated from a wild-type \( mt^- \) progeny, a wild-type-sized \( mat3^-2 \) smt2 \( mt^- \) progeny, and a small \( mat3^-2 \) \( mt^- \) progeny and probed with two nuclear-specific probes, \( ezy^-1 \) and aryl sulfatase, a 6.8-kb BamHI chloroplast DNA probe, and a 5.6-kb BamHI/SalI mitochondrial DNA probe. (A and B) Total DNA isolated from replicate samples.

The Biparental Inheritance Phenotype, But Not the Cell-size Defect, Is Correlated with the Reduced Chloroplast DNA Content of \( mat3^-2 \) Cells

Treatment of wild-type \( mt^- \) cells with FdUr generates cells that mimic two of the three characteristics of \( mat3^-2 \) cells: a reduced chloroplast DNA content and a disruption of uniparental inheritance (Wurtz et al., 1977; Matagne and Beckers, 1983). Moreover, a direct correlation has been shown to exist between the chloroplast DNA content of the \( mt^- \) parent and the percentage of zygotes that inherit chloroplast DNA from the \( mt^- \) parent; as the amount of chloroplast DNA contributed to the zygote by the \( mt^- \) parent is decreased, the number of zygotes that transmit \( mt^- \) chloroplast DNA to their progeny is increased (Wurtz et al., 1977; Matagne and Beckers, 1983; Matagne and Mathieu, 1983). A biparental inheritance phenotype is thus strongly correlated with a reduced chloroplast DNA content in both \( mat3^-2 \) and FdUr-treated \( mt^- \) gametes.

To examine the relationship between chloroplast DNA content, chloroplast DNA inheritance patterns, and cell size, suppressed and unsuppressed \( mat3^-2 \) strains were treated with increasing concentrations of FdUr. As expected, treatment of wild-type-sized (suppressed) \( mat3^-2 \) cells with FdUr generates cells with reduced amounts of chloroplast DNA (Table 4), comparable to previously reported levels (Wurtz et al., 1977). Moreover, when these suppressed \( mat3^-2 \) \( mt^- \) parents with less chloroplast DNA are mated to a \( mt^- \) strain carrying \( kan^- \), the percentage of zygotes that inherit \( kan^- \) increases as the amount of chloroplast DNA contained within the \( mt^- \) parent decreases (Table 4). Curiously, this same FdUr treatment did not lead to a further reduction in the chloroplast DNA content of the small \( mat3^-2 \) cells, nor in a significant change in their subsequent transmission patterns (Table 4), suggesting that some chloroplast DNA replication may be FdUr insensitive. Importantly, suppressed \( mat3^-2 \) cells with an FdUr-induced reduction in chloroplast DNA remain wild type in size (Figure 6), indicating that the cell-size defect of \( mat3^-2 \) cells is not simply due to a reduced chloroplast DNA content.

**DISCUSSION**

A number of models have been developed to explain the uniparental inheritance of organelle genomes in C. reinhardtii (reviewed in Sears and VanWinkle-Swift, 1994). A key feature of each model is the central regulatory role played by the mating-type locus: chloroplast genomes are transmitted almost exclusively by the \( mt^- \) parent while mitochondrial genomes are transmitted almost exclusively by the \( mt^- \) parent. In
Table 4. Effect of FdUr treatment on chloroplast DNA content and chloroplast genome inheritance of mat3 strains

<table>
<thead>
<tr>
<th>mat3 parent</th>
<th>mM FdUr</th>
<th>Relative chloroplast DNA content</th>
<th>Chloroplast inheritance patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%UP⁺</td>
<td>%BP</td>
</tr>
<tr>
<td>mat3-2 smt2 mt⁺</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>100</td>
<td>0</td>
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<td>31</td>
</tr>
<tr>
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<td>20</td>
</tr>
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<td>mat3-2 mt⁺</td>
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</tr>
<tr>
<td></td>
<td>1.0</td>
<td>29</td>
<td>44</td>
</tr>
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</table>

C. reinhardtii, the mating-type locus is a complex region of suppressed recombination (Gillham, 1978) spanning almost 1 megabase of DNA (Ferris and Goodenough, 1994). The mt⁺ and mt⁻ loci are homologous except for a central domain of about 190 kb in which chromosomal rearrangements have occurred and sequences unique to one or the other mating-type are found (Ferris and Goodenough, 1994). Genes encoding the protector of mt⁺ chloroplast DNA and the destroyer of mt⁻ chloroplast DNA have been proposed to reside within the mt⁺ locus while genes required for mitochondrial inheritance are postulated to reside within the mt⁻ locus (Gillham, 1994). Identification of the mt⁺-linked mutation mat3 seemed to substantiate this hypothesis: when the mt⁺ parent carries the mat3 mutation, mt⁻ chloroplast DNA is not destroyed in the zygote and meiotic progeny can inherit chloroplast genomes from both parents (Gillham et al., 1987b). Thus it seemed likely that the mat3 gene encoded either the zygote-specific nuclease itself or else a regulator of this nuclease (Gillham et al., 1987b). Our re-examination of the phenotypes associated with the mat3 mutation suggests that contrary to expectations, the mat3 gene likely plays an indirect role in the uniparental inheritance of the chloroplast genome.

Suppression of the mat3 Phenotype

The possibility that the mat3 gene is involved in aspects of the life cycle other than chloroplast DNA transmission is suggested by the fact that the biparental inheritance phenotype is quickly suppressed. Because Chlamydomonas reproduces in the laboratory via mitotic divisions and not by its optional sexual cycle, this observation indicates that the mat3 mutation creates a phenotype selected against during vegetative growth. Under exponential growth conditions, mat3 cells do not display a growth defect. When mat3 cells are deprived of nitrogen and enter stationary (G0) phase, however, they appear handicapped in their ability to resume cycling, allowing suppressed cells to take over the culture. This behavior is most apparent when stationary phase mat3 cells are transferred to liquid culture. We found that mat3 can be suppressed by mutations at two distinct loci, smt1 and smt2, suggesting that the mat3 gene product may act as part of a complex such that compensating mutations in either smt1 or smt2 restore functionality to the complex when a defective mat3 gene product is present. The suppressors confer no obvious mutant phenotypes on their own.

Size Defect of mat3 Cells

A primary defect of the mat3 mutation is the generation of vegetative cells and gametes that are substantially smaller than wild type and contain reduced...
amounts of both mitochondrial and chloroplast DNA. However, the cell-size defect displayed by mat3 cells is not a result of their reduced levels of organelle DNA. We and others (Hosler et al., 1989) have shown that a decrease in the relative amount of chloroplast DNA due to treatment with FdUrd has no effect on cell size. Similarly, Alexander et al. (1974) has shown that cells remain wild-type sized after incubation with acriflavin, a treatment that generates cells with substantial deletions in the mitochondrial genome (Gillham et al., 1987a). This reduction in mitochondrial DNA is quickly lethal, however, and the resulting colonies remain quite small, a phenotype referred to as minute (Alexander et al., 1974).

The simplest explanation for the small size of mat3 cells is that the wild-type gene product is required for cell-size regulation. A defining feature of the cell cycle of many flagellated green algae like Chlamydomonas is that vegetative cells larger than a given size threshold generate multiple mitotic progeny (Craigie and Cavalier-Smith, 1982). In these instances, a cell first progresses through a cycle composed of G1, S, M, and cytokinesis to generate two mitotic progeny encased within the mother wall. These progeny then dispense with a second G1 phase and enter directly into a second round of DNA synthesis and division to form four mitotic progeny (Coleman, 1982; McAteer et al., 1985). Depending on the size of the original mother cell, these four progeny are either released from the mother wall or else enter into a third and perhaps even a fourth round of synthesis and division. The loss of subsequent G1 phases means that essentially no growth occurs during these later rounds of division. If the mat3 gene product was involved in regulating the number of mitotic divisions per cell, a mutated gene product might result in cells that produced a greater number of small mitotic progeny; however, we found no evidence for such a defect. Under the growth conditions tested, the mat3 cells behave as wild type and form only four or eight mitotic progeny per mother cell.

Alternatively, small mat3 cells might be generated by a change in the rate of progression through the cell cycle. In all eukaryotes, cell size is evaluated during the cell cycle at two checkpoints, one at the G1/S transition and a second at the G2/M transition (Sherr, 1994; D’Urso and Nurse, 1995). Cells that lose the ability to regulate progression through these checkpoints, and thus coordinate cell growth and cell division, will divide when they are too small. A number of yeast cell-cycle mutants have been identified that display such a small-size phenotype. For example, the wee1 gene, originally identified in Schizosaccharomyces pombe (Russell and Nurse, 1987), encodes a protein kinase that acts as a negative regulator of entry into mitosis, and the whi1 gene of Saccharomyces cerevisiae encodes a positive regulator of commitment to division (Nash et al., 1988). In wee1 mutants, the G2 phase is shortened while in whi1 mutants, the G1 phase is shortened. In both instances, these cell-cycle defects result in a small cell-size phenotype even though the overall duration of the mutant cell cycles remains unchanged. The normal Chlamydomonas cell cycle is essentially devoid of a G2 phase, with cell growth occurring predominantly during G1 (McAteer et al., 1985). Thus, the mat3 gene product would likely act during the early portion of the cycle. The fact that mat3 cells have difficulty negotiating stationary phase also suggests that the defect occurs during G1.

If the mat3 gene does prove to be involved in cell-cycle regulation, it is not obvious why a mutation in such a gene should result in cells with a reduced amount of chloroplast DNA. The multiple copies of the organelle genome are replicated throughout the cell cycle (Turmel et al., 1980), with an apparently stochastic timing in the replication of any given DNA molecule (Birky, 1994). Therefore, one might expect chloroplast DNA content to depend solely on generation time, and because the doubling time of a mat3 cell is comparable to wild type, these small cells would be expected to contain a wild-type content of organelle DNA. Instead, we clearly observed a correlation between cell size and organelle DNA content—small mat3 cells have less organelle DNA than wild type, and suppressed mat3−2 cells, which are larger than wild-type, have more organelle DNA than wild type—suggesting that replication of organelle DNA is somehow coupled to cell size, most likely due to a coupling to cell-cycle progression. Unfortunately, essentially nothing is known about this relationship; nuclear genes required for the control of chloroplast division have only recently been identified in Arabidopsis (Pyke and Leech, 1992, 1994; Pyke et al., 1994), but none of these genes have been cloned and it is not yet clear how they function.

Relation Between the mat3 Mutation and Uniparental Inheritance

The observation that the mat3 mutation disrupts both cell-size control and uniparental inheritance suggests that a size-control gene required during the vegetative stage of the life cycle is somehow able to influence chloroplast DNA destruction, a zygote-specific phenomenon. One possible explanation is that the mat3 gene might encode a kinase or a phosphatase that has the dual function of regulating cell cycle progression during vegetative growth and activating the chloroplast DNA nuclease during zygote formation. Alternatively, the mat3 gene product might act as a general transcription factor required for expression of genes involved in both cell-cycle regulation and chloroplast inheritance.
The more likely explanation, however, for the coupling between cell-size control and uniparental inheritance is that the mat3 gene is only indirectly involved in the transmission of chloroplast DNA. It is clear that the amount of chloroplast DNA contributed to the zygote by the mt+ parent influences subsequent transmission of mt- chloroplast DNA. We propose that the effect of the mat3 mutation on uniparental inheritance occurs before zygote formation by generating mt+ cells with a reduced amount of chloroplast DNA. We further propose that Chlamydomonas is able to detect the amount of chloroplast DNA contributed to the zygote by the mt+ parent. When this amount falls below a threshold level, the destruction process is disrupted, thus sparing the mt- chloroplast genome and ensuring that meiotic progeny will inherit chloroplast DNA. Because the extent of the biparental inheritance phenotype associated with mat3 is variable (Gillham et al., 1987b; this study), we hypothesize that the inhibition of destruction can be modulated; the less chloroplast DNA contributed to the zygote by the mt+ parent, the more completely destruction is prevented. The fact that cyt1, a gene hypothesized to be involved in mt- chloroplast DNA degradation (Armbrust et al., 1993), is expressed under these conditions suggests that multiple factors may be required for destruction. The key implication of our model is that Chlamydomonas is somehow able to monitor chloroplast DNA content, an ability that may have evolved during the initial endosymbiosis of the chloroplast.

Identification of the cell size defect and the reduced amount of organelle DNA associated with the mat3 mutation presents a unique opportunity to examine the potential relationship between cell cycle control and chloroplast DNA replication. Moreover, it should be possible to confirm whether the Chlamydomonas zygote can count chloroplast genomes by selecting for zygote lethals that are unable to turn off zygote-specific destruction regardless of the amount of chloroplast DNA contributed by the mt+ parent. And finally, the location of a size-control gene like mat3 within the mating-type locus raises intriguing evolutionary questions because genes within this locus fail to recombine into the opposite mating type; therefore, a mutation in a gene like mat3 could in theory generate anisogamy. Further examination of the role played by mat3 will require the characterization of the wild-type gene, a goal that is possible now that the mt locus has been cloned (Ferris and Goodenough, 1994).

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