Induction and Characterization of Mitochondrial DNA Mutants in *Chlamydomonas reinhardtii*

R. F. Matagne,* M.-R. Michel-Wolwertz,* C. Munaut,* C. Duyckaerts,‡ and F. Sluse‡

*Laboratoire de Génétique des microorganismes, Département de Botanique and ‡Laboratoire de Bioénergétique, Service de Biochimie et Physiologie Générales, Université de Liège, Sart Tilman, B-4000 Liège, Belgium

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)[1] 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 which before dying are able to undergo 8–9 mitotic divisions in the light and form very small colonies, called minutes. As these mutants do not die immediately, they have been used in crosses with wild-type cells to analyze the inheritance of the minute phenotype. A complex non-Mendelian transmission was observed which led Alexander et al. (1) to conclude that the mutations might arise from alterations of mit DNA. This was recently confirmed by Gillham et al. (9) who showed that the induction of minute mutations is accompanied by specific loss of mit DNA. In addition, the non-Mendelian inheritance of the minute phenotype could be explained in terms of transmission of mit DNA by the mating type 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

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1. *Abbreviations used in this paper*: AF, acriflavin; KCN, cyanide; M, minimal medium; mit DNA, mitochondrial DNA; SHAM, salicylhydroxamic acid; TAP, Tris-acetate phosphate medium.

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 $\times$ 10^{-6} einsteins $\times$ m^{-2} $\times$ 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 $\times$ 10^5 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 $\times$ 10^2, 2 $\times$ 10^3, and 2 $\times$ 10^4 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 (10^7 cells/ml). Total oxygen consumption was recorded during 10-15 min while the rate remains constant. Cyanide-(KCN) sensitive and salicylhydroxamic acid- (SHAM) sensitive respirations 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 O_2 $\times$ min^{-1} $\times$ 10^{-7} cells taking into account that 1 ml TAP medium contains 210 nmol O_2.

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 $\times$ 30 s of cells suspended in 0.03 M phosphate buffer, pH 7.4, 0.1% BSA) was assayed in 0.1 M Tris-HCl 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 mit 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 mit DNA (12; *P1* in Fig. 3) and (b) the pCrm CE1 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 mit 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
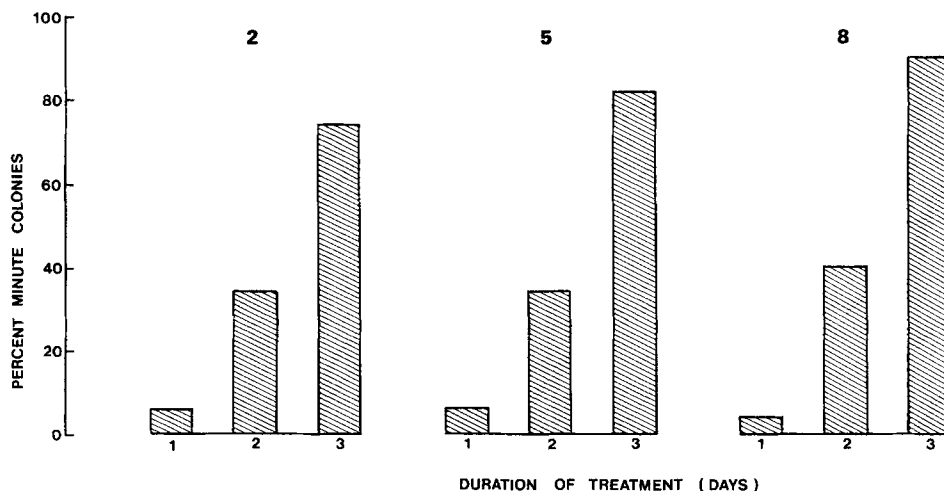

Figure 1. Percentages of minute colonies (minute colonies/min + viable colonies) induced by AF (2, 5, or 8 μg/ml) during 1-3 d. The percentages were determined after growth for 7 d in mixotrophic conditions. No minute colony was observed in control.
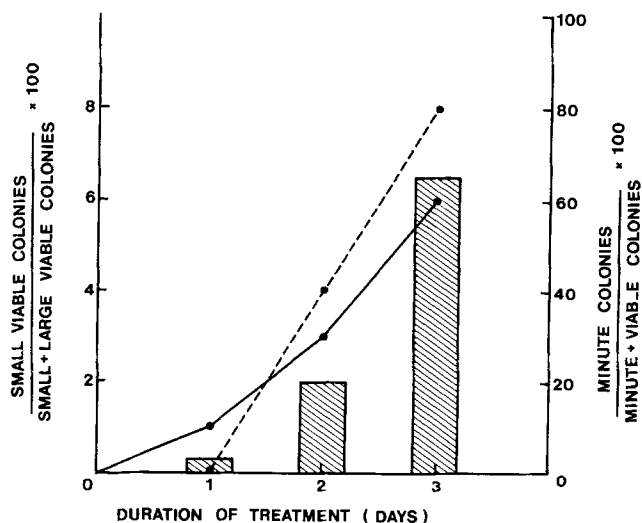
Figure 2. Percentages of small size viable colonies induced by AF (5 μg/ml, 1-3 d). After treatment, known numbers of cells were plated on TAP agar medium. Plates were incubated under continuous light for 7 d (————) or in the dark (7 d) then transferred to the light (7 d) (- - - -). Histogram gives the percentages of minute colonies neoformed after transfer from darkness to light.

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 photoautotrophic 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 μg/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 interfertile 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 × 10⁷ viable cells (10⁷ 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).
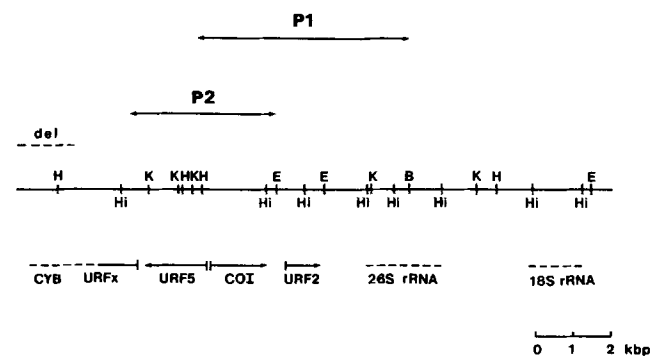

Figure 3. Restriction map of the 15.8 kb linear mitochondrial DNA of *C. reinhardtii*. Restriction enzyme sites: *B*, Bam HI; *E*, Eco RI; *H*, Hpa I; *Hi*, Hind III; *K*, Kpn I. Genes: *COI*, cytochrome oxidase subunit I; *CYB*, apocytochrome b; *18S* and *26S RNA*, small and large subunit ribosomal RNA; *URF2* and *URF5*, unassigned open reading frames homologous to mammalian mitochondrial genes; *URFx*, unidentified open reading frame (from references 2, 3, 13, 16). *P1*, Bam HI-Sal I fragment present in probe 1; *P2*, Eco RI-Cla I fragment present in probe 2 (Sal I and Cla I sites are not indicated in the figure). *del*, probable location of the deletion (see text).

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^+ × mutant 1 mt^-	26 dk⁺/157 dk⁻
Wild-type mt^+ × mutant 2 mt^-	28 dk⁺/151 dk⁻
Mutant 1 mt^+ × wild-type mt^-	239 dk⁺/1 dk⁻
Mutant 2 mt^+ × wild-type mt^-	240 dk⁺/0 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 $\times$ min⁻¹ $\times$ 10⁻⁷ cells of *dum-1* and *dum-2*, respectively) than in the wild type (32 nmol cytochrome c oxidized $\times$ min⁻¹ $\times$ 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 P1 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 dele-

tion 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* acriflavin 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 photoautotrophs. 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

Table II. Respiratory Rate (nmol O₂ $\times$ min⁻¹ $\times$ 10⁻⁷ cells) of Wild Type and Mutants Measured at 30°C in the Dark in 2 ml TAP Medium Containing 10⁷ Cells/ml. (At 5-Min Intervals, the Inhibitors Were Added.)

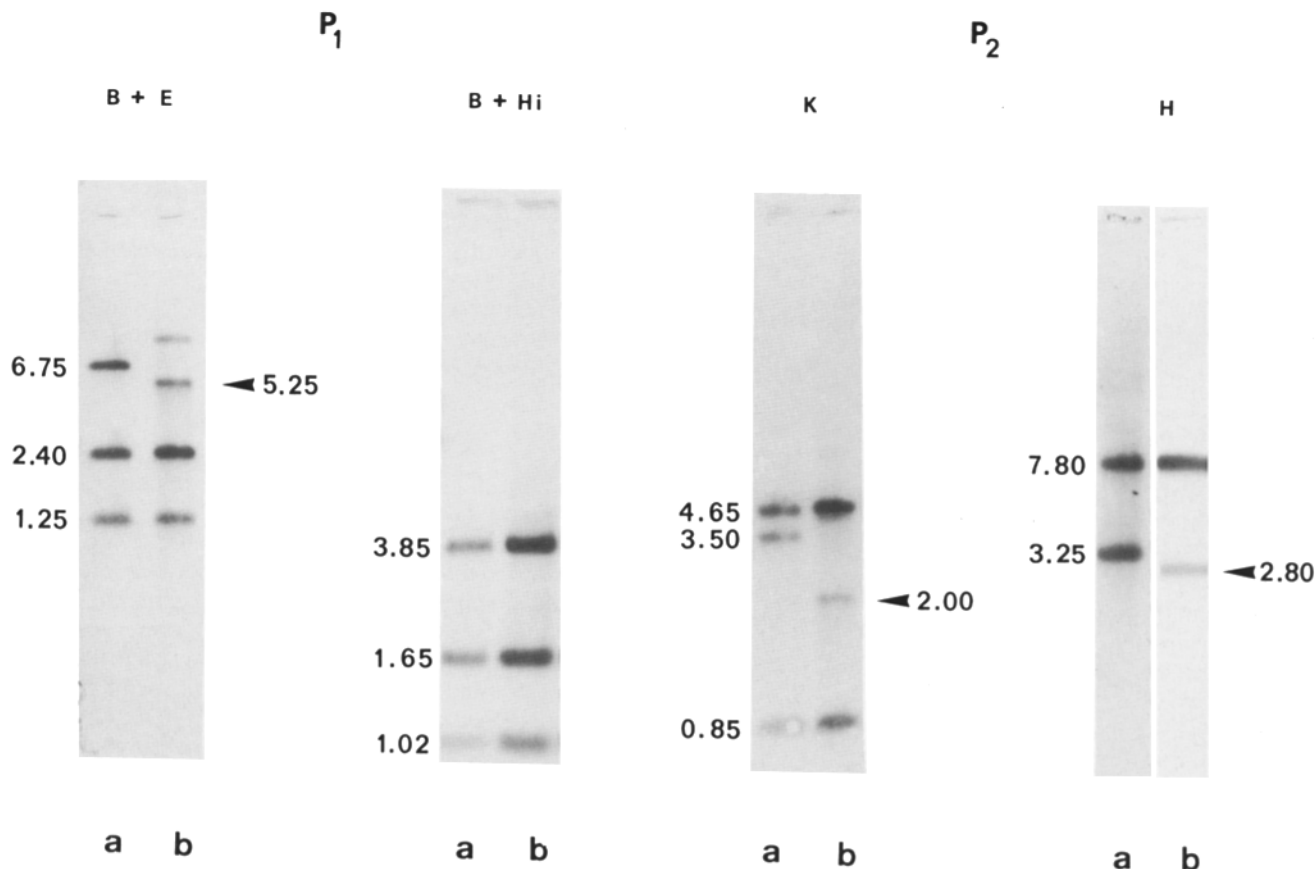|	Total respiratory rate	Respiratory rate after addition of			
		KCN	KCN followed by SHAM	SHAM	SHAM followed by KCN
Wild type mt^-	23.2	8.1	2.9	23.4	1.4
Dum-1 mt^-	3.7	3.7	0.9	0.4	0.3
Dum-2 mt^-	8.8	8.8	1.3	1.3	1.3

Figure 4. Hybridization patterns obtained with *P1* and *P2* probes after electrophoresis followed by Southern blotting of wild type (lanes *a*) and *dum-1* (lanes *b*) total DNA digested by the indicated restriction enzymes. Only the fragments equal or larger than 0.85 kb are visible in the figure. Arrows indicate the size of the new fragments present in the mutant. Abbreviations like in Fig. 3.

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 COI, 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.

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