Biochemical, genetic and molecular characterization of new respiratory-deficient mutants in *Chlamydomonas reinhardtii*

Marie-Pierre Dorthu[1], Suzanne Remy[1], Marie-Rose Michel-Wolwertz[1], Laurence Colleaux[3], Didier Breyer[1], Marie-Claire Beckers[1], Serge Englebert[1], Claire Duyckaerts[2], Francis E. Sluse[2] and René F. Matagne[1]*

[1] *Laboratoire de Génétique des microorganismes, Département de Botanique, B22 (* author for correspondence) and* [2] *Laboratoire de Bioénergétique, Centre Interdisciplinaire de Biochimie normale et pathologique de l'Oxygène, B6, Université de Liège, Sart-Tilman, B-4000 Liège, Belgium;* [3] *Unité de Génétique moléculaire des Levures, Département de Biologie Moléculaire, Institut Pasteur, 25 rue du Docteur Roux, F-75724 Paris Cedex 15, France*

Abstract

Eight respiratory-deficient mutants of *Chlamydomonas reinhardtii* have been isolated after mutageni treatment with acriflavine or ethidium bromide. They are characterized by their inability to grow or the very reduced growth under heterotrophic conditions. One mutation (Class III) is of nuclear origi whereas the seven remaining mutants (Classes I and II) display a predominantly paternal mt^- inheri ance, typical of mutations residing in the mitochondrial DNA. Biochemical analysis has shown that a mutants are deficient in the cyanide-sensitive cytochrome pathway of the respiration whereas the alter native pathway is still functional. Measurements of complexes II + III (antimycin-sensitive succinate cytochrome *c* oxido-reductase) and complex IV (cytochrome *c* oxidase) activities allowed to conclud that six mutations have to be localized in the mitochondrial apocytochrome *b* (COB) gene, one in th mitochondrial cytochrome oxidase subunit I (COI) gene and one in a nuclear gene encoding a compc nent of the cytochrome oxidase complex. By using specific probes, we have moreover demonstrated th; five mutants (Class II mutants) contain mitochondrial DNA molecules deleted in the terminal en containing the COB gene and the telomeric region; they also possess dimeric molecules resulting fror end-to-end junctions of deleted monomers. The two other mitochondrial mutants (Class I) have n detectable gross alteration. Class I and Class II mutants can also be distinguished by the pattern (transmission of the mutation in crosses.

An *in vivo* staining test has been developed to identify rapidly the mutants impaired in cyanide-sensitiv respiration.

The functional interactions between chloroplasts and mitochondria are still poorly understood despite the central role played by photosynthesis and respiration in plant cell metabolism. Mutants impaired in these major functions constitute useful tools for studying the interactions between the two organelles. The unicellular alga *Chlamydomonas reinhardtii* is the only green organism in which mutations affecting photosynthesis and respiration have been isolated. These mutations are located in the nucleus, in the chloroplast genome or in the mitochondrial genome. Each type of mutation can easily be distinguished by its pattern of transmission in crosses: whereas the mutations located in the nuclear genome are inherited following a Mendelian mode, those residing in the chloroplast DNA or in the mitochondrial DNA are almost exclusively inherited from the mt^+ parent (uniparental maternal or UP$^+$ inheritance) or from the mt^- parent (uniparental paternal or UP$^-$ inheritance) respectively (15).

Many nuclear and chloroplast mutations determining an alteration of the photosynthetic function and making the alga dependent on an exogenous carbon source (acetate) for growth, have been characterized (for a review, see [8]). On the other hand, a few nuclear mutations determining the loss of some respiration functions have been identified [23]. These mutants are unable to grow heterotrophically (darkness + acetate) and survive only under phototrophic conditions. After acriflavine mutagenic treatment, we recently isolated two mutants unable to grow in the dark (dk$^-$) because of the presence of a ca. 1.5 kb deletion in the mitochondrial DNA [15]. The deletion is localized in the terminal part of the genome containing the apocytochrome b (COB) gene. In crosses, the two mutations were inherited from the mt^- parent.

We here describe the isolation and characterization of eight new dk$^-$ mutants in *Chlamydomonas*. Seven of them possess a mutation which is uniparentally mt^- inherited and is thus located in the mitochondrial genome. The mutants are defective in the cyanide-sensitive respiration and

cinate-cytochrome c oxidoreductase or in tochrome oxidase. Five mutants possess tered mitochondrial genome and contain di types of DNA molecules which are deleted region containing the COB gene. An *in vi* orimetric test allowing to detect rapidly r tory mutants and to analyze the segregation dk$^-$ phenotype is also describe

Material and methods

Strain and culture conditions

The wild-type strains mating type plus (*mt* minus (*mt$^-$*) are derived from strain 137 cells were grown in liquid medium or o plates (15 g/l Gibco agar) under cool whi rescent light (0.94 mE m^{-2}s^{-1}) or in the 25 °C. Two culture media were used: n (M) medium [21] or Tris-acetate pho (TAP) medium [7].

Mutagenesis

The cells from synchronized liquid culture *mt$^-$* wild-type strain were grown for 1–3 light or darkness in TAP liquid medium c ing acriflavine (AF, 6–8 μg/ml) or ethidiu mide (EB, 3–6 μg/ml). After washing, 2 × 2×10^4 cells were plated on TAP agar n and incubated under mixotrophic (light tate) or heterotrophic (darkness + acetat ditions. Obligate phototrophic mutants (phenotype, unable to grow in the dark) w tected as earlier described [15].

Genetic analysis

Except stated otherwise, the zygotes were rated for 1 day in the light followed by 5 the dark on M agar plates. After matu blocks of agar carrying 50–100 zygote transferred to fresh M agar plates and trea

induced by exposure to light for 16–24 h and haploid spores were plated at random to yield isolated clones.

In one experiment (described in Table 2), the period of maturation in the dark varied from 3 to 10 days; the zygotes were transferred to fresh plates and incubated under light to germinate *in situ.*

Whole-cell respiration

Samples containing 2×10^7 cells were resuspended in 2 ml M medium (for cultures grown phototrophically) or in 2 ml M medium containing 2 mg/ml sodium acetate (for cultures grown mixotrophically). Respiration was measured at 30 °C in the dark with a Clark electrode (Gilson oxygraph). 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/min per 10^7 cells taking into account that 1 ml 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.

Enzyme assays

Mitochondrial enzyme activities were measured spectrophotometrically at 30 °C in whole cell homogenates, using an Aminco DW-2 spectrophotometer. The homogenates were prepared by sonication (3 × 30 s) of cells suspended at a cell density of 1.5×10^8 cells/ml in 0.03 M phosphate buffer pH 7.4, plus 0.1% bovine serum albumin [23].

The complex IV or cytochrome c oxidase activity was assayed in 0.1 M Tris-HCl buffer pH 7.4 containing 50 μM reduced cytochrome c

pared according to Moller and Palmer [17]. In order to make the inner mitochondrial membran accessible to cytochrome c, 5 μl aliquots o 250 mM deoxycholate were added until a maxi mal cytochrome c oxidase activity was obtained The final concentration of deoxycholate wa about 2 mM. The oxidation of cytochrome c wa followed at 550–540 nm. The change of absor bance was linear over a period of 5 min. The re action was totally inhibited by 2 mM potassiur cyanide.

Succinate-cytochrome c oxidoreductase wa assayed in 0.1 M Tris-HCl buffer pH 7.4 contair ing 2.1 μM rotenone and 2 mM potassium cya nide to block complex I and complex IV respec tively, and 20 mM sodium succinate as substrate. Reaction was initiated by addition oxidized cytochrome c (50 μM final) and its re duction was measured at 550–540 nm. The ac tivity inhibited by addition of malonate (20 mI final) was considered as total succinate-cy tochrome c oxidoreductase activity. The antim cin-sensitive succinate-cytochrome c oxidoredu tase or complex II + III activity was measure from the decrease of activity observed after th addition of antimycin A (6 μM final) to the rea tion mixture.

In order to reduce the experimental variabili due to variations in cell disruption by ultrasound the enzyme activities were expressed relatively the lactate dehydrogenase activity which was a sayed in phosphate buffer pH 7.0 containii 0.24 mM NADH and 10 mM sodium pyruvate a substrate. The mean specific lactate dehydr genase activity, measured at 350–375 nm, w 20 nmol NADH oxidized/min per 10^7 cells.

Tetrazolium overlay technique for detecting respii tion deficiency

The test procedure described for yeast [18] h been adapted to *Chlamydomonas* as follows. Alg colonies are transferred with tooth-picks or fresh TAP agar plates (9 cm in diameter) a grown under light for 2–3 days. The test medii

phate buffer pH 7.0, containing 0.5 mg/ml 2,3,5-triphenyltetrazolium chloride (TTC). As TTC is reduced chemically by heating, it is added to the agar medium after cooling at 55 °C. The test is performed by pouring 15 ml of TTC agar at 50 °C over plates bearing the colonies. The plates are incubated at 30 °C in the dark; after 3–5 h, the wild-type colonies have become purple whereas the respiratory mutants remain green.

Molecular analysis of mitochondrial DNA

The mitochondrial genome of *C. reinhardtii* is made of 15.8 kb linear DNA molecules. The nucleotide sequences encoding apocytochrome *b* (COB), subunit 1 of cytochrome *c* oxidase (COI), subunits 1, 2, 4, 5 and 6 of NADH dehydrogenase (NAD1, NAD2, NAD4, NAD5 and NAD6) and RTL protein, the small and large rRNAs and three tRNAs (Trp, Met and Glu) have been determined (for most recent data, see [13, 16]).

To characterize physically the mitochondrial DNA of the mutants, four molecular probes were used (Fig. 1): (a) P$_1$ or pULG-R1 plasmid containing the 5.5 kb *Bam* HI-*Sal* I fragment of the mitochondrial DNA [14]; (b) P$_2$ or pCrm CE1 plasmid containing the 3.87 kb *Cla* I-*Eco* RI fragment [22]; (c) P$_3$ or pUC12 plasmid including a 1.65 kb fragment which contains the 1171 last nucleotides of NAD4 and the 450 first nucleotides of COB (LC8/LC9 in Colleaux *et al.* [4]); (d) P$_4$, a pUC13 plasmid containing the 1146 nucleotides of COB (this laboratory); (e) P$_5$, a fragment am-

cleotides of COB and the ca. 500 bp foll sequence (LC11/LC12 in Colleaux *et al.* [4]

Total DNA was digested with appropri striction enzymes and the fragments separa electrophoresis on agarose gels [14]. After S ern blotting, the mitochondrial DNA frag were detected by using probes labelled digoxygenin (Boehringer).

Results

Phenotypical and genetic analysis

Eight dk$^-$ mutants were isolated after trea of wild-type *mt*$^-$ cells with AF (mutants 16 173) or EB (mutants 194, 196, 200, 202, They were characterized by their inability t under heterotrophic conditions (or re growth in case of strains 173 and 196) an slower growth under mixotrophic cond Under photoautotrophic conditions, the r colonies were also slightly smaller than th type.

Each mutant was crossed to the wild-typ strain and the individual meiotic progeny analyzed for their capacity to grow in the d each cross, one dk$^-$ *mt*$^+$ mutant clone w lected and crossed to wild-type *mt*$^-$ cells.

Three classes of mutants were identified basis of the pattern of transmission of th character in reciprocal crosses (Table 1).

– Class I mutants: for these mutants, the mission was almost exclusively unipa

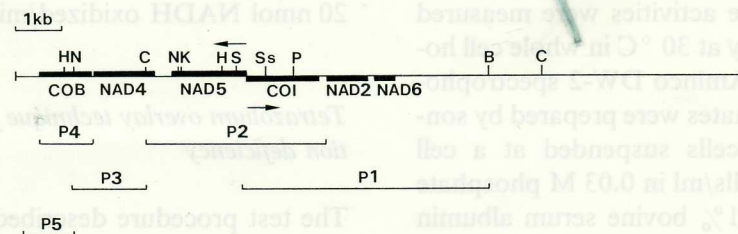*Fig. 1.* Partial restriction map of the 15.8 kb linear mitochondrial DNA of *C. reinhardtii*. Restriction enzyme sites: B, *Bar Cla* I; H, *Hpa* I; K, *Kpn* I; N, *Nco* I; P, *Pvu* II; S, *Sal* I; Ss, *Sst* I. In case of C, H, K, N and P, only the sites important study are indicated. Genes: COB, apocytochrome *b*; COI, cytochrome *c* oxidase subunit I; NAD 2,4,5 and 6, subunits of cytochrome *c* oxidoreductase. P1–5: segments present in the probes (see text). For most recent data, see [~~12, 15~~]. [13, 16].

Class	Reference number of the mutant mt^- isolate (and mutagen used)	Phenotype of the mt^- parent in crosses	Segregation (%)	Name of the mutant
I	173 (AF)	mutant	97 dk$^-$: 3 dk$^+$	dum-6
		wild	100 dk$^+$: 0 dk$^-$	
	202 (EB)	mutant	100 dk$^-$: 0 dk$^+$	dum-15
		wild	99 dk$^+$: 1 dk$^-$	
II	168 (AF)	mutant	77 dk$^-$: 23 dk$^\pm$	dum-3
		wild	100 dk$^+$: 0 dk$^-$	
	170 (AF)	mutant	81 dk$^-$: 19 dk$^+$	dum-4
		wild	100 dk$^+$: 0 dk$^-$	
	194 (EB)	mutant	82 dk$^-$: 18 dk$^+$	dum-11
		wild	100 dk$^+$: 0 dk$^-$	
	200 (EB)	mutant	72 dk$^-$: 28 dk$^+$	dum-14
		wild	100 dk$^+$: 0 dk$^-$	
	204 (EB)	mutant	82 dk$^-$: 18 dk$^+$	dum-16
		wild	99 dk$^+$: 1 dk$^-$	
III	196 (EB)	mutant	46 dk$^-$: 54 dk$^+$	dn-12
		wild	52 dk$^+$: 48 dk$^-$	

paternal (mt^-), and thus typical of mitochondrial mutations. Following the nomenclature used earlier [15], the mutants were named dum-6 and dum-15 (dark uniparental transmission by the minus parent).

– Class II mutants (dum-3, dum-4, dum-11, dum-14 and dum-16): in this case, the transmission was more frequently paternal than maternal in the crosses dk$^-$ mt^- × dk$^+$ mt^+ and almost exclusively paternal in the reciprocal crosses dk$^+$ mt^- × dk$^-$ mt^+. Moreover, the percentage of zygote germination after 1 day of exposure to light was generally lower in the former crosses (40–80%) than in the latter ones (>90%). This result is similar to that obtained with the dum-1 and dum-2 mutants previously isolated [15].

– Class III was represented by one mutant which exhibited a typical Mendelian segregation and was named dn-12 (dark nuclear).

Another trait, common exclusively to dum-1, dum-2 and Class II mutants (and their subclones) was the capacity to segregate mitotically viable cells (90–98%) and cells (2–10%) which divide

8–9 times under light to produce lethal minut white colonies.

To understand more about the transmissio patterns observed in reciprocal crosses involvin the Class II mutants, additional crosses betwee dum-4 and wild-type strain were performed. I this experiment, the zygotes were maturated i the dark for periods ranging from 3 to 10 day then transferred to fresh medium to germinat in situ (see Material and methods).

In the cross dum-4 mt^- × wild-type mt^+, th total percentage of germinating zygotes wa around 70–80%. However, the proportion of zy gotes having germinated 24 h after transfer to ligh decreased with increasing the period of matura tion in the dark (Table 2). After 10 days of ma uration, only 14% of zygotes germinated earl (24 h) whereas the remaining zygotes produce spores only after 2–3 days of exposure to ligh The rare 10-day-old zygotes germinating ear gave rise to dk$^+$ meiotic spores, which indicate that all of them transmitted the marker of mate nal (mt^+) origin. On the contrary, the zygote whose germination was delayed (86%), displaye

zygotes germinating after 1 day of exposure to light (early germination), depending on the duration of zygote maturation in the dark. For early (1 day) and late (2–3 days) germinating zygotes, the percentages of zygotes giving rise to dk$^+$ cells are also given.

Duration of zygote maturation (days)	% of zygotes germinating early	% of zygotes giving dk$^+$ progeny	
		early-germinating zygotes	late-germinating zygotes
3	100	6	–
6	70	20	2
10	14	100	0

a typical paternal transmission. When the zygote maturation was limited to 3 days, all zygotes germinated early and most often transmitted the character of the paternal (mt^-) parent. The situation was intermediate for zygotes maturated for 6 days in the dark.

In the wild-type mt^- × dum-4 mt^+ reciprocal cross, the percentage of zygote germination was high (90–99%) in all cases and occurred within 24 h of exposure to light. Moreover, independently of the duration of maturation period, the transmission was exclusively paternal (100 dk$^+$: 0 dk$^-$).

It thus appears that in the cross between dum-4 mt^- and wild-type mt^+, the duration of the period of zygote maturation considerably influences the mode of transmission of the dk$^-$ character: a clear-cut paternal inheritance is observed only when the zygotes are maturated for a short period of time (3 days) in the dark.

Respiratory chain activity

As many higher plants, *C. reinhardtii* possesses two respiratory chains: the classical cytochrome cyanide-sensitive pathway and an alternative pathway that branches from the main chain at the level of ubiquinone. This alternative pathway is insensitive to KCN but sensitive to SHAM [15, 23].

When the wild-type cells were grown under photoautotrophic conditions (minimal medium +

tween 4.9 and 6.9 nmol O$_2$/min per 10^7 ce sults from 4 experiments). The uptake of was not significantly modified in the differe tant strains (data not shown).

After growth under mixotrophic con (TAP + light), the total respiratory rate i type was about 23 nmol O$_2$/min per 10^7 cel ble 3). The addition of SHAM did not mo respiration rate, which indicates that all th tron flow can transit through the cytoc pathway. In contrast, the addition of KCN reduced the respiration to 41%. The addi SHAM after cyanide (or the reverse) stror duced but did not totally abolish the oxyge sumption.

In mutants (Table 3), the total respiratic generally lower than in the wild type, witl ever some variability which might reflect logical differences between the different c [23]. As opposed to the wild type, the n (except *dum*-6 and *dn*-12) had a respiratic sensitive to SHAM and poorly sensitive to This indicates that the reduced respiratic observed in these mutants is due to the alt of the cyanide-sensitive pathway where SHAM-sensitive pathway is still function

In *dn*-12 mutant, the situation was inte ate: the respiration was more sensitive to $ but less sensitive to KCN than in wild-t *dum*-6, the respiration was insensitive to $ but moderately sensitive to cyanide.

Antimycin-sensitive succinate-cytochrome c reductase and cytochrome c oxidase activiti

In homogenates of wild-type cells, the act the succinate-cytochrome c oxidoreducta 14 times smaller than the activity of dehydrogenase taken as a reference (T About eighty percent of this activity was s to antimycin A, an inhibitor which bloc respiratory chain by interacting with tochrome b [19]. This antimycin-sensitive nate-cytochrome c oxidoreductase activity sponds to the activity of complexes

tions. Respiration was measured before and after the addition of the inhibitors (SHAM and KCN). For total respiratory rate the limit values obtained from 2–4 experiments are given in parenthesis.

Mutant class	Strain	Total respiratory rate	% respiratory rate			
			+ SHAM	+ SHAM + KCN	+ KCN	+ KCN + SHAM
–	wild type	22.8 (18.0–25.5)	100	13	41	15
I	dum-6	19.2 (18.1–21.1)	96	18	77	17
	dum-15	8.5 (7.5–10.2)	21	19	87	19
II	dum-3	11.8 (6.6–16.0)	21	18	90	21
	dum-4	13.1 (12.9–13.2)	21	18	84	19
	dum-11	10.0 (6.6–13.0)	30	21	82	22
	dum-14	10.2 (6.0–14.1)	15	13	90	15
	dum-16	5.5 (5.2–5.8)	24	18	100	24
III	dn-12	9.1 (8.9–9.4)	52	13	67	14

Table 4. Succinate-cytochrome *c* oxidoreductase and cytochrome *c* oxidase activities in wild-type and mutant strains. The data expressed relatively to lactate dehydrogenase activity, represent the mean of 2–3 experiments for mutants and 7 experiments for the wild type (S.D. in parenthesis).

Mutant class	Strain	Relative activity ($\times 10^{-3}$) of		cytochrome *c* oxidase
		succinate-cytochrome *c* reductase ($\times 10^{-3}$)		(complex IV)
		total	antimycin-sensitive (complexes II + III)	
–	wild type	70 (± 20)	54 (± 20)	3.4 (± 1.4)
I	dum-6	67	54	0.8
	dum-15	26	12	2.4
II	dum-3	23	9	2.1
	dum-4	21	0	3.4
	dum-11	26	4	3.4
	dum-14	28	5	3.1
	dum-16	36	9	3.2
III	dn-12	65	55	0.4

of the respiratory chain. Expressed relatively to the number of cells, the activity of complexes II + III was about 1 nmol oxidized cytochrome *c* per minute per 10^7 cells (see Material and methods).

The total and antimycin-sensitive activities in *dum-6* and *dn-12* were similar to the wild type (Table 4). In the other mutants, the total activity was lower and almost insensitive to antimycin A which indicates that the activity of complexes II + III was very low or null (Table 4).

The activity of cytochrome *c* oxidase (com

12 and *dum-6*, the enzyme activity was reduced (Table 4).

As indicated by their transmission pattern in crosses, the *dum* mutations must be localized in the mitochrondrial genome. Since the mitochondrial DNA of *Chlamydomonas* encodes one component of complex III (apocytochrome *b*), one component of complex IV (subunit I of cytochrome oxidase) and no component of complex II (succinate-ubiquinone oxidoreductase), it can be assumed that the *dum* mutations are located in the COB (mutants *dum-3, 4, 11, 14, 15* and *16*) or in the COI gene (mutant *dum-6*). As the *dn-12* strain has a reduced cytochrome oxidase activity, the mutation could affect a nuclear gene coding for a subunit (different from subunit I) of the cytochrome *c* oxidase complex.

An in vivo *staining test for detecting respiratory mutants*

The biochemical analysis of the dk⁻ mutants indicate that they are defective in some enzyme activity involved in the cyanide-sensitive respiration pathway.

In anaerobic conditions, the electrons of the respiratory chain can be transferred by cytochrome oxidase to 2,3,5-triphenyltetrazolium chloride (TTC) which is reduced to red formazan [20]. In yeast, a TTC overlay technique has been developed to distinguish respiration deficient mutants from wild-type colonies [18]. As our mutants are probably blocked at the level of complex III or complex IV, we assumed that the TTC diagnosis test could be applied to distinguish between mutant and wild-type strains. The overlay technique described in Material and methods was found to be useful for that purpose. After incubation for 3–5 h in the presence of TTC, the wild-type colonies became red-purple whereas the mutant colonies remained green. An intermediate coloration was obtained with the *dum-6* and *dn-12* mutant. The test was also successfully applied to the analysis of the meiotic progeny obtained from crosses between wild-type and mutant strains (data not shown).

because under light, both mutant and wild nies rapidly develop a purple coloration, pr because of the reduction of TTC by the synthetic electron transport system.

The TTC overlay technique thus consti rapid method to isolate mutants impaired cytochrome respiration pathway and may in photosynthetic activities.

Molecular analysis of the dum *mutants*

The phenotype and the pattern of inherit the *dum* mutants indicate that the mutatic localized in the mitochondrial genome. A flavine and ethidium bromide induce de and alterations in the mitochondrial DNA and *Chlamydomonas*, we performed molecu bridization analysis of the *dum* mutants. different probes containing mitochondrial fragments (see Material and methods).

No alteration was found in the two mutants, *dum-6* and *dum-15* (data not shov contrast, all Class II mutants contained tw of mitochondrial DNA molecules: deleted cules whose molecular weight was betw and 15 kb and molecules whose size was than in wild-type cells (more than 25 kb, of 15.8 kb) (Fig. 2). In order to determine v the two types of molecules can segregate cally to give rise to clones homoplasmic of the other types of mitochondrial DN *dum-4* strain was subcloned several times subclones, the two types of molecules per

The total DNA of Class II mutants v gested with several restriction enzymes a resulting fragments were analyzed by hyb tion after electrophoresis and Southern b

All Class II mutants except *dum-11* will considered. After digestion with *Sst* I and ization with P₂, the large 10.4 kb fragmei cal of wild-type and two new fragments (t *dum-3*) were detected in the mutants (Tabl Fig. 1). Two or three fragments of abnorn were also detected with P₃ after digest *Pvu* II (Table 5). Both with *Sst* I and *Pvu*

15.8 kb →

10.4 kb →

5.4 kb →

Fig. 2. Hybridization patterns obtained with P1 probe after DNA electrophoresis on 0.3% agarose gel followed by Southern blotting of Class II mutants. Lanes 1, 3–7: undigested DNA of wild-type, *dum-3, 4, 11, 14* and *16*, respectively. Lane 2: wild-type DNA digested by *Sst* I.

of the fragments was smaller than in the wild type (= fragment with a deletion) whereas the other, or one of the two others in case of *dum-3*, was about two times larger than the deleted fragment. After digestion with *Sal* I or *Cla* I, only the wild-type fragment (11.0 or 8.5 kb, respectively) was detected with P_1 (data not shown). These data indicate the fragments of abnormal size present in *dum-3*, *dum-4*, *dum-14* and *dum-16* result from alterations located at the left of the *Cla* I site of NAD4. The fragments recognized by P_3 after restriction by *Kpn* I and *Nco* I (Table 5) confirm this conclusion. Moreover, the P_5 probe does not hybridize with the left-side *Sal* I fragment of the mutants (data not shown), indicating that the end of COB gene and most of the left telomeric region is deleted. It can thus be concluded that *dum-3*, *dum-4* and *dum-14* carry mitochondrial DNA molecules which have a 1.6–1.7 kb deletion extending from 300–400 bp downstream from the *Nco* I site in COB to the left end of the mitochondrial molecule. As the P_5 probe does not contain the terminal single stranded short sequence of the telomere (L. Colleaux, personal communication), we do not know whether this sequence is retained

hybridization experiments after digestion of wild-type (WT) and mutant DNAs by various endonucleases.

Enzyme used	Probe	Strain	Obtained fragments (kb)
Sst I	P_2	WT	10.4 + 5.4
		dum-3	10.4 + 3.8 + 6.6 + 7.6
		dum-4	10.4 + 3.9 + 7.4
		dum-11	10.4 + 4.7 + 15
		dum-14	10.4 + 3.8 + 7.6
		dum-16	10.4 + 4.1 + 8.0
Pvu II	P_3	WT	6.1
		dum-3	4.4 + 7.4 + 8.6
		dum-4	4.4 + 8.7
		dum-11	5.4 + 6.0
		dum-14	4.4 + 9.2
		dum-16	4.7 + 9.6
Kpn I	P_3	WT	3.5
		dum-3	1.9 + 2.7 + 3.7
		dum-4	1.9 + 3.7
		dum-11	2.8 + 6.0
		dum-14	1.9 + 3.7
		dum-16	2.1 + 4.4
Nco I	P_3	WT	2.3
		dum-3	2.0 + 2.7 + 4.0
		dum-4	1.9 + 3.6
		dum-11	2.3
		dum-14	1.9 + 4.0
		dum-16	2.1 + 4.4

in the mutants. In *dum-16*, the deletion is shorter (1.3–1.4 kb) but located in the same region.

All mutants (except *dum-11*) also contain a fragment whose size is approximately twice the size of the deleted fragment. From the results of Table 5, this is most easily explained by assuming that the molecules larger than 25 kb correspond to dimers produced by the linkage of monomers at the deleted ends. The third abnormal fragment present in *dum-3* could purchase to dimers resulting from the union in opposite orientation of a molecule carrying a 1.6–1.7 kb deletion and a smaller molecule deleted in the same region (size of the deletion 2.6–3.0 kb). The *dum-3* mutant would thus contain deleted monomers and two types of dimers.

The situation was different in *dum-11*. This strain possesses molecules which have a deletion

gion, at the left of *Nco* I (Table 5). Molecules of higher molecular weight are also present in the strain (Fig. 2) but they do not result from the end-to-end union of two equally deleted molecules, as deduced from the size of the second abnormal fragment (15.0 kb with *Sst* I and P_2 probe; 6.0 kb with *Pvu* II and P_3 probe; see Table 5) detected in the mutant. Other experiments were necessary to understand the origin of this second fragment. We notably found that a same additional fragment was detected by P_1 on one hand, by P_3 or P_4 on the other hand. It was the case for a 14 ($\pm$ 1) kb *Bam* HI fragment, a 15 ($\pm$ 1) kb *Sst* I fragment, a 14 ($\pm$ 1) kb *Sal* I fragment, a 7.6 ($\pm$ 0.4) kb *Cla* I fragment and 10 ($\pm$ 1) kb *Hpa* I fragment (Table 6). The simpler model to explain the results obtained with *dum-11* is to postulate that in addition to monomers deleted in the terminal part of COB, the mutant contains dimeric molecules resulting from the union in inverted orientation of two unequally deleted monomers (Fig. 3).

Discussion

In *Chlamydomonas*, acriflavine and ethidium bromide induce with very high efficiency lethal mu-

Table 6. Restriction fragments detected on Southern blots by hybridization experiments after digestion of wild-type and *dum-11* DNAs by various endonucleases.

Enzyme used	Probe	Obtained fragments (kb)	
		wild type	dum-11
Bam HI	P_1	10.2	9.4 + 14
	P_4	10.2	9.3 + 14
Sst I	P_1	5.4 + 10.4	4.6 + 10.4 + 15
	P_4	5.4	4.5 + 15
Hpa I	P_1	7.8	7.8 + 10.3
	P_4	1.0 + 3.4	3.4 + 10.3
Sal I	P_1	11	11 + 14.5
	P_3	4.8	3.9 + 14.5
Pvu II	P_3	6.1	5.2 + 6.0
Cla I	P_1	8.5	8.5 + 7.8
	P_3	2.8	2.1 + 7.6

missing [6]. As in yeast (see [5]) these i lating dyes also induced viable mutants wh deficient for respiration ([15] and the work). Among the eight mutants here des (Table 7), one (*dn-12*; Class III) is mutate nuclear gene whereas the others contain a tion in the mitochondrial genome.

The Class I mutants (*dum-6* and *dum-* characterized by their predominantly pater heritance in crosses and the absence of det alteration in their mitochondrial DNA. (basis of biochemical analysis, *dum-6* and *c* mutants are defective in cytochrome oxida antimycin-sensitive succinate-cytochrome dase, respectively. By considering the genes are encoded by the mitochondrial genon mutated genes must be COI in *dum-6* anc in *dum-15*. The nature of the mutations ha determined by DNA sequencing. Howev fact that *dum-6* can grow very slowly und erotrophic conditions and retains some cy sensitive respiration (Table 3), is indicati the COI gene must be partially active. The the value of cytochrome *c* oxidase activ ported for this mutant (0.8, against 3.4 wild-type; Table 4) must be significantly d from zero. The same is true for *dn-12* which very slowly in the dark and has a cytoch oxidase activity of 0.4. It can thus be con that the mutations present in *dum-6* and *dr* not produce gross alterations of the e polypeptides.

The *dum-15* mutant, as the Class II n deleted for COB (see below), does no heterotrophically and is totally missi cyanide-sensitive respiration (Table 3): th ual antimycin-sensitive succinate-cytoch oxidoreductase activities measured in al mutants are probably not significantly d

The five Class II mutants share the prop have a deletion in the left terminal part mitochondrial genome (Table 7). They a similar to the *dum-1* and *dum-2* mutants ously described. We do not know whether nucleotides of the telomeric end are retai the deleted molecules are stable enough
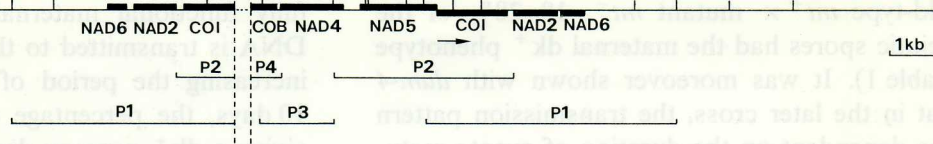
Fig. 3. Proposed structure of the high molecular weight mitochondrial DNA molecules present in the *dum-11* strain (see text) Abbreviations as in Fig. 1.

Table 7. Summary of the characteristics of the isolated mutants.

Class	Mutant	Mutagen used	Biochemical defect	Genome affected	Type of alteration
I	*dum-6*	AF	cytochrome *c* oxidase (complex IV)	mitochondrial	mutation in COI gene
	dum-15	EB	complex III	mitochondrial	mutation in COB gene
II	*dum-3, dum-4, dum-14*	AF or EB	complex III	mitochondrial	1.6–1.7 kb deletion in COB and left end of the genome (monomers + dimers)
	dum-16	EB	complex III	mitochondrial	1.3–1.4 kb deletion in COB and left end of the genome (monomers + dimers)
	dum-11	EB	complex III	mitochondrial	0.7 kb deletion in the end of COB and left end of the genome (monomers + dimers)
III	*dn-12*	EB	cytochrome *c* oxidase (complex IV)	nuclear	mutation in a nuclear CO gene

maintained and visualized by molecular hybridization. In addition to deleted molecules, the five stains possess molecules of higher molecular weight. The restriction mapping analyses allowed to conclude that these large size molecules result from end-to-end junction of two equally deleted molecules (palindromic dimers: *dum-3, dum-4, dum-14, dum-16*) or two molecules which have different deletions (*dum-3, dum-11*). Hence, each strain possesses both monomeric molecules and one or two types of dimeric molecules which are not able to segregate after subcloning, as demonstrated with *dum-4* mutant.

The presence of several types of molecules seems to be a general property of deletion mutants since DNAs of higher molecular weight have also been found in *dum-1* and *dum-2* (Collignon

and Matagne, unpublished). These molecules had not been identified in our previous publication [15]. Such a molecular polymorphism is similar to that described for several ρ^- mutants of yeast where numerous circular molecules corresponding to multimeric series were identified [11, 12]. With these authors, it can be proposed that the probable coexistence of monomers and dimers in a same cell would result from illegitimate recombination events, by a mechanism which involves the joining of mitochrondrial DNA segments at precisely defined sites and does not require an extensive sequence homology [12].

The Class II mutations share also the property to be differently inherited in reciprocal crosses When mutant *mt*$^+$ cells were mated with wild type *mt*$^-$ cells, the transmission was almost ex

wild-type mt^+ × mutant mt^-, 18–28% of the meiotic spores had the maternal dk$^+$ phenotype (Table 1). It was moreover shown with *dum-4* that in the later cross, the transmission pattern was dependent on the duration of zygote maturation in the dark. When the maturation period was reduced to 3 days, all zygotes germinated within 18 h after transfer to light and gave a typical paternal transmission; when the zygotes remained 10 days in the dark, only a small proportion germinated early to give dk$^+$ progeny whereas the majority took 2 to 3 days to germinate and give a paternal transmission. These findings could be related to respiration and photosynthetic activities during zygospore maturation and germination. During maturation, chloroplast appears to disintegrate and chlorophyll is lost [3]. In parallel, a decrease of mitochondrial mass occurs and mature zygotes contain nearly half the mitochondrial content of vegetative and gametic cells [2]. It was also shown that during zygote maturation in the dark, the mitochondrial DNA of mt^+ origin was progressively but not totally eliminated. A total elimination was obtained only after induction of germination [1]. Taking these observations altogether, we postulated that in the course of maturation, the mt^+ mitochondria were specifically degraded [1]. Hence, at the time of germination, the zygote would contain a majority of mitochondria of mt^- origin and no functional chloroplast. During the first hours of germination, no photosynthesis occurs and a respiratory activity totally sensitive to cyanide increases [9]. Therefore, the energy required to start germination must rely on the mitochondrial cytochrome pathway. One moreover knows that some nuclear mutants deficient in cyanide-sensitive respiration [23] but also the deletion *dum-11* mutant (M.-P. Dorthu, unpublished) contain mitochondria whose ultrastructure shows gross alterations. It can be postulated that in a cross wild-type mt^+ × deletion mutant mt^-, the majority of the zygotes matured for 3 days in the dark would still retain some wild-type mitochondria of maternal origin able to induce a rapid germination but unable to transmit their DNA to the meiotic progeny. The

fully functional maternal mitochondria DNA is transmitted to the meiotic proge increasing the period of maturation to 10 days, the percentage of exceptional : giving a dk$^+$ progeny does not change d cally (14–15%; Table 2); the other zygotes progressively lose their metabolically act genetically inactive mitochondria, makin unable to germinate rapidly. If, after 5 maturation in the dark, one analyzes o early germination zygotes (as we did in T one artificially selects the zygote populatio contains the fully functional wild-type m mitochondria transmitting their DNA. In ciprocal cross: mutant mt^+ × wild-type n zygotes would contain a majority of wi mitochondria and would thus germinate In relation to our observations, it has to be out that when mt^- cells treated by acrifla produce almost 100% of minute lethal c are mated with mt^+ untreated cells, germ was good but took 2 to 3 days instead of th 16–24 h (6). The hypothesis that the wild-mutant mitochondria of maternal origin a gressively eliminated during the maturatio zygospores could be tested by electron copy analyses.

Another trait common to all deletion r is their capacity to segregate mitotically 2– cells which divide 8–9 times before dyir minute colonies produced are phenot identical to those which are induced by vine or ethidium bromide treatment and l tochondrial DNA [6]. Their spontaneou duction by mitotic segregation might indic the deleted mitochondrial DNA is unstal that a small proportion of mitotic products non functional mitochondria.

This sets the problem of the role of mit dria in *Chlamydomonas*. In yeast, cells mitochondrial DNA are viable and their is sustained by fermentation metaboli *Chlamydomonas*, the minute mutants tha lost their mitochondrial DNA are unviable indicates that some mitochondrial functi not dispensable, even when the cells are g

mitochondrial mutants isolated up to now are mutated in a gene coding for apocytochrome *b* or a subunit of cytochrome *c* oxidase. No mutation localized in one of the five NAD genes or in a rRNA gene of the mitochondrial genome has been identified. Such mutants would be defective for complex I or for complexes I, III and IV and would thus be unable to regenerate NAD^+ from NADH produced in the Krebs cycle. Wiseman *et al.* [23] suggested that in the presence of SHAM to inhibit cyanide-insensitive alternative pathway, mutants deficient in cyanide-sensitive respiration may be lethal because NADH produced in the Krebs cycle could not be oxidized. Husic and Tolbert [10] showed that in the presence of SHAM, the nuclear *dk-97* mutant defective for cytochrome *c* oxidase accumulates tricarboxylic acid cycle intermediates during photosynthetic $^{14}CO_2$ fixation. The glycolate produced during photosynthesis also accumulates in the mutant, which suggests that its oxidation can be mediated by the SHAM-sensitive pathway [10]. The importance of the alternative pathway is also demonstrated by the inhibitory effect of 0.3 mM SHAM on mixotrophic growth of the *dum-3* mutant; the alternative pathway can also be essential when the cyanide-sensitive pathway is functional since in the wild-type strain, SHAM (0.3 mM) is inhibitory under heterotrophic growth and reduces growth under mixotrophic conditions (M.-P. Dorthu and R.F. Matagne, personal observations). Moreover, under photoautotrophic growth, mutant and wild-type cells have the same total respiratory rate ([10] and present work) whereas under mixotrophic conditions, the respiratory rate is increased in wild-type but not in mutants. This indicates that without exogenous carbon source, the alternative pathway is sufficient to transfer all the electrons to oxygen whereas it is no more the case when acetate is present in the growth medium.

Further experiments on mutant or wild-type cells to analyze the functional activities of chloroplast and mitochondria will bring fundamental information upon the relations between photosynthesis and respiration.

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