Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 7447-7450, August 1991
Genetics

Cytoduction in *Chlamydomonas reinhardtii*

(chloroplast gene transmission/sexual cycle/"heteroplasmons")

RENÉ F. MATAGNE, CLAIRE REMACLE, AND MONIQUE DINANT

Genetics of Microorganisms, Department of Botany, B22, University of Liege, Sart Tilman, B-4000 Liege, Belgium

Communicated by David J. L. Luck, May 15, 1991 (received for review February 25, 1991)

ABSTRACT **After conjugation between *Chlamydomonas* gametes of opposite mating type, a transient dikaryon is formed. The two nuclei fuse within 4-6 hr after mating. The young diploid zygote differentiates into dormant zygospore competent to complete meiosis, or more rarely (2-10% of cases) it undergoes mitosis to produce a stable diploid progeny. We here bring genetical, biochemical, and cytological evidence that among the mitotic zygotes, a large proportion of them undergo cytokinesis without fusion of the nuclei—a process that has been termed "cytoduction." By using appropriate genetic markers, haploid cytoductants that possess the nuclear genotype of one parent and the chloroplast marker of the other parent can easily be isolated. Genetical analysis and hybridization experiments moreover show that many haploid cytoductants transmit the chloroplast DNA molecules of both parents and that, as in diploids, these DNA copies occasionally recombine. This process of cytoduction extends the life cycle of *Chlamydomonas* and provides new tools for its genetic analysis.**

In the unicellular alga *Chlamydomonas reinhardtii*, genetical analysis can be performed at both haploid and diploid levels.

The sexual cycle occurs by an orderly progression of sequential events. When vegetative cells are deprived of nitrogen, they differentiate into morphologically identical mating type plus (mt^+) and minus (mt^-) gametes, each of which possesses a haploid nucleus and a unique cup-shape chloroplast. After gamete fusion, a transient dikaryon is formed. The two nuclei and the two chloroplasts fuse within 4-6 hr after mating (1). The zygote then differentiates into a thick-walled zygospore that after maturation undergoes meiosis to give rise to four or eight haploid spores. The genes residing in the chloroplast are most often inherited from the mt^+ parent only, the chloroplast DNA of mt^- origin being specifically eliminated before the fusion of the two organelles (2, 3).

A small proportion (2-10%) of zygotes, called "vegetative zygotes," do not differentiate into zygospores. Instead, they divide mitotically and give rise to stable diploid clones (4). In these diploids, the chloroplast genes of the two parents are often conserved and, through mitotic divisions, they segregate and occasionally recombine (5, 6).

By using appropriate nuclear and chloroplast markers, we demonstrate here that a third class of zygotes is produced. When the transient dikaryon is formed, cell division can occur before the fusion of the two nuclei—a process that has been termed "cytoduction" (see *Discussion*). Haploid cells possessing the nuclear genome of one parent but the chloroplast marker of the other parent ("haploid cytoductants") can be recovered. Chloroplast fusion without nuclear fusion can also occur, since haploid clones recombinant for chloroplast DNA markers have been isolated. This process of cytoduction constitutes a novel tool to identify extrachromosomal genes, to transfer these genes into a new nuclear background, and to analyze the action of nuclear genes through the cytoplasm.

MATERIALS AND METHODS

Strains and Culture Media. Strains used in the present study are listed in Table 1. Some of them carry nuclear mutations determining auxotrophy for acetate, arginine or *p*-aminobenzoic acid and/or chloroplast mutations conferring resistance to spectinomycin (*spr*), streptomycin (*sr*), or erythromycin (*er*). Strains 7, 8, 154, and 155 are derived from the 137c wild-type strains (1 and 2), which carry *nit-1* and *nit-2* nuclear mutations, making them unable to utilize nitrate as the sole nitrogen source. Strain j contains the *nit-b* (unmapped) nuclear marker: it was isolated by mutagenic treatment of the wild-type (CC-1373) *Chlamydomonas smithii* mt^+ strain (7).

The location of the different nuclear markers is the following (6): mt^+ and mt^-, linkage group VI; *nit-1*, l.g. IX; *nit-2*, l.g. III; *ac-14, arg-2, pab-2*, l.g. I.

The cells were routinely grown on agar plates containing minimal medium (HSM) (6) or acetate-containing medium (TAP) (6), supplemented with arginine (100 μg/ml) or *p*-aminobenzoic acid (1 μg/ml) when required. In some cases, KNO_3 (400 μg/ml) was used instead of NH_4Cl (400 μg/ml) as the sole nitrogen source. To prepare the selective medium, spectinomycin (100 μg/ml), streptomycin (300 μg/ml), or erythromycin (100 μg/ml) was added after sterilization, when the medium had cooled to approximately 50°C.

Crosses and Isolation of Diploids and Haploid Cytoductants. For crosses, gametes of opposite mating types were mixed for 1 hr, and the suspensions containing mating products and unmated cells were plated on appropriate selective medium. The plates were incubated for 10 days under continuous light (6000 lux) to recover the mitotic progeny of the diploids or cytoductants. (In these experimental conditions, the zygotes cannot undergo maturation and meiosis). The phenotype of the resulting colonies was determined by replica-plating onto different appropriate media.

Determination of Mating Type. Cells of the different clones to be analyzed were suspended in distilled water (2×10^7 cells per ml) and mixed with an equal amount of wild-type mt^+ or mt^- cells. The zygote pellicle formed at the surface of one of the test tubes was observed after 24 hr.

Determination of DNA Content and Nucleoid Number. The following rapid spectrofluorometric method was developed to determine the amount of DNA per cell.

A known number of cells (5×10^6 to 10^7) were suspended in 0.5 ml of TEN buffer (100 mM Tris/10 mM EDTA/1 mM NaCl, pH 7.4). The cells were chilled, disrupted at 0°C by ultrasound (MSE [London] Ultrasonic Disintegrator; 30 sec) and then 1 ml of cold ethanol was added to precipitate DNA. After incubation overnight at −20°C, the extracts were centrifuged at 15,000 × *g* for 5 min, and the supernatant was discarded. The pellet was dried by centrifugation under vacuum and resuspended in 1 ml of TEN buffer. After incubation for 1 hr at room temperature, the suspension was

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Table 1. Nuclear and chloroplast markers of the strains used (see text)

Strain number	Nuclear markers	Chloroplast marker
1	*nit-1 nit-2 mt*$^+$	—
2	*nit-1 nit-2 mt*$^-$	—
7	*nit-1 nit-2 ac-14 mt*$^+$	—
8	*nit-1 nit-2 ac-14 mt*$^-$	—
154	*nit-1 nit-2 arg-2 mt*$^+$	*spr*
155	*nit-1 nit-2 arg-2 mt*$^-$	*spr*
38	*pab-2 ac-14 mt*$^-$	*sr*
86	*pab-2 ac-14 mt*$^-$	*spr*
j	*nit-b mt*$^+$	*er*

The j strain is *C. smithii*, the other strains are *C. reinhardtii*.

centrifuged, and 10 μl of Hoechst 33258 (100 μg/ml) was added to the supernatant. The DNA was estimated by measuring the emitted fluorescence (8). The reference curve was established with purified DNA of *Chlamydomonas*.

The chloroplast nucleoids were counted (30 cells in each case) at the fluorescent microscope after staining with 4',6-diamidino-2-phenylindole (DAPI) as described (9).

Hybridization Experiments. DNA extraction, digestion, electrophoreses, and Southern blotting were performed as described (10). Specific chloroplast DNA fragments were detected after hybridization of *Bam*HI + *Eco*RI fragments with the pBR313 + Ba4 probe obtained from J.-D. Rochaix (University of Geneva). The Ba4 fragment [7 kilobases (kb)] of Rochaix (11), equivalent to fragments 11 and 12 in Boynton and Gillham's laboratory, covers a region containing the 16S, 7S, 3S, and part of the 23S rRNA genes of the chloroplast DNA of *C. reinhardtii*.

The probe was labeled with digoxigenin (Boehringer Mannheim).

RESULTS

The *nit-1 nit-2 arg-2 spr mt*$^+$ strain (strain 154) was crossed with the *nit-1 nit-2 ac-14 mt*$^-$ strain (strain 8), and the mating mixture was plated onto agar containing TAP medium and spectinomycin. None of the parental strains can survive on the selective medium. Diploid products having inherited the *spr* chloroplast marker from the *mt*$^+$ parent and also any haploid cell carrying the *ac-14* nuclear marker from the *mt*$^-$ parent and the *spr* chloroplast marker from the *mt*$^+$ parent will be able to grow on selective medium. To distinguish between both possible types of clones, 223 colonies were replica-plated onto agar containing HSM and TAP media. Only 62% of the clones grew on HSM/agar (Table 2): they correspond to prototrophic diploids formed by complementation between the *arg-2* and *ac-14* markers. Ten diploid colonies were selected at random and tested for their mating type: as expected, all were mating type minus because in diploids heterozygous for the *mt* locus, the *mt*$^-$ marker is dominant (4). The 38% remaining clones were auxotrophic

Table 2. Crosses, selective media used, and percentages of clones able to grow after replica-plating onto minimal agar medium

Cross	Selective medium	Colonies analyzed, no.	Clones growing on minimal medium, %
154 × 8	TAP + Spec	223	62
155 × 7	TAP + Spec	71	42
154 × 38	TAP + PABA/Spec	142	20
	HSM + Arg/Strep	165	55

Spec, spectinomycin; Strep, streptomycin.

for acetate. Hence, they must be haploid or aneuploid for chromosome I, which carries the *ac-14* marker. Again, ten colonies were selected at random and tested for their mating behavior: all were mating type minus, which indicates that they also had inherited the *mt*$^-$ marker (chromosome VI) from the same parent (strain 8).

Two types of clones, *ac*$^+$ and *ac*$^-$, were also obtained in the reciprocal cross (155 × 7) after selection on the same medium (Table 2). Ten of the presumptive diploids (*ac*$^+$) were all phenotypically *mt*$^-$, whereas 10 *ac*$^-$ clones were all mt$^+$. Thus, these 10 auxotrophs carry one chromosome I (*ac-14* marker) and one chromosome VI (*mt*$^+$ marker), originating from the parental strain 7 only.

A third cross was performed involving the *nit-1 nit-2 arg-2 spr mt*$^+$ strain (154) and the *pab-2 ac-14 sr mt*$^-$ strain (38). The mitotic progeny was first selected on agar containing TAP medium, *p*-aminobenzoic acid, and spectinomycin (Table 2). As in the previous crosses, many clones could not grow after replica-plating on agar containing HSM medium, which indicates that they were auxotrophs for *p*-aminobenzoic acid and/or acetate. To determine which markers were present, the auxotrophs were tested for mating type and for growth on different selective media. All of them were phenotypically nit$^+$ pab$^-$ ac$^-$ mt$^-$ (data not shown) and have thus inherited chromosome I (*pab-2 ac-14*), chromosome VI (*mt*$^-$), chromosome IX (*nit-1*$^+$), and chromosome III (*nit-2*$^+$) of the same parent (strain 38). The remaining clones representing 20% of the total were all prototrophs and phenotypically mt$^-$ as expected for diploid clones.

Thus, the auxotrophs obtained in the three crosses resulted from a cosegregation of nuclear markers carried by the same parent. This is most easily explained by assuming that the auxotrophs have inherited the haploid nucleus from one parent and the chloroplast-resistant marker from the other parent.

These observations were confirmed when the mating mixture of the third cross (154 × 38) was first plated on agar containing HSM medium, arginine, and streptomycin, and the isolated clones were tested further for growth on agar containing HSM medium (Table 2). The *arg*$^+$ diploid clones were all phenotypically mt$^-$, whereas the *arg*$^-$ presumptive haploids were all mt$^+$ (data not shown). In this cross, we also observed that after plating the same amounts of cells, more colonies grew on agar containing TAP medium, *p*-aminobenzoic acid, and spectinomycin than on agar containing HSM medium, arginine, and streptomycin. Expressed in percentages of zygospores encysted in the agar, they represented 7.8% and 1.7%, respectively. If no auxotrophic marker effect is involved, this indicates that the clones carrying the chloroplast marker of *mt*$^+$ origin are more frequent than those carrying the chloroplast marker of the *mt*$^-$ parent.

To prove that the auxotrophic clones were really haploid, several colonies obtained in the cross 154 × 38 were analyzed for their DNA content. Wild-type haploid strains (137c) and phenotypically wild-type diploid colonies isolated in the same cross were used as control (Table 3). The values found in the controls fully agree with those reported in the literature (6). As assumed, the auxotrophic clones had unambiguously a haploid DNA content. Identical results (Table 3) were obtained with clones isolated from the cross 86 × j (see below).

Like haploid cells, diploids possess a single chloroplast, but the mean number of nucleoids is about twice that found in haploids (12). The values reported in Table 3 confirm the conclusions concerning the ploidy of the various clones.

Taken altogether, these data indicate that the original isolated auxotrophs correspond to cytoductants that have received the haploid nucleus of one parent and the chloroplast marker of the other. To determine whether some cytoductants have received the chloroplast markers of both parents, the 142 clones selected from the cross 154 × 38 on

agar containing TAP medium, *p*-aminobenzoic acid, and spectinomycin were replica-plated on agar containing all of those ingredients plus streptomycin. Eleven of the 28 diploid clones (39%) and 48 of the 114 haploid clones (42%) could grow in the presence of both antibiotics. Thus, a similar proportion of vegetative diploids and cytoductants possess recombinant *sr spr* chloroplast DNA molecules, which indicates that fusion of chloroplasts occurs even in the absence of nuclear fusion.

This was confirmed in the cross between the *pab-2 ac-14 spr mt*$^-$ strain (86) of *C. reinhardtii* with the *nit-b er mt*$^+$ strain (j) of *C. smithii*. The fusion products were selected on agar containing TAP medium, *p*-aminobenzoic acid, spectinomycin, and erythromycin. Both parental strains are selected against on that medium because each of them is sensitive to one of the antibiotics. By contrast, any *spr er* recombinant cell containing either a diploid nucleus or a haploid nucleus from one or the other parent will grow on the selective medium. After replica-plating of 238 colonies on TAP (nitrate nitrogen source)/*p*-aminobenzoic acid, HSM (nitrate nitrogen source), and HSM (ammonium nitrogen source), and subsequent mating type analyses, we found that 97 clones were diploid, 130 clones were *nit-b mt*$^+$, 3 clones were *pab-2 ac-14 mt*$^-$, and 8 clones were composed of a mixture of *nit-b mt*$^+$ and *pab-2 ac-14 mt*$^-$ cells. Again, five of the putative cytoductants selected at random had a DNA content and a nucleoid number typical of a haploid strain (Table 3).

The chloroplast DNAs of *C. reinhardtii* and *C. smithii* display different restriction patterns. In particular, some polymorphism has been detected in the inverted repeats containing the 16S and 23S rRNA genes (13). In *Chlamydomonas*, chloroplast mutations conferring resistance to spectinomycin and erythromycin have been mapped in the 16S and 23S rRNA genes, respectively (14). By assuming that the *spr* and *er* markers used in this study are located in the same genes, the comparison of the published maps (13) allows one to predict that the recombinant molecules carrying the *spr* and *er* markers most probably contain the three *Bam*HI–*Eco*RI fragments equivalent in size to those of *C. reinhardtii* as far as the 16S–23S region is concerned. (As a matter of fact, the segment in which recombination events have to occur to produce new restriction fragments is very short as compared with the length of the *spr er* fragment; see for example figure 1 in ref. 13). The data of Fig. 1 fit with this prediction: whatever nucleus is present, both the diploid and haploid isolates possess the chloroplast DNA fragments similar to those of *C. reinhardtii*. Moreover, Fig. 1 shows that some cytoductants also have in a lower amount the fragments typical of *C. smithii* and thus are probably heteroplasmic for recombinant and for *C. smithii* chloroplast DNA molecules.

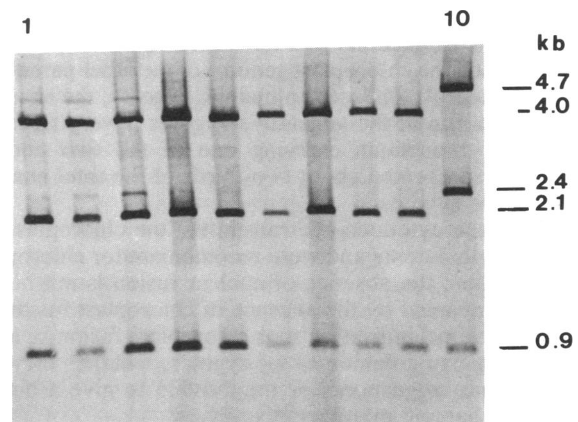

FIG. 1. Hybridization of the pBR313-Ba4 probe with *Bam*HI/*Eco*RI-digested DNAs isolated from clones obtained in the cross 86 × j. Lanes: 1, 2, 5, and 6, *nit-b mt*$^+$ cytoductants; 3 and 4, *pab-2 ac-14 mt*$^-$ cytoductants; 7 and 8, diploids; 9, control *C. reinhardtii* (strain 86); 10, control *C. smithii* (strain j).

DISCUSSION

The term cytoduction was used by Zakharov *et al.* (15) to describe the consequences of the fusion of cytoplasms without nuclear fusion in zygotes of *Saccharomyces cerevisiae*. This aberration occurs at low frequency and gives rise to haploid "heteroplasmons"—cytoductants that contain the cytoplasmic components of both parents and the nuclear genotype of one parent. Several mutations that prevent nuclear fusion after conjugation allow isolation of cytoductants at high frequency (16, 17).

In *Chlamydomonas*, nuclear fusion in newly formed zygotes can be inhibited by colchicine and other drugs that affect the microtubule stability (18). This inhibition was demonstrated genetically by the production of four viable haploid meiotic products from conjugation between haploid and diploid strains. Without colchicine treatment, the absence of nuclear fusion in meiotic zygotes is very rare or absent, as deduced from the observations of Dutcher (18).

We here demonstrate that the absence of nuclear fusion spontaneously occurs in those particular zygotes that, instead of differentiating into zygospores, divide mitotically. For these zygotes, the transient heterokaryons that are formed soon after mating can evolve into two directions: either the two nuclei fuse and the zygotes will give rise to a stable diploid progeny or cytokinesis takes place before the nuclear fusion ("cytoduction"). In this last case, the zygotes will produce some haploid progeny carrying the nuclear genotype of one parent and some haploid progeny carrying the genotype of the other parent.

Table 3. Amount of DNA per cell and mean number of chloroplast nucleoids per cell in various clones presumed haploid or diploid

Cross	Phenotype	Ploidy	Clones analyzed, no.	DNA per cell, pg	Nucleoids per cell
154 × 38	pab$^-$ ac$^-$ nit$^+$ mt$^-$	Haploid?	2	0.14; 0.15	9.4; 9.5
	arg$^-$ mt$^+$	Haploid?	3	0.16; 0.16; 0.17	9.6; 9.7; 9.9
	wild-type mt$^-$	Diploid	3	0.34; 0.39; 0.31	18.8; 20.1; 20.9
86 × j	pab$^-$ ac$^-$ nit$^+$ mt$^-$	Haploid?	3	0.21; 0.18; 0.17	—
	nit$^-$ mt$^+$	Haploid?	2	0.18; 0.19	—
	wild-type mt mt$^-$	Diploid	2	0.35; 0.34	—
—	137c wild-type mt$^+$	Haploid	1	0.15	9.8
	137c wild-type mt$^-$	Haploid	1	0.17	9.3

By using appropriate nuclear and extrachromosomal markers, haploid cytoductants that have received the nucleus of one parent and the chloroplast genome of the other parent are easily selected. As opposed to meiotic zygotes, the absence of nuclear fusion in the vegetative zygotes is very frequent since the cytoductants carrying one of the two nuclear genotypes represented about two-thirds of the total number of vegetative zygotes.

Many of the cytoductants transmitted the chloroplast genomes of both parents and were recombinant for chloroplast markers. Thus, the absence of nuclear fusion is not necessarily accompanied by the absence of chloroplast fusion. In fact, we have no indication that chloroplast fusion is not a general property common to all zygotes, whether they differentiate into zygospores or they divide to give a diploid progeny or haploid cytoductants.

The percentage of diploids and haploid cytoductants that are recombinant for chloroplast markers are similar (about 40%). This suggests that the patterns of chloroplast gene transmission can be identical in the two classes of zygotes and thus independent of nuclear fusion.

In the cross 86 × j, the cytoductants were selected in such a way that each parental nuclear genotype could grow. Surprisingly, only a small proportion of isolated colonies were composed of both types of haploid cells, the large majority of cytoductants yielding *nit-b* mt^+ progeny only. Additional experiments are needed to demonstrate whether this bias is related to the mating-type locus or results from an auxotrophic marker effect.

As in yeast, the production of cytoductants extends the life cycle of *Chlamydomonas* and provides new tools for its genetic analysis. In particular, it allows rapid identification of traits that are inherited through the cytoplasm. Further experiments are needed to show whether the chloroplast genes are actually transmitted in the haploid cytoductants as in the diploids and to analyze how the mitochondrial genome is inherited in the absence of nuclear fusion.

This research was supported by a grant from the Belgian Fonds de la Recherche Fondamentale et Collective (F.R.F.C.) (2.4521.89). C.R. is research assistant of the National Foundation for Scientific Research (Belgium).

1. Cavalier-Smith, T. (1970) *Nature (London)* **228**, 333–335.
2. Kuroiwa, T., Kawano, S., Nishibayashi, S. & Sato, C. (1982) *Nature (London)* **298**, 481–483.
3. Munaut, C., Dombrowicz, D. & Matagne, R. F. (1990) *Curr. Genet.* **18**, 259–263.
4. Ebersold, W. T. (1967) *Science* **157**, 447–449.
5. Gillham, N. W. (1978) in *Organelle Heredity* (Raven, New York), p. 602.
6. Harris, E. H. (1989) in *The Chlamydomonas Sourcebook* (Academic, San Diego), p. 780.
7. Matagne, R. F. & Beckers, M.-C. (1987) *Plant Sci. Lett.* **49**, 85–88.
8. Labarca, C. & Paigen, K. (1980) *Anal. Biochem.* **102**, 344–352.
9. Matagne, R. F. & Hermesse, M.-P. (1981) *Genetics* **99**, 371–381.
10. Matagne, R. F., Rongvaux, D. & Loppes, R. (1988) *Mol. Gen. Genet.* **214**, 257–262.
11. Rochaix, J.-D. (1987) *FEMS Microbiol. Rev.* **46**, 13–14.
12. Matagne, R. F. & Hermesse, M.-P. (1980) *Arch. Int. Physiol. Biochim.* **88**, B291–292.
13. Newman, S. M., Boynton, J. E., Gillham, N. W., Randolph-Anderson, B. L., Johnson, A. M. & Harris, E. H. (1990) *Genetics* **126**, 875–888.
14. Harris, E. H., Burkhart, B. D., Gillham, N. W. & Boynton, J. E. (1989) *Genetics* **123**, 281–292.
15. Zakharov, J. A., Yurchencko, L. V. & Yarovot, B. F. (1969) *Genetika* **5**, 136–141.
16. Conde, J. & Fink, G. R. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3651–3655.
17. Kim, J., Ljungdahl, P. O. & Fink, G. R. (1990) *Genetics* **126**, 799–812.
18. Dutcher, S. K. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3946–3950.