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Detection of chloroplast DNA by using fluorescent monoclonal anti-bromodeoxyuridine antibody and analysis of its fate during zygote formation in *Chlamydomonas reinhardtii*

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Summary. A monoclonal anti-bromodeoxyuridine antibody conjugated to fluorescein was used to detect the chloroplast nucleoids after specific incorporation of bromodeoxyuridine (BUdR) into the chloroplast DNA of *Chlamydomonas reinhardtii*. The incorporation of BUdR was enhanced by simultaneous treatment with fluorodeoxyuridine (FUdR). The method was applied to analyze the fate of chloroplast DNA in zygotes resulting from mating between BUdR-treated gametes (mt^+ or mt^-) and untreated gametes of opposite mating-type. In crosses between wild-type strains, the nucleoids of mt^+ origin remained in the large majority of zygotes whereas those of mt^- origin most often disappeared within the first hours following copulation. In crosses of the type *mat*-3 $mt^+ \times$ wild-type mt^- (the *mat*-3 mutation permits a high transmission of chloroplast genes from the mt^- parent), the nucleoids of mt^- origin were generally not eliminated which indicates that the *mat*-3 mutation prevents the selective destruction of paternal chloroplast DNA in the zygote.

Key words: Chloroplast DNA – Monoclonal anti-bromodeoxyuridine antibody – *Chlamydomonas*

Introduction

The single chloroplast of the unicellular green alga *Chlamydomonas reinhardtii* contains approximately 100 circular DNA copies at the end of cell division (Turmel et al. 1980). These copies are organized into about ten nucleoids easily detectable by fluorescence microscopy after staining with the DNA-specific dye 4′,6-diamidino-2-phenylindole (DAPI) (Kuroiwa et al. 1981).

In crosses between mating-type plus (mt^+) and minus (mt^-) gametes, more than 90% of zygotes transmit only the chloroplast genes of mt^+ or maternal origin to the meiotic progeny (Gillham 1978) despite the fusion of the two parental chloroplasts in the zygotes. Searching for the molecular basis of the uniparental maternal mode of transmission of chloroplast genes, Sager and coworkers labelled the chloroplast DNA with a radioactive precursor and concluded that the DNA of mt^- origin was selectively lost in the zygote, a few hours after its formation (Sager and Lane 1972; Burton et al. 1979). However, similar analyses performed in another laboratory led to different conclusions (Chiang 1971, 1976).

By using the DAPI staining technique, Kuroiwa et al. (1982) and Tsubo and Matsuda (1984) provided microscopic evidence that the nucleoids of one parent disappear within the first hours after mating, before the fusion of the two chloroplasts in the zygote. To determine the origin of the nucleoids which were eliminated, these authors used gametes differing either by the size of the flagella (Kuroiwa et al. 1982) or by the chlorophyll fluorescence (Tsubo and Matsuda 1984). They concluded that only the nucleoids of mt^- or paternal origin were eliminated in the young zygote, thus confirming the observations of Sager and coworkers. However, the DAPI technique does not permit analysis of the origin of the nucleoids in a large number of zygotes. Moreover, the distinction between maternal and paternal nucleoids becomes impossible after the two chloroplasts have fused, i.e., about 4–5 hours after mating.

We here describe an immunocytochemical method which allows the unambiguous identification of the nucleoids of each parent and the determination of their fate in the zygotes, even after fusion of the two chloroplasts. The method utilizes the specific labelling of the chloroplast nucleoids of one or the other parent with 5-bromodeoxyuridine (BUdR) and its detection in situ with a monoclonal anti-BUdR antibody conjugated to fluorescein. Thymidine, or its analogue BUdR, specifically labels the chloroplast DNA of vegetative or gametic cells in *Chlamydomonas* (Chiang et al. 1975) because the thymidine kinase necessary to phosphorylate the nucleosides is only present in this organelle (Swinton and Hanawalt 1972; Swinton and Chiang 1979). The immunocytochemical method was applied to analyze the

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fate of nucleoids in zygotes obtained in crosses between mt^+ and mt^- wild-type cells and in crosses between mt^+ *mat*-3 mutant and mt^- wild-type cells. As recently reported by Gillham et al. (1987), the *mat*-3 mutation, which is tightly linked to the mt^+ locus (linkage group VI), permits high transmission of the chloroplast markers from the mt^- parent and thus disrupts the preferential maternal inheritance generally observed for these markers. We demonstrate here that in contrast to wild-type, the *mat*-3 mutation prevents the selective destruction of nucleoids of paternal origin within the first hours of zygote formation. Part of these results have been published as a preliminary note (Munaut et al. 1988).

Materials and methods

Strains. The wild-type (WT) strain 137c, mating-type plus or minus, and the *mat*-3 (cc-1995 *mat*-3-2 allele isolated by Gillham et al. 1987) mating-type plus strain were used in this study.

Media and growth conditions. The cells were maintained routinely on TAP agar plates (Gorman and Levine, 1965). Liquid cultures were grown in TAP medium under an alternating 12 h-light (3000 lux by cool white fluorescent light) and 12 h-dark cycle at 24 °C. In these conditions, cells (4×10^5 cells/ml) divided synchronously at the end of the dark period. Treatments with BUdR began at the beginning of the light period. Gametogenesis was induced by transferring the cells at the end of the dark period to nitrogen-free medium for 18 h under continuous light. Zygotes were obtained by mixing gametes (10^7 cells/ml) of opposite mating-types.

DAPI staining. The cells were fixed in cold ethanol for 30 min. After washing with distilled water, they were suspended (10^8 cells/ml) in 0.1 µg/ml DAPI for 30 min at 37 °C, then washed again with water. All observations were made with a BH-RFL Olympus fluorescence microscope equipped with a HBO 100 W mercury lamp, a UG-1 excitation filter and a 435 nm suppression filter.

Staining with the FITC-conjugated monoclonal antibody. The anti-BUdR monoclonal antibody conjugated to fluorescein isothiocyanate (FITC) was purchased from Becton-Dickinson, Mountain View, California. About 3×10^7 cells grown in the presence of BUdR were washed 2–3 times with ethanol (removal of the chlorophylls) then maintained in cold ethanol for 30 min. After centrifugation, the pellet was hydrolysed with 4N HCl:70% ethanol (1:1 v/v) for 1 h at room temperature. Hydrolysis was stopped by immersion of the cells in 2 ml 0.1 M $Na_2\ B_4O_7$ pH 8.5. The cells were resuspended in 2 ml PBS pH 7.2 containing 0.5% Tween 20. An aliquot containing 4×10^6 cells was centrifuged and the pellet resuspended in 40 µl PBS-Tween and 10 µl of monoclonal anti-BUdR reagent conjugated to FITC. After incubation for 1 h in the dark (room temperature), the cells were washed and then resuspended in 50 µl PBS. The observations of the chloroplast nucleoids were made with the fluorescence microscope equipped with a IF 490 excitation filter and a 530 nm suppression filter. In some cases, additional staining of the nucleus was achieved by adding propidium iodide (final concentration:3–5 µg/ml) to the suspension.

Results

Incorporation of BUdR

Treatment of cells with BUdR (0.1–1.0 mM) for 24 h did not perturb the growth rate: in our experimental conditions, cells divided twice both in treated and control cultures (data not shown). Similarly, the mean number of nucleoids per cell (9.8±1.6), determined after DAPI staining, was not modified by the treatment.

To determine whether BUdR had been incorporated into the chloroplast nucleoids, samples of cells were stained with the antibody conjugated to fluorescein. The epifluorescent microscopic analysis revealed the green fluorescence characteristic of FITC at the level of the nucleoids whereas the nucleus was not stained. The nucleus could be visualized only after additional staining with propidium iodide (orange fluorescence). The FITC fluorescence of the nucleoids was, however, faint and only detectable in approximately 10% of the cell population. This poor labelling could reflect a low incorporation of BUdR into chloroplast DNA, as observed with radioactive thymidine (Swinton and Hanawalt 1972; Swinton and Chiang 1979).

Action of FUdR and its reversal by BUdR

To increase the incorporation of the analogue, we treated the cells simultaneously with BUdR and 5-fluorodeoxyuridine (FUdR). In *Chlamydomonas,* FUdR is thought to block chloroplast DNA synthesis by inhibiting de novo synthesis of thymidylate from deoxyuridylate (Wurtz et al. 1977). Thus, FUdR treatment should favor the incorporation of BUdR via the salvage pathway.

When the cells were maintained for 24 h in the presence of FUdR, the growth rate was not modified. However, the mean number of nucleoids per cell decreased with increasing concentration of the analogue (Table 1). At the concentrations 1 and 5 mM, the nucleoids were reduced in size and fluorescence intensity.

Addition of BUdR reversed the inhibitory effect of FUdR and restored the normal number of nucleoids (detected by DAPI staining) when the BUdR/FUdR molar ratio was approximately 0.5 or higher (Table 2).

Incorporation of BudR in the presence of FUdR

To determine whether the incorporation of BUdR was actually increased by treatment with FUdR, the cells were stained with the anti-BUdR antibody conjugated to FITC after cultivation for 24 h in the presence of both analogues. At the chosen concentrations (BUdR: 0.2–0.5 mM; FUdR: 0.2–1.0 mM), the nucleoids were visible in all cells and their mean number per cell was about nine (data not shown), a value very close to that obtained after DAPI staining. No fluorescence was detected in cells

Table 1. Mean numbers of nucleoids per cell (±SD) after growth for 24 h in the presence of FUdR at various concentrations (DAPI staining; 50 cells analyzed in each case)

FUdR concentration (mM)				
10^{-3}	10^{-2}	10^{-1}	1	5
9.8±1.5	8.2±1.4	7.4±1.6	5.9±1.4	5.2±1.0

Table 2. Mean numbers of nucleoids per cell (±SD) after growth for 24 h in the presence of FUdR and BUdR (DAPI staining; 50 cells analyzed in each case)

FUdR concentration (mM)	BUdR concentration (mM)				
	0	0.1	0.2	0.5	1.0
0.2	6.6 (±1.2)	9.0 (±1.7)	9.3 (±1.5)	9.5 (±1.4)	–
0.5	6.0 (±1.2)	8.4 (±1.5)	9.0 (±1.6)	9.3 (±1.5)	9.4 (±1.3)
1.0	5.3 (±1.1)	7.5 (±1.7)	–	9.2 (±1.8)	9.5 (±1.5)

treated with FUdR alone. The brightest FITC fluorescence was obtained with the combination 0.2 mM BUdR + 0.5 mM FUdR. This fluorescence was, however, much less brilliant and stable than that obtained with DAPI and due to quenching of fluorescence during the relatively long exposure times, it was not possible to obtain photomicrographs in which all nucleoids were visible.

Fate of nucleoids in the zygotes analyzed by DAPI staining and by the BUdR labelling technique

In order to analyze the fate of maternal and paternal nucleoids in zygotes, the cells of one or the other parent were grown for a cell cycle in the presence of 0.2 mM BUdR + 0.5 mM FUdR. Gametogenesis was induced by transfer for an additional period of 18 h to nitrogen-free medium containing the two analogues. Crosses were made between BUdR-treated cells and untreated cells in both mating-type combinations.

Crosses between mt^+ and mt^- wild-type strains were first analyzed. The mean nucleoid number determined by DAPI staining decreased significantly after gametogenesis: the gametes of mt^+ and mt^- parental strains contained 5.2 and 5.4 nucleoids respectively, values which are lower than those obtained with vegetative cells (see above). At 30 min after mixing of both gametes, the frequency of tetraflagellated zygotes was about 80%. These zygotes contained an average of 9.7 nucleoids, i.e., a value which is close to the sum of maternal and paternal nucleoids (Table 3). This number declined rapidly to reach half that value between 1 and 5 h after mating. The 24 h zygotes contained one or two large and bright nucleoids probably resulting from the fusion of the smaller nucleoids. These data, obtained after DAPI staining, confirm the observations of Kuroiwa et al. (1982) and Tsubo and Matsuda (1984) and the quantitative analysis performed by Birky et al. (1984).

The results obtained after staining with the antibody conjugated to FITC were dramatically different for mt^+ and mt^- BUdR-treated crosses (Table 3). In the cross BUdR-treated mt^+ × untreated mt^-, only the nucleoids of mt^+ (maternal) origin were detected with the antibody. About 5–6 nucleoids were counted in the zygotes 30 min after cell fusion and this number was maintained for 5 h.

Table 3. Mean number (±SD) of nucleoids per zygote obtained in the cross: wild-type mt^+ × wild-type mt^-. The nucleoids were visualized after staining with DAPI or with the FITC-conjugated antibody. The asterisk indicates the parent which was labelled with BUdR. The percentages of 5 h and 24 h zygotes containing 1–2 nucleoids or no nucleoids are shown in brackets (50 zygotes analyzed in each case)

Age of zygotes (h)	DAPI staining	FITC-antibody staining	
		WT* mt^+ × WT mt^-	WT mt^+ × WT* mt^-
0.5	9.7±1.7	5.7±1.8	3.9±1.9
1	6.5±1.9	5.6±1.1	2.0±1.8
5	4.8±1.8	5.3±1.4	1–2 (4%)
			0 (96%)
24	1.6±0.6	1.8±1.4 (96%)	2 (2%)
		0 (4%)	0 (98%)

Table 4. Mean numbers (±SD) of nucleoids in zygotes from the cross *mat*-3 mt^+ × wild-type mt^-. The nucleoids were visualized after staining with DAPI or with the FITC-conjugated antibody. The asterisk indicates the parent which was labelled with BUdR. The percentages of 5 h and 24 h zygotes containing 1–2 nucleoids or no nucleoids are shown in brackets (50 zygotes analyzed in each case)

Age of zygotes (h)	DAPI staining	FITC-antibody staining	
		mat-3* mt^+ × WT mt^-	*mat*-3 mt^+ × WT* mt^-
0.5	10.4±1.6	5.2 ±1.8	5.2±1.3
1	10.1±1.1	4.9 ±2.3	5.0±1.8
5	10.6±2.0	4.5 ±2.1	5.0±1.6
24	1.9±0.7	1.95±1.1 (88%)	2.3±1.2 (44%)
		0 (12%)	0 (56%)

After 24 h, 96% of the zygotes had about two labelled nucleoids. In the reciprocal cross, untreated mt^+ × BUdR-treated mt^-, the number of paternal nucleoids was already lower (3.9) within 30 min after mating and decreased rapidly with time. After 5 h, no nucleoids were visible in 96% of the cells. Comparison of the results obtained for two crosses suggests that the one or two large nucleoids present in the zygotes 24 h after mating most often originate from the mt^+ parent.

The same analysis was performed in the cross *mat*-3 mt^+ × wild-type mt^-. As mentioned in the Introduction, the *mat*-3 mutation linked to the mt^+ allele allows the transmission of the paternal chloroplast genes at relatively high frequencies.

In contrast to the control wild-type crosses, the number of nucleoids visualized in the *mat*-3 heterozygotes after DAPI staining did not change during the first 5 h following copulation (Table 4). This number represents the sum of nucleoids present in both gametes (*mat*-3 mt^+: 5.6±1.4; wild-type mt^-: 5.2±1.3). After 24 h, the cells contained one or two large and bright nucleoids.

Staining with the antibody coupled to FITC showed that the nucleoids of both parental origins were conserved at least for the first 5 h of zygote formation. After

24 h, 88% of the zygotes had chloroplast DNA of maternal origin and 44% had chloroplast DNA of paternal origin.

Discussion

We have developed an immunocytochemical method allowing in situ visualization of the chloroplast DNA of *Chlamydomonas* after incorporation of BUdR. The incorporation was sufficient to detect all nucleoids only when the cells were simultaneously treated with FUdR. In the chloroplast of *Chlamydomonas,* deoxythymidine monophosphate (dTMP) is probably synthesized following two routes: a de novo synthesis by conversion of deoxyuridine monophosphate into dTMP through the action of thymidylate synthetase and a salvage pathway in which thymidine kinase transforms endogenous or exogenous thymidine into dTMP (or BUdR into dBUMP) (Swinton and Hanawalt 1972). In bacteria, the de novo synthesis is blocked by FUdR which, after phosphorylation by the thymidine kinase, binds covalently to thymidylate synthetase (Santi and Mc Henry 1972; Santi et al. 1974). In *Chlamydomonas,* the reduction in chloroplast DNA content induced by FUdR, and its reversal by thymidine, probably result from a similar block in the de novo pathway (Wurtz et al. 1977). Our results show that the effect of FUdR is also reversed by BUdR. The improved incorporation of BUdR in the chloroplast nucleoids is easily explained by a block of the de novo synthesis by FUdR.

The detection of BUdR-labelled DNA with monoclonal antibody has been previously demonstrated for mammalian cells (Dombrowicz et al. 1988) which contain large amounts of DNA. We have applied this approach to visualize single nucleoids in the chloroplast of *Chlamydomonas*. Assuming that each nucleoid contains an average of ten copies of 190 kb DNA molecules (see Introduction), i.e., about 2×10^{-3} pg DNA, the method apparently allows detection of DNA in amounts some 3000 times lower than that found in a human diploid cell.

The BUdR labelling technique was used to analyze the fate of chloroplast DNA of maternal and paternal origin in zygotes. Crosses between mt^+ and mt^- wild-type strains were analyzed first. By DAPI staining, we confirmed the previous observations of Birky et al. (1984): before mating, the gametes of both mating-types contain similar numbers of nucleoids, usually five (which represent about half the number found in vegetative cells); after mating, the reduction of nucleoid number begins rapidly and in zygotes aged for 5 h, the number of nucleoids is about half that found in the very young zygotes.

With the FITC-conjugated antibody, we found that the nucleoids of mt^+ origin are maintained whereas those of mt^- origin are eliminated within the first hours following the mixing of gametes. This fully confirms the data of Kuroiwa et al. (1982) and Tsubo and Matsuda (1984) who observed the elimination of mt^- nucleoids before the fusion of the two chloroplasts in the zygotes. The presence of the bromouracil abnormal base in the chloroplast DNA does not prevent the elimination process from occurring. Our results, moreover, demonstrate that the elimination of mt^- nucleoids occurs in most, but not all, zygotes since 5 and 24 h after mating, 2–4% of zygotes contain paternal chloroplast DNA in amounts sufficient to be visualized by the antibody. This observation agrees with the genetical data which show that exceptional zygotes transmitting the paternal chloroplast markers to their progeny represent 1–10% of the total population (Gillham 1978).

The labelling technique was then applied to zygotes obtained from the cross between *mat*-3 mt^+ and wild-type mt^- strains. As mentioned in the Introduction, the *mat*-3 mutation modifies the pattern of chloroplast gene inheritance by increasing the percentage of zygotes transmitting the chloroplast marker from the mt^- parent. Our results demonstrate that this high proportion of exceptional zygotes results from the almost complete absence of specific elimination of mt^- chloroplast DNA. Five hours after mixing the gametes, the zygotes contain nucleoids of both mt^+ and mt^- origin.

After 24 h, the interpretation of labelling becomes difficult since fusion of nucleoids probably occurs between 5 and 24 h. The 24 h-zygotes possessing mt^+ chloroplast DNA represent 88% (Table 4). These zygotes contain either mt^+ DNA copies only (uniparental maternal zygotes) or both mt^+ and mt^- DNA copies (biparental zygotes). Thus, the remainder (12%) should correspond to uniparental paternal zygotes in which the maternal DNA has been eliminated (or is present in too low an amount to be detected). Similarly, the uniparental maternal zygotes represent 56% and the biparental zygotes (100 − 12 − 56 =) 32%. Interestingly, the relative frequencies of these three classes are similar to the proportions of zygotes transmitting chloroplast genetic markers from one, from the other, or from both parents (Gillham et al. 1987). Since, after 24 h, the zygotes possessing mt^+ chloroplast DNA (88%) are twice as frequent as those containing mt^- DNA (44%), this would mean that the specific elimination of paternal DNA during the first hours following mating is not completely abolished. Between 5 and 24 h, multiple rounds of random processes (such as random replication of chloroplast genomes, random degradation or random pairing and conversion: see for example, Matagne and Beckers 1983; Matagne and Mathieu 1983) applied to mt^+ and mt^- DNA copies present in different proportions result in zygotes more frequently containing maternal than paternal chloroplast DNA.

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