Abstract. In addition to lethal minute colony mutations which correspond to loss of mitochondrial DNA, acriflavin induces in Chlamydomonas reinhardtii a low percentage of cells that grow in the light but do not divide under heterotrophic conditions. Two such obligate photoautotrophic mutants were shown to lack the cyanide-sensitive cytochrome pathway of the respiration and to have a reduced cytochrome c oxidase activity. In crosses to wild type, the mutations are transmitted almost exclusively from the mating type minus parent. A same pattern of inheritance is seen for the mitochondrial DNA in crosses between the two interfertile species C. reinhardtii and Chlamydomonas smithii. Both mutants have a deletion in the region of the mitochondrial DNA containing the apocytochrome b gene and possibly the unidentified URFx gene.

The green unicellular alga Chlamydomonas reinhardtii constitutes the best model for classical and molecular genetic studies of chloroplast biosynthesis and function. This mainly results from the availability of a large collection of nuclear and non-Mendelian mutants affected in photosynthesis or synthesis of chloroplast components. Mutations localized in the mitochondrial DNA (mit DNA) would be very useful for similar studies on the mitochondria and for the analysis of functional relations between the two organelles. However, attempts to isolate mit DNA mutations have been so far unsuccessful in Chlamydomonas.

In baker's yeast, the intercalating dye acriflavin (AF) induces vegetative petite mutations with 100% efficiency (7). The petite mutants are conditional lethal which survive only when grown on a fermentable carbon source. On a carbon source that must be respired, they die because essential elements of the mitochondria, such as cytochrome oxidase or cytochrome b, are missing. AF-induced mutants either lack mit DNA or have mit DNA molecules containing large deletions (for a review, see reference 8). Alexander et al. (1) used AF to isolate mutants in Chlamydomonas. They found that the dye induces, with nearly 100% efficiency, mutant cells through vegetative growth. Stable dark minus (dk-) mutant cells derived from the two clones were only obtained in crosses to wild type but the results were obscured by the fact that the mutant cells segregated phenotypically wild minus (mt-) parent exclusively (9), as observed in crosses between the two interfertile species C. reinhardtii and Chlamydomonas smithii (4).

Viable mutants affected in mitochondrial function have also been isolated in Chlamydomonas after mutagenic treatment with nitrosoguanidine (19, 20). Contrary to wild type, the mutants were unable to grow in heterotrophic conditions (darkness + acetate) and were defective in cytochrome oxidase activity. Most of these obligate photoautotrophs were mutated in nuclear genes (19). Two mutants however gave a non-Mendelian, random biparental pattern of transmission in crosses to wild type but the results were obscured by the fact that the mutant cells segregated phenotypically wild cells through vegetative growth. Stable dark minus (dk-) mutant cells derived from the two clones were only obtained after acquisition of secondary nuclear mutations (20).

We here describe the isolation of two conditional mutants in which the lesions are localized in the mitochondrial genome. The mutants, induced by AF, are obligate photoautotrophs. Both are defective in cyanide-sensitive respiration and possess deletions in their mitochondrial genomes. The mutations are most often inherited in a non-Mendelian, uniparental paternal fashion (i.e., from the mating type minus or mt- parent only), as is mit DNA in interspecific crosses between C. reinhardtii and C. smithii (see above). To our knowledge, this is the first report of induction of viable
mutations affecting the mitochondrial genome in a photosynthetic organism.

**Materials and Methods**

**Strain and Culture Conditions**

The wild-type strains mating type plus (mt+) and minus (mt−) are derived from strain 137c of *C. reinhardtii*.

The cells were grown on agar plates (15 g/liter bacto-agar Difco) under cool white fluorescent light (90 × 10−6 einsteins × m−2 × s−1) or in the dark at 25°C. Two media were used: minimal (M) medium containing mineral salts only (18) and Tris-acetate phosphate (TAP) medium (10) containing acetate as a carbon source.

Cells were also grown in TAP liquid medium (100 ml in 300-ml flasks) with continuous agitation under a 12-h light/12-h dark regime to induce synchronization of cell divisions (occurring in the middle of the dark period).

**Acriflavin Treatment**

The cells from synchronized liquid cultures were sampled at the beginning of the light period. They were inoculated (2 × 10⁵ cells/ml) into flasks containing 100 ml TAP medium added with 2–8 μg/ml acriflavin (AF; Sigma Chemical Co., St. Louis, MO). AF was always prepared freshly and sterilized by filtration through 0.22 μm Millipore filters. Cells were treated for 1–3 d in the dark. Aliquots were sampled at 24-h intervals and after counting with the aid of a Coulter counter, 2 × 10², 2 × 10³, and 2 × 10⁴ cells were plated on TAP agar solid medium and incubated under mixotrophic (light + acetate) or heterotrophic (darkness + acetate) conditions.

**Genetic Analysis**

The zygotes resulting from crosses were matured for 5 d under continuous light on nitrogen-free minimal agar plates according to VanWinkle-Swift (17). After maturation, blocks of agar carrying 50–100 zygotes were transferred to plates containing fresh M medium and, after meiosis, the spores were plated at random to yield isolated colonies.

**Whole Cell Respiration**

Respiratory rates of whole cells (harvested at the end of the log phase of growth) were measured at 30°C in the dark with a Clark electrode (Gilson oxygraph) in 2 ml TAP medium (10⁷ cells/ml). Total oxygen consumption was recorded during 10–15 min while the rate remains constant. Cyanide- (KCN) sensitive and salicylhydroxamic acid- (SHAM) sensitive respiratory rates were determined in parallel assays by addition at 5-min intervals of 1 mM KCN and 1 mM SHAM (or the reverse). Respiratory rates were expressed in nmol O₂/min⁻¹ × 10⁻⁷ cells taking into account that 1 ml TAP medium contains 210 nmol O₂.

SHAM was purchased from Sigma Chemical Co. and dissolved in ethanol; KCN was dissolved in 17 mM HCl at 0°C in a stoppered flask and used immediately.

**Cytochrome c Oxidase Activity**

Cytochrome c oxidase activity of homogenates (prepared by sonication 3 × 30 s of cells suspended in 0.03 M phosphate buffer, pH 7.4, 0.1% BSA) was assayed in 0.1 M Tris-Cl buffer, pH 7.4, containing 0.15% Triton X-100, 50 μM reduced cytochrome c as a substrate. The oxidation of cytochrome c was followed at 550–540 nm in an Aminco DW-2 spectrophotometer. Reduced cytochrome c was prepared by reduction with ascorbate.

**Molecular Analysis of Mitochondrial DNA**

To characterize physically the mt DNA of the mutant strains in comparison with wild type, two molecular probes were used: (a) the pULG-R1 plasmid containing the 5.8 kb Bam HI–Sal I fragment of the mt DNA (12; P1 in Fig. 3) and (b) the pCrn CEI plasmid containing the 3.87 kb Cla I–Eco RI fragment (16; P2 in Fig. 3).

Total DNA was digested with appropriate restriction enzymes and the resulting restriction fragments separated by electrophoresis on agarose gels. The gels were Southern blotted and the mt DNA fragments were detected by using biotinylated probes, according to procedures described earlier (12).

**Results**

**Effects of AF**

Wild-type mt− cells were grown for 3 d in the dark in the absence or in the presence of AF (2–8 μg/ml). After treatment, the cell suspensions were diluted, plated on TAP (acetate containing) agar medium, and incubated in the light. The viability, estimated on the capacity of the treated cells to divide under mixotrophic conditions, was not substantially modified by the AF treatment: in all cases, the plating efficiency was >60%. However, at the three concentrations tested, the fraction of the cell population giving rise to minute colonies increased with the duration of treatment (Fig. 1). Among the cells giving rise to green viable colonies, some formed colonies of small size, possibly due to a reduction of the cell division rate. The fraction of these small colonies also increased with the time of treatment. Fig. 2 (solid line) gives the results obtained for a concentration of 5 μg/ml AF.

When the treated cells were plated on TAP medium and grown heterotrophically for 7 d, only a small fraction of them formed colonies, all green and viable. After transfer to light, new colonies appeared, most of them of minute phenotype (histogram in Fig. 2), the others were green and viable (broken line in Fig. 2). Hence, cells developing into minute or viable green colonies could survive at least for 1 wk in

![Figure 1. Percentages of minute colonies (minute colonies/min + viable colonies) induced by AF (2, 5, or 8 μg/ml) during 1–3 d. The percentages were determined after growth for 7 d in mixotrophic conditions. No minute colony was observed in control.](https://jcb.rupress.org/content/jcb/108/12/1222/F1)

Downloaded from jcb.rupress.org on August 17, 2017
the dark without dividing but grew only after transfer to light.

One can imagine that among those viable cells unable to grow under heterotrophic conditions or that grow more slowly than wild-type cells under mixotrophic conditions, some are obligate photautotrophic mutants which, like the dk- mutants, are unable to use acetate because certain mitochondrial functions are missing. According to this hypothesis, AF might induce deletions in mitochondrial genes coding for enzymes of the respiratory chain (e.g., cytochrome oxidase or cytochrome b; see Fig. 3). Such deletion mutants would be expected to be unable to grow heterotrophically.

Small viable green colonies were thus isolated and tested for their ability to grow in the dark on acetate-containing medium. Out of 50 colonies analyzed, two were found to display the sought for dk- phenotype. Both were obtained after treatment for 3 d by 8 \( \mu \text{g/ml} \) AF and incubation for 1 wk in the dark before transfer to light. The two clones were compared to wild type for growth under mixotrophic (TAP medium) and photoautotrophic (M medium) conditions. The mutant colonies produced on TAP were smaller than the wild-type colonies whereas no significant difference was found on M agar medium.

**Genetic Analysis of the dk- Clones**

The two clones were crossed to wild-type mt+ cells. The percentages of zygotes which after maturation were able to germinate after 24 h incubation on fresh M medium ranged between 20 and 50%. The individual meiotic progeny were analyzed for their capacity to grow heterotrophically. Whereas the segregation of the mt+ and mt- nuclear alleles (linkage group VI) was 1:1 as expected (data not shown), a majority of the meiotic products were unable to grow in the dark (Table I). In each cross, one dk- mt+ mutant clone was selected and crossed to wild-type mt+ cells. In this case, the zygote germination was 100% and meiotic progeny were able to grow heterotrophically (Table I). Hence, the capacity to grow in the dark is inherited according to a non-Mendelian fashion. The transmission of the dk- phenotype carried by the mt+ parent was frequent when that parent was mutant while the dk+ phenotype was transmitted to almost all progeny in the reciprocal cross. This pattern of inheritance is similar to that observed for mit DNA in crosses between the two interspecific species *C. reinhardtii* and *C. smithii* (4, 12) and the reverse of that found for markers located in the chloroplast genome (mt+ or maternal inheritance) (for a review, see reference 8).

The two mutants were named *dum-1* and *dum-2* (dark uniparental transmission by the minus parent).

**Mitotic Segregation and Reversion of the dum Mutants**

The two mutants were plated on agar medium (TAP or M) at low density to obtain individual colonies. After 8 d of incubation in the light, the plates were examined under the dissecting microscope. In addition to green colonies, minute colonies representing \(~10\)% of the total were observed.

Two subclones of the original *dum mt* mutant and the *dum mt* isolates also segregated mitotically dk- cells (\(~90\)%), and cells which formed minute colonies (\(~10\)%). It thus seems that the production of cells which form minute colonies constitutes an intrinsic property of the *dum* mutants.

The capacity for reversion of the *dum* mutants was tested by plating a total of \(14 \times 10^7\) viable cells \(10^6\) cells/plate) on TAP agar medium and incubating the plates in the dark for 15 d. No revertants were obtained in this experiment.

**Respiratory Chain Activity**

As in many plants, the respiratory chain of *Chlamydomonas* is composed of the classical cyanide-sensitive (cytochrome) pathway and a second pathway that branches from the main chain and is insensitive to cyanide but sensitive to SHAM (11, 15).

![Restriction map of the 15.8 kb linear mitochondrial DNA of *C. reinhardtii*. Restriction enzyme sites: B, Bam HI; E, Eco RI; H, Hpa I; Hi, Hind III; K, Kpn I. Genes: COI, cytochrome oxidase subunit I; CYB, apocytochrome b; 18S and 26S rRNA, small and large subunit ribosomal RNA; URF2 and URF5, unassigned open reading frames homologous to mammalian mitochondrial genes; *dum* mutants.](https://jcb.rupress.org/content/122/13/1223)
Table I. Segregation of the dk+ and dk- Phenotypes in Reciprocal Crosses between Wild-Type (dk+) and Mutant (dk-) Strains

| Crosses                      | Segregation of capacity to grow in the dark |
|------------------------------|---------------------------------------------|
| Wild-type mt+ x mutant 1 mt- | 26 dk+/157 dk-                              |
| Wild-type mt+ x mutant 2 mt- | 28 dk+/151 dk-                              |
| Mutant 1 mt- x wild-type mt+ | 239 dk+/1 dk-                               |
| Mutant 2 mt- x wild-type mt+ | 240 dk-/10 dk-                              |

Total respiratory rates, measured in the dark with the endogenous substrates, were lower in dum-1 and dum-2 than in the wild-type strain (16 and 38%, respectively; see Table II). In the wild type, the total respiration was inhibited 65% by cyanide whereas the further addition of SHAM reduced the percentage to 12%. In contrast, the respiratory rate of the two mutants was insensitive to cyanide but remained sensitive to SHAM. These results indicate that both mutants have a lower respiratory rate because cyanide-sensitive respiration is absent or greatly reduced.

This conclusion was corroborated by the data obtained when SHAM was added before KCN. In the wild type, SHAM alone did not modify the respiration, perhaps because the alternative pathway does not function when the cytochrome main pathway is not saturated, as hypothesized by Bahr and Bonner (15). In the two mutants however, the addition of SHAM strongly reduced the respiration rate. The further addition of cyanide largely affected the respiration of the wild type but not that of the mutants.

The activity of cytochrome c oxidase was measured on cell homogenates. The activity was much lower in the mutants (3.7 and 3.6 nmol cytochrome c oxidized × min⁻¹ × 10⁻⁷ cells of dum-1 and dum-2, respectively) than in the wild type (32 nmol cytochrome c oxidized × min⁻¹ × 10⁻⁷ cells). These activities were completely inhibited by 1 mM KCN. Thus, a deficiency in cytochrome c oxidase may be responsible, at least partially, for the low cyanide-sensitive respiration found in the two mutants.

Analysis of the Mitochondrial Genome

The phenotype and the pattern of inheritance of the dum mutants strongly suggest that the mutations alter the mit DNA. As AF induces large deletions in the mitochondrial genome of yeast, we searched for the presence of such deletions in the mutants.

The mitochondrial genome of C. reinhardtii is a small (15.8 kb) linear DNA molecule present in 40-50 copies per cell (3, 13, 14). Several genes and restriction sites have been identified in the genome (Fig. 3). To analyze the mit DNA of the two mutants, total DNA was digested with Bam HI + Eco RI or Bam HI + Hind III. Fig. 4 shows the fragments detected on Southern blots by the PI probe (Fig. 3) for the wild type and the dum-1 strain. The larger fragment obtained after Bam HI + Eco RI digestion was smaller in dum-1 than in wild type, indicating that a deletion of ~1.5 kb is present in the segment containing CYB, URFx, URF5, and COI genes. (In dum-1, a fragment of higher molecular weight, resulting from the partial digestion of the mit DNA, was also present.) In contrast, the Bam HI–Hind III fragments were identical in both mutant and wild-type strains; thus, the deletion is located at the left side of the Hind III site present in URFx (Fig. 3). Digestion with Kpn I and hybridization with the P2 probe confirmed that the deletion is present in the segment containing CYB and URFx (Fig. 4). Finally, the detection of the Hpa I fragments with P2 indicates that the segment covering a part of CYB, URFx, and a part of URF5 is shorter in the mutant (2.8 instead of 3.25 kb).

The same restriction patterns were found for dum-2 mt- as well as for the two mutant mt+ isolates obtained from crosses.

The conclusion is that both mutants contain a deletion of ~1.5 kb, probably terminal, including the Hpa I site located in CYB-URFx genes (del in Fig. 3).

Discussion

The two dum mutations described here exhibit a paternal (mt+)-mode of transmission, characteristic of the inheritance of the mit DNA observed in crosses between C. reinhardtii and C. smithii (4, 12). In crosses between wild-type strains and dum mutants, the uniparental paternal transmission was almost absolute when the mt+ parent was wild type, whereas when the mt- parent was mutant, ~15% meiotic products transmitted the marker from the wild mt+ parent. In this latter cross, many zygotes did not germinate, maybe as a result of their lack of cyanide-sensitive respiration. The rare zygotes possessing the wild phenotype and a high rate of germination would have a selective advantage, increasing the probability to find dk+ meiotic progeny. A low germination of dum meiotic products would still amplify the phenomenon.

The present work demonstrates that in Chlamydomonas acriflavine induces not only loss of mit DNA with subsequent lethality (9) but also nonlethal deletions in the organelle genome. The two mutants isolated here are deleted in the portion of the genome that contains the apocytochrome b (CYB) gene and possibly a short sequence of the unidentified gene URFx. The size of the deletions (~1.5 kb) are rather small in comparison to those generally observed in yeast (6). The restriction analyses suggest that the deletions are probably terminal. The capacity of both mutants to give rise by mitotic segregation to ~10% of minute colonies could result from the absence of the terminal region, making the mit DNA relatively unstable.

As a consequence of the deletion, the two mutants lack cyanide-sensitive cytochrome pathway and become obligate photautotrophs. The cytochrome c oxidase deficiency observed in the two mutants is rather puzzling since the deletions do not affect the COI gene. One can postulate that the
deletions affect a gene that encodes (1) another subunit of the cytochrome oxidase (URFx?), (2) a product controlling the transcription or the translation of cytochrome oxidase gene(s), or (3) a product necessary for the assembly and the activity of the cytochrome oxidase. Another less likely possibility is that both mutants possess a second mutation located in CO1, greatly reducing cytochrome oxidase activity.

Neither mutant uses acetate in the dark but both grow at near wild-type rate under photoautotrophic conditions. This indicates that the cytochrome pathway and subsequent oxidative phosphorylation are dispensable in the light, sufficient ATP being provided by photosynthetic activities. Like the dk- nuclear mutants (19), the dum mutants however possess a normal alternative SHAM-sensitive pathway. As discussed by Wiseman et al. (19), in the absence of cytochrome pathway, the alternative pathway might be essential to shuttle electrons from the Krebs cycle and regenerate NAD+ from NADH. The minute mutants may die because they have lost the ability to carry out both cyanide-sensitive and SHAM-sensitive pathways (9). Deletions in URF2 and URF5 (two open reading frames homologous to mammalian mitochondrial genes whose products were recently found to be components of the NADH:ubiquinone reductase [5]) might be lethal for the cell since the ubiquinone pool is common to both pathways. Similarly, mutations localized in ribosomal RNA genes will prevent protein synthesis in the organelle and induce lethality. In contrast, deletions inside the gene coding for subunit I of cytochrome oxidase (COI) are expected to lead to obligate photoautotrophy since the dk- nuclear mutants isolated by Wiseman et al. (19) lack that enzyme. This brings the question why the two mutants apparently possess the same deletion. As both were isolated in the same experiment (AF 8 μg/ml, 3 d of treatment), they might result from the same mutational event. However, two additional mutants recently isolated in independent experiments were shown to possess a similar deletion in the same region of the genome (S. Remy and R. Matagne, unpublished observations). The isolation and analysis of new mutations leading to obligate photoautotrophy will tell us more about the possible location of deletions in the mit DNA.

We wish to thank Mr. Jean Monfort and Mrs. Corinne Boule for technical assistance; Mrs. Viviane Gielen and Mr. Yves Lemoine for manuscript preparation; and Mr. Pierre Parkinson for photography. Drs. E. Pratje and G. Michaelis, who provided the pCrm CEI plasmid, have also to be acknowledged.

Received for publication 31 August 1988 and in revised form 31 October 1988.

References
1. Alexander, N. J., N. W. Gillham, and J. E. Boynton. 1974. The mitochondrial genome of Chlamydomonas. Induction of minute colony mutations by acriflavine and their inheritance. Mol. Gen. Genet. 130:275–290.
2. Boer, P. H., and M. W. Gray. 1986. The URF5 gene of Chlamydomonas reinhardtii mitochondria: DNA sequence and mode of transcription.
EMBO (Eur. Mol. Biol. Organ.) J. 5:21-28.
3. Boer, P. H., L. Bonen, R. W. Lee, and M. W. Gray. 1985. Genes for respiratory chain proteins and ribosomal RNAs are present on a 16-kilobase-pair DNA species from Chlamydomonas reinhardtii mitochondria. Proc. Natl. Acad. Sci. USA. 82:3340-3344.
4. Boynton, J. E., E. H. Harris, B. D. Burkhart, P. M. Lamerson, and N. W. Gillham. 1987. Transmission of mitochondrial and chloroplast genomes in crosses of Chlamydomonas. Proc. Natl. Acad. Sci. USA. 84:2391-2395.
5. Chomyn, A., P. Mariottini, M. W. T. Cleeter, C. I. Ragan, A. Matsuno-Yagi, Y. Hatefi, R. F. Doolittle, and F. Attardi. 1985. Six unidentified reading frames of human mitochondrial DNA encode components of the respiratory chain NADH dehydrogenase. Nature (Lond.). 314:592-597.
6. Dujon, B. 1981. Mitochondrial genetics and functions. In Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance. J. N. Strathern, E. W. Jones, and J. R. Broach, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 505-635.
7. Ephrussi, B., H. Hottinguer, and Y. Chimenes. 1949. Action de l'acriflavine sur les levures. I. La mutation "petite colonie." Ann. Inst. Pasteur (Paris). 76:351-364.
8. Gillham, N. W. 1978. Organelle Heredity. Raven Press, New York. 602 pp.
9. Gillham, N. W., J. E. Boynton, and E. H. Harris. 1987. Specific elimination of mitochondrial DNA from Chlamydomonas by intercalating dyes. Curr. Genet. 12:41-47.
10. Gorman, D. S., and R. P. Levine. 1965. Cytochrome F and plastocyanin: their sequence in the photosynthetic electron transport chain of Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA. 54:1665-1669.
11. Henry, M.-F., and E.-L Nyns. 1975. Cyanide-insensitive respiration and alternative pathway. Subcell. Biochem. 4:1-65.
12. Matagne, R. F., D. Rongvaux, and R. Loppes. 1988. Transmission of mitochondrial DNA in crosses involving diploid gametes homozygous or heterozygous for the mating-type locus in Chlamydomonas. Mol. & Gen. Genet. 214:257-262.
13. Pratje, E., S. Schrierrer, and B. Dujon. 1984. Mitochondrial DNA of Chlamydomonas reinhardtii: the DNA sequence of a region showing homology with mammalian URF 2. Curr. Genet. 9:75-82.
14. Ryan, R., D. Grant, K.-S. Chiang, and H. Swift. 1978. Isolation and characterization of mitochondrial DNA from Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA. 75:3268-3272.
15. Siedow, J. N., and D. A. Berthold. 1986. The alternative oxidase: a cyanide-resistant respiratory pathway in higher plants. Physiol. Plant. 66:569-573.
16. Vahrenholz, C., E. Pratje, G. Michaelis, and B. Dujon. 1985. Mitochondrial DNA of Chlamydomonas reinhardtii: sequence and arrangement of URF5 and the gene for cytochrome oxidase subunit I. Mol. & Gen. Genet. 201:213-224.
17. Varwinkle-Swift, K. P. 1977. Maturation of algal zygotes: alternative experimental approaches for Chlamydomonas reinhardtii (Chlorophyceae). J. Phycol. 13:225-231.
18. Surzycki, S. 1971. Synchronously grown cultures of Chlamydomonas reinhardtii. Methods Enzymol. 23A:87-73.
19. Wiseman, A., N. W. Gillham, and J. E. Boynton. 1977. Nuclear mutations affecting mitochondrial structure and function in Chlamydomonas. J. Cell. Biol. 73:56-77.
20. Wiseman, A., N. W. Gillham, and J. E. Boynton. 1977. The mitochondrial genome of Chlamydomonas. II. Genetic analysis of non-Mendelian obligate photoautotrophic mutants. Mol. & Gen. Genet. 150:109-118.