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

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Received 16 August 1991; accepted in revised form 2 December 1991

Key words: *Chlamydomonas*, cytochrome *b* gene, cytochrome oxidase gene, mitochondrial genome, respiratory-deficient mutants

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 alte native pathway is still functional. Measurements of complexes II + III (antimycin-sensitive succinat cytochrome *c* oxido-reductase) and complex IV (cytochrome *c* oxidase) activities allowed to conclu 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 compo 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 fro 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 o 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 deletec
region containing the COB gene. An *in vi*
orimetric test allowing to detect rapidly r
tory mutants and to analyze the segregatio
dk^- phenotype in crosses 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 tre

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* prepared according to Moller and Palmer [17]. In order to make the inner mitochondrial membran[e] accessible to cytochrome *c*, 5 μl aliquots [o]f 250 mM deoxycholate were added until a maxi[m]al cytochrome *c* oxidase activity was obtaine[d]. The final concentration of deoxycholate wa[s] about 2 mM. The oxidation of cytochrome *c* wa[s] 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 potassiu[m] cyanide.

Succinate-cytochrome *c* oxidoreductase wa[s] assayed in 0.1 M Tris-HCl buffer pH 7.4 contai[n]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 [of] 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 mM final) was considered as total succinate-cy[]tochrome *c* oxidoreductase activity. The antimy[]cin-sensitive succinate-cytochrome *c* oxidoredu[c]tase or complex II + III activity was measure[d] from the decrease of activity observed after th[e] addition of antimycin A (6 μM final) to the rea[c]tion mixture.

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

Tetrazolium overlay technique for detecting respi[ra]tion deficiency

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

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.* [[...]

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[...]

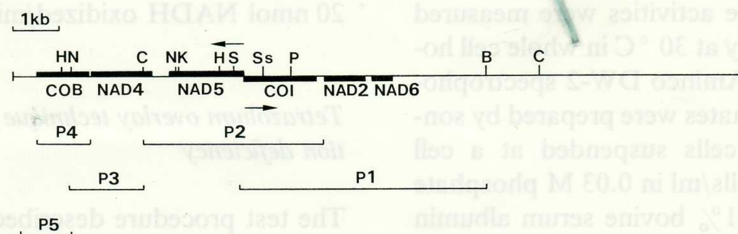

Fig. 1. Partial restriction map of the 15.8 kb linear mitochondrial DNA of *C. reinhardtii*. Restriction enzyme sites: B, *Bam* [...] *Cla* I; H, *Hpa* I; K, *Kpn* I; N, *Nco* I; P, *Pvu* II; S, *Sal* I; Ss, *Sst* I. In case of C, H, K, N and P, only the sites important [...] study are indicated. Genes: 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].

(100–300 meiotic clones analyzed in each cross).

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

paternal (mt^-), and thus typical of mitochondrial mutations. Following the nomenclature used earlier [15], the mutants were named *dum-6* and *dum-15* (*d*ark *u*niparental transmission by the *m*inus 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* (*d*ark *n*uclear).

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 earl 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 mod 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) stro duced but did not totally abolish the oxyge sumption.

In mutants (Table 3), the total respirati generally lower than in the wild type, wit ever some variability which might reflect logical differences between the different c [23]. As opposed to the wild type, the m (except *dum-6* and *dn-12*) had a respiratio sensitive to SHAM and poorly sensitive to This indicates that the reduced respiratio 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 blo respiratory chain by interacting with tochrome *b* [19]. This antimycin-sensitive nate-cytochrome *c* oxidoreductase activit sponds to the activity of complexes

tions. Respiration was measured before and after the addition of the inhibitors (SHAM and KCN). For total respiratory rat
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 + SHA
–	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 dat expressed relatively to lactate dehydrogenase activity, represent the mean of 2–3 experiments for mutants and 7 experiments fo the wild type (S.D. in parenthesis).

Mutant class	Strain	Relative activity ($\times 10^{-3}$) of		
		succinate-cytochrome *c* reductase		cytochrome *c* oxida (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	8	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 typ (Table 4). In the other mutants, the total activi was lower and almost insensitive to antimycin which indicates that the activity of complex 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 wilc 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 l in photosynthetic activities.

Molecular analysis of the dum *mutants*

The phenotype and the pattern of inherita 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 c 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, i of 15.8 kb) (Fig. 2). In order to determine w the two types of molecules can segregate cally to give rise to clones homoplasmic f of the other types of mitochondrial DN *dum-4* strain was subcloned several times subclones, the two types of molecules pe

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 P_2, the large 10.4 kb fragme 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 P_3 after digest *Pvu* II (Table 5). Both with *Sst* I and *Pvu*