# INDUCTION AND SEGREGATION OF CHLOROPLAST MUTATIONS IN VEGETATIVE CELL CULTURES OF CHLAMYDOMONAS REINHARDTII[1]

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ABSTRACT

The single chloroplast of the alga *Chlamydomonas reinhardtii* contains at least 100 copies of the chloroplast chromosome. It is not known how the chloroplast (or cell) becomes homoplasmic for a mutation that arises in one of these copies. Under suitable selection conditions, clones with chloroplast mutations for streptomycin resistance induced by methyl methanesulfonate can be recovered with direct plating after mutagenesis. Using an adaptation of the LURIA-DELBRÜCK fluctuation test, mutagenized cultures grown on nonselective liquid medium for seven to nine doublings show negligible proliferation of cells capable of forming such mutant colonies. In contrast, cells among the same cultures with reduced nuclear mutations conferring streptomycin resistance reveal considerable clonal propagation prior to plating on selection medium. Reconstruction growth-rate experiments show no reduced growth of cells with chloroplast mutations relative to either wild-type cells or to those with nuclear mutations. We propose that newly arising chloroplast mutations and their copies are usually transmitted to only one daughter cell for several cell generations by reductional divisions of the chloroplast genome. In the absence of recombination and mixing, such a reductional partition of chloroplast alleles would readily permit the formation of homoplasmic lines without the need for selection.

ALL eukaryotic cells so far examined have multiple copies of their chloroplast and/or mitochondrial chromosomes (reviewed by GILLHAM 1978). Despite this fact, cell lines homoplasmic for a chloroplast or mitochondrial mutation can appear among the mitotic descendants of at least some cells that initially contained single mutations in only one of these copies (reviewed by BIRKY 1978; GILLHAM 1978; KIRK and TILNEY-BASSETT 1978).

An important step toward understanding how homoplasmic lines originate is to establish the pattern(s) by which an induced mutant allele is transmitted to daughter cells during vegetative division. If there is good reason to believe that the mutant allele, or the cells containing it, are not at a significant replicative disadvantage under the conditions of growth employed, we can ask if this allele initially segregates in a random or nonrandom fashion. With random segrega-

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tion, copies of the mutant allele would sometimes go to the same daughter cell and sometimes to both daughter cells; with nonrandom segregation, one or the other pattern would prevail for the first several divisions following induction of the mutation.

The haploid unicellular alga *Chlamydomonas reinhardtii* is well suited for investigating the induction and vegetative transmisson of chloroplast mutations. It possesses only a single chloroplast (SAGER and PALADE 1954), so that complications associated with multiple organelles can be avoided. Also, non-Mendelian mutations for streptomycin resistance in this alga provide a convenient chloroplast genetic marker. All such mutations, so far mapped, are localized in one of four loci on a single non-Mendelian linkage group (HARRIS *et al.* 1977), and there is substantial evidence that this linkage group resides in the chloroplast (see review by GILLHAM 1978). Chloroplast mutations for streptomycin resistance in this alga confer moderate to high-level resistance to streptomycin on solid medium and are thus phenotypically distinct from Mendelian (nuclear) streptomycin-resistant mutants, which confer only low-level resistance to this antibiotic. The correlation between high-level resistance (at least 500 μg/ml streptomycin sulfate) and inheritance pattern was originally described by SAGER (1954) and has since been confirmed with numerous streptomycin-resistant mutations that have either arisen spontaneously (GILLHAM and LEVINE 1962; SAGER and TSUBO 1961) or were induced with the alkylating mutagens nitrosoguanidine (MNNG) (GILLHAM 1965; LEE and JONES 1973) or methyl methanesulfonate (MMS) (HAWKS and LEE 1976). In addition, certain chloroplast streptomycin-resistant mutants have been recovered after MNNG mutagenesis that are reported to be sensitive to high levels of antibiotic (LEE *et al.* 1973). However, using screening conditions similar to those employed in this study, we have reexamined one of these mutants (*sm-3a*, now termed *sr-u-sm-3a*, HARRIS *et al.* 1977) and we routinely detect slight but positive growth on 500 μg/ml of antibiotic, whereas nuclear streptomycin-resistant mutants on the same plates always score as sensitive (LEE and SAPP 1978). In the present study, the mutations resistant to low and high levels of streptomycin are designated *str-50* and *str-500*, respectively.

Chloroplast mutants for streptomycin resistance induced with MNNG or MMS can be recovered on low levels of streptomycin (50 to 100 μg/ml) with plating directly after mutagenesis, but these mutants can not normally be recovered on the concentration of streptomycin (500 μg/ml) to which they are ultimately resistant (GILLHAM 1969; LEE, unpublished). The apparent linear relationship between MNNG dose and the recovery of both nuclear and chloroplast mutants on low levels of antibiotic suggests that one mutational event is sufficient for their detection under such selection conditions (LEE 1971).

In this study, we followed the transmission of chloroplast (*str-500*), and nuclear (*str-50*)-coded, streptomycin-resistant mutations during the first several cell divisions following their induction with MMS. Mutagenesis was performed on wild-type haploid cells harvested from cultures synchronized by growth under alternating 12-hr light, 12-hr dark periods. At the cell-cycle stage

employed (near the onset of light), cells had just completed division and contain about 100 copies of the chloroplast chromosome per cell. This estimate is based on the average cellular content of chloroplast DNA in such cells being about $2.2 \times 10^{-14}$g (WHITEWAY and LEE 1977; TURMEL, LEMIEUX and LEE, in preparation) and on the molecular weight of individual chloroplast DNA molecules being about $1.3 \times 10^8$ daltons (BEHN and HERRMANN 1977; HOWELL, HEIZMANN and GELVIN 1977; ROCHAIX 1977).

The transmission of chloroplast and nuclear mutations during vegetative cell division was followed by an adaptation of the classical fluctuation test (LURIA and DELBRÜCK 1943). Starting cell concentrations and culture numbers were employed to insure that several cultures received one cell with an induced chloroplast or nuclear streptomycin-resistance mutation, but that no cultures were likely to receive more than one. The results show little evidence for the proliferation of cells, preceding action of the antibiotic, that are capable of forming chloroplast mutant colonies on low-level streptomycin agar medium. In contrast, within many of the same culture tubes, there was a marked proliferation of cells capable of forming nuclear mutant colonies. We explain the nonproliferation of cells with chloroplast mutations by a model in which these mutations and their copies are usually transmitted to the same chloroplast (and daughter cell) for the first several divisions following their induction.

Some of these results have been described in a preliminary report (HAUGHN and LEE 1978).

## MATERIALS AND METHODS

*Strain:* The wild-type (*137c*, *mt*+) strain of *C. reinhardtii* employed in this study was obtained from N. W. GILLHAM and J. E. BOYNTON of Duke University.

*Media and culture:* The inorganic macronutrient components of the growth media were based on the high salt (HS) or 3/10 HS media of SUEOKA, CHIANG and KATES (1967) or on the minimal medium (M) of LEVINE and EBERSOLD (1958). Trace metal and chelated iron stocks were prepared in accordance with JONES (1962), and 1 ml of each stock was added per liter of culture medium. HS or M media supplemented with hydrated sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) at 2 g/l are designated HSA and MA, respectively. Solid media contained 15 g/l of Difco Bacto agar. Illumination was provided by "cool white" fluorescent bulbs, and all cultures were maintained under asceptic conditions at 25°.

Liquid cultures in 3/10 HS medium were synchronized by alternating 12-hr light, 12-hr dark periods as previously described (LEE and JONES 1973). The culture density and light intensity were adjusted so that cell number increased about 4-fold every 24 hr.

*Mutagenesis:* Cells from synchronized cultures were harvested for mutagenesis just before the onset of the light period when the liberation of daughter cells was completed and the cell number was $10^6$ to $2 \times 10^6$/ml. Mutagenesis with MMS (Aldrich Chemical Company, 97% minimum purity) was performed as previously described (HAWKS and LEE 1976). Cells were suspended in 0.03M, pH 7.0, phosphate buffer to a density of $10^7$ cells/ml. MMS was added to make a concentration of 24 mM, and the suspension was placed at a 25° shaking water bath, in darkness, for 30 min. Cells were harvested and washed with phosphate buffer by filtration (LEE and JONES 1973) before and after mutagenesis and resuspended in HS medium for plating.

*Assay and characterization of streptomycin-resistant mutants:* The incidence of streptomycin-resistant mutants was determined by a modification of the embedded method employed by LEE and JONES (1973). Aliquots (up to 1.0 ml) containing $10^6$ to $4 \times 10^6$ cells were delivered to 20-mm diameter test tubes containing 15 ml of 45°-molten HSA agar medium, and the well-

mixed contents of each tube were poured into 100 × 15 plastic disposable Petri dishes. Upon solidification, a second 15 ml layer of melted agar medium without cells was added as an overlay. After 15 to 20 hr of illumination (500 ft-c), 0.2 ml of Millipore sterilized steptomycin sulfate (SCHWARTZ-MANN, 740 units/mg) in 0.01 M phophate buffer and at a concentration of 15 mg/ml was uniformly spread over the surface of each plate (final concentration, 100 μg/ml). Resistant clones were scored after 15 days of additional incubation under 500 ft-c. With further incubation, there was no increase in the recovery of mutants. Streptomycin-resistant mutants were individually transferred by sterile toothpicks onto HSA agar plates and, after 1 week of culture under 500 ft-c, were again transferred in the same manner to MA agar plates containing 50 to 500 μg of streptomycin per ml. After 2 weeks under 200 ft-c, those inocula that grew and remained green only on the lower concentration of streptomycin were termed *str-50*, while those that grew and remained green on both concentrations were termed *str-500*. The Mendelian- and non-Mendelian-inherited streptomycin-resistant mutants described in Table 3 of LEE and SAPP (1978) were routinely monitored as controls.

*Fluctuation experiments:* Control and mutagenized cell populations were suspended in HSA liquid medium to a cell density of about $10^4$ cells per ml. One ml aliquots from each population were distributed into 13 × 100 mm closure-capped test tubes and placed under 1,500 ft-c until the cell number reached $10^6$ to $4 \times 10^6$ cells per tube. The contents of each tube were then plated by embedding in HSA agar medium, and streptomycin was added after 15 to 20 hr, as described above. In the same way, the incidence of streptomycin-resistant mutants was assayed in the control and MMS-treated populations prior to dilution and growth in the culture tubes.

*Reconstruction experiments:* Five *str-50* and 5 *str-500* mutants recovered from 10 independent cultures following MMS mutagenesis were used. Equivalent cell numbers from each of the *str-50* mutant lines were pooled, and the pooled suspension was mixed with wild-type cells in HSA medium so that about 10% of the cells were resistant. *Str-500* mutants were mixed with wild-type cells in a similar manner. Both cultures were then diluted in HSA medium to about $10^4$ cells/ml and 10 aliquots of 1.0 ml from each culture were distributed to 13 × 100 mm test tubes. The tubes were incubated under conditions employed in the fluctuation experiments until the cell number reached about $2 \times 10^6$ cells/ml. With the streptomycin overlay method, the proportion of resistant mutants was determined prior to and subsequent to this period of growth. In this instance, however, the addition of streptomycin was delayed until 24 hr after plating so that both sensitive and resistant colonies could be scored from the same plates.

## RESULTS

*Survival and recovery of streptomycin-resistant mutants after MMS treatments:* Cells from three mutagenesis experiments were employed in the fluctuation studies described below. Directly after MMS treatments and prior to growth in the culture tubes, cells were plated for assays of percentage survival and the incidence of streptomycin resistant mutants. Survival in the first two experiments, which employed 24 mM MMS, was greater than 50%; whereas, in the third experiment, which employed 96 mM MMS, it was about 6% (Table 1). After all control treatments, survival was at least 80%. Higher frequencies of both mutant types, relative to control values, were recovered immediately following MMS treatment (Table 1). In experiment 3, a technical failure prevented the initial assay of streptomycin-resistant mutants.

*Fluctuation analysis of streptomycin-resistant mutants:* The fluctuation analysis of *str-50* and *str-500* mutants among cultures from experiments 1, 2 and 3 are presented in Table 2. Cell density in the culture tubes increased about 150- to 300-fold (about seven to eight doublings) in experiments 1 and 2 and 650-fold

TABLE 1

*Recovery of* str-50 *and* str-500 *mutants and survival following control and MMS treatments*

| Experiment | MMS concentration | % Survival Control | % Survival MMS |
|---|---|---|---|
| 1 | 24 mM | 90.2 ± 6.0 | 58.3 ± 3.3 |
| 2 | 24 mM | 83.5 ± 4.0 | 71.8 ± 7.6 |
| 3 | 96 mM | 81.4 ± 4.2 | 6.2 ± 1.0 |
| Experiment | Mutant phenotype | Incidence per $10^6$ (control) | Viable cells plated (MMS) |
| 1 | *str-50* | 1.3 ± 1.0 | 31.2 ± 4.9 |
| | *str-500* | 1.1 ± 0.9 | 21.2 ± 3.0 |
| 2 | *str-50* | 11.7 ± 2.7 | 19.2 ± 6.9 |
| | *str-500* | 0.0 | 8.4 ± 1.1 |

Values represent the mean and standard error.

(about nine doublings) in experiment 3. After this period of growth, the frequency of *str-50* mutants per tube showed considerable fluctuation. This is evident both by direct inspection of the data, which reveal several cultures yielding very high numbers of *str-50* mutants, and by the extremely high interculture variance of these mutants within each experiment. The frequency of *str-500* mutants showed little evidence of fluctuation from culture to culture; in all experiments, the variance in the recovery of these mutants was close to the mean and hence differed from the *str-50* mutants. Only tubes revealing 11 and seven *str-500* mutants among control and MMS-treated cultures of experiment 1 represent significant ($p < 10^{-3}$) departures from Poisson expectations. Resistance-level tests were performed on all mutant colonies from cultures revealing up to 20 mutants, and on 20 mutants from each culture yielding more than this number. In all but one of the 23 cultures with more than 20 total mutants, no more than two, and in the majority of instances, no *str-500* mutants were detected among the 20. One culture from the MMS series of experiment 2 yielding 46 total mutants may have contained a non-Poisson but low number of *str-500* mutants; six of the 20 mutants characterized were of the *str-500* phenotype. For purposes of data tabulation in Table 2, we assumed that all mutants from these 23 cultures were of the *str-50* type. Certainly, the small number of *str-500* mutants that contribute to these values will have no important influence on the conclusions drawn. Without characterizing every mutant from these cultures, however, we were unable to obtain estimates of *str-500* mutant numbers suitable for Table 2. Hence, *str-500* mutant numbers are not reported for the 23 cultures showing large numbers of *str-50* mutants. Nevertheless, these numbers were small and did not differ significantly from those recovered from the 149 cultures showing small numbers of *str-50* mutants.

The absence of tubes showing large numbers of *str-500* mutations in these experiments may have resulted from no cultures receiving a cell with an *str-500* mutation prior to liquid growth. This possibility cannot be examined for the control cultures because the initial assay of these mutants does not discriminate

## TABLE 2

*Fluctuation analysis of* str-50 *and* str-500 *mutants*

| | | 1-control | 2-control | 1-MMS | 2-MMS | 3-MMS |
|---|---|---|---|---|---|---|
| Volume of culture | | 1.0 ml | 1.0 ml | 1.0 ml | 1.0 ml | 1.0 ml |
| Volume plated | | 1.0 ml | 1.0 ml | 1.0 ml | 1.0 ml | 1.0 ml |
| Viable cells per ml at start | | $9.0 \times 10^3$ | $8.4 \times 10^3$ | $5.7 \times 10^3$ | $7.2 \times 10^3$ | $2.3 \times 10^3$ |
| Viable cells per ml at plating | | $2.5 \times 10^6$ | $1.6 \times 10^6$ | $1.8 \times 10^6$ | $1.2 \times 10^6$ | $1.5 \times 10^6$ |
| Relative increase in viable cell number | | 278x | 190x | 315x | 167x | 650x |
| str-50 | Mutants obtained per culture | | Number of cultures | | | |
| | 0 | 11 | 1 | 8 | 0 | 43 |
| | 1 | 8 | 3 | 6 | 1 | 9 |
| | 2 | 2 | 3 | 2 | 5 | 0 |
| | 3 | 0 | 2 | 4 | 3 | 0 |
| | 4 | 0 | 2 | 1 | 2 | 1 |
| | 5 | 0 | 4 | 1 | 6 | 0 |
| | 6 | 0 | 5 | 0 | 3 | 0 |
| | 7 | 0 | 2 | 0 | 1 | 0 |
| | 8 | 0 | 1 | 1 | 1 | 1 |
| | 9 | 0 | 0 | 0 | 1 | 0 |
| | 10 | 0 | 0 | 0 | 2 | 0 |
| | 11 | 0 | 0 | 0 | 1 | 0 |
| | 12 | 0 | 1 | 0 | 0 | 0 |
| | 13 | 0 | 1 | 0 | 0 | 0 |
| | 26 | 0 | 0 | 0 | 1 | 0 |
| | 29 | 0 | 0 | 1 | 0 | 0 |
| | 33 | 0 | 0 | 1 | 0 | 0 |
| | 38 | 0 | 0 | 0 | 2 | 0 |
| | 45 | 0 | 0 | 0 | 0 | 1 |
| | 46 | 0 | 0 | 0 | 2 | 0 |
| | 52 | 0 | 0 | 1 | 0 | 0 |
| | 60 | 0 | 0 | 0 | 0 | 1 |
| | 83 | 0 | 0 | 1 | 0 | 0 |
| | 93 | 0 | 0 | 0 | 0 | 1 |
| | 98 | 0 | 0 | 1 | 0 | 0 |
| | 112 | 0 | 0 | 1 | 1 | 0 |
| | 114 | 0 | 0 | 0 | 0 | 1 |
| | 121 | 0 | 0 | 1 | 0 | 0 |
| | 122 | 0 | 0 | 1 | 0 | 0 |
| | 186 | 1 | 0 | 0 | 0 | 0 |
| | 212 | 0 | 1 | 0 | 0 | 0 |
| | 216 | 0 | 0 | 0 | 1 | 0 |
| | 247 | 0 | 0 | 0 | 1 | 0 |
| | 763 | 0 | 0 | 0 | 0 | 1 |
| Total number of cultures | | 22 | 26 | 31 | 34 | 59 |
| Mean number of mutants obtained per culture | | 9.0 | 12.8 | 21.3 | 26.4 | 18.6 |
| Variance | | 1,492 | 1,597 | 1,465 | 3,093 | 9,986 |
| str-500 | Mutants obtained per culture | | Number of cultures | | | |
| | 0 | 10 | 0 | 7 | 1 | 40 |
| | 1 | 6 | 3 | 9 | 1 | 12 |
| | 2 | 3 | 4 | 4 | 4 | 2 |
| | 3 | 0 | 3 | 2 | 0 | 0 |
| | 4 | 1 | 3 | 0 | 8 | 0 |
| | 5 | 0 | 2 | 0 | 4 | 0 |
| | 6 | 0 | 6 | 0 | 3 | 0 |
| | 7 | 0 | 2 | 1 | 4 | 0 |
| | 8 | 0 | 1 | 0 | 1 | 0 |
| | 9 | 0 | 1 | 0 | 0 | 0 |
| | 11 | 1 | 0 | 0 | 0 | 0 |
| Total number of cultures | | 21 | 25 | 23 | 26 | 54 |
| Mean number of mutants obtained per culture | | 1.30 | 4.36 | 1.30 | 4.42 | 0.30 |
| Variance | | 5.73 | 5.11 | 2.30 | 4.01 | 0.29 |

between those preexisting in the culture and those which arose after plating and selection. Attention is therefore focused on the MMS-treated cultures where the initial level of induced *str-500* mutants, after correction for the recovery of mutants in the controls, permits an estimate of the minimum number of cultures expected to contain an *str-500* mutation prior to growth in the culture tubes. For comparison, this analysis was also performed for *str-50* mutants. The calculations in Table 3 predict that seven to eight of the MMS-treated cultures, from experiments 1 and 2 combined, should have received an MMS-induced *str-50* mutation prior to growth and therefore should have shown non-Poisson numbers of these mutations after growth. In fact, 11 of 65 cultures in these two experiments yielded more than 40 *str-50* mutants, although in many of these tubes the numbers of mutants were significantly smaller than predicted on the basis of the relative increases in viable cell number. Among the 48 control cultures, 46 revealed 13 or fewer *str-50* mutants, and the remaining two revealed mutant numbers similar to the relative increases in viable cell number. From similar calculations, 5.6 of the 65 MMS-treated cultures, from these two experiments, on average, should have received a cell with an *str-500* mutation prior to growth. However, none of the 49 cultures for which precise *str-500* numbers are available yielded more than eight *str-500* mutants, thus indicating a maximum three-fold proliferation of cells capable of forming such resistant colonies. Also, as discussed above, there was no evidence for large numbers of these mutants among the remaining 16 cultures showing large numbers of *str-50* mutants. It is unlikely that all 65 MMS-treated cultures from experiments 1 and 2 initially contained no induced *str-500* mutations by chance alone ($\rho < 0.003$).

*Reconstruction growth-rate experiments:* One possible explanation for the absence of fluctuation in the recovery of *str-500* mutations is that cells receiving such mutations grow more slowly than both wild-type cells and those receiving *str-50* mutations. Since it is not technically possible to test this hypothesis rigor-

TABLE 3

*Number of MMS-treated cultures expected to contain one cell with an* str-50 *or* str-500 *mutation prior to the start of growth in liquid*

| | Incidence of induced mutants per $10^6$ viable cells* | Number of viable cells delivered to all fluctuation tubes combined | Expected number of mutants delivered to all fluctuation tubes combined |
|---|---|---|---|
| *str-50* | | | |
| Experiment 1 | 29.9 ± 5.0 | $1.77 \times 10^5$ | 5.29 |
| Experiment 2 | 7.5 ± 7.4 | $2.45 \times 10^5$ | 1.84 |
| | | | 7.13 |
| *str-500* | | | |
| Experiment 1 | 20.1 ± 3.1 | $1.77 \times 10^5$ | 3.56 |
| Experiment 2 | 8.4 ± 1.1 | $2.45 \times 10^5$ | 2.06 |
| | | | 5.62 |

* Corrected for control values.
Calculations are based on data from Tables 1 and 2.

TABLE 4

*Growth of* str-50 *and* str-500 *mutants in competition with wild-type cells**

| | At start | | At finish | | |
|---|---|---|---|---|---|
| | Number of viable cells | % Mutant | Number of viable cells | % Mutant | Relative increase viable cell number |
| *str-50* | $1.0 \times 10^4$ | $8.6 \pm 0.2$ | $1.9 \times 10^6$ | $10.1 \pm 1.5$ | 190× |
| *str-500* | $9.6 \times 10^4$ | $12.8 \pm 0.4$ | $2.0 \times 10^6$ | $18.5 \pm 2.4$ | 208× |

* *Str-50* and *str-500* populations were each derived from five independent mutants (see METHODS).

ously, we isolated fully expressed mutants of both types and measured their growth against wild-type cells under conditions similar to those employed in the fluctuation experiments. Cultures derived from five *str-50* and five *str-500* mutants (see METHODS) were mixed with wild-type cells, and the percent of each phenotypic class was determined before and after a period of growth equivalent to that provided in the fluctuation experiments. The results in Table 4 show no significant change in the proportion of either *str-50* or *str-500* mutants with respect to wild-type during growth. Therefore, neither mutant class shows a slower growth rate than wild-type in these competition experiments.

## DISCUSSION

Using an adaptation of the LURIA-DELBRÜCK fluctuation test, we were unable to find convincing evidence for the clonal propagation of cells with chloroplast streptomycin-resistant mutations (*str-500*). Control and MMS-treated cultures were grown for seven to nine doublings in the absence of streptomycin before they were plated for the assay of resistant mutants. Although a few significant departures from Poisson expectation were observed, these fluctuations were only one- or two-fold higher than the normal background level of these mutants. In contrast, the recovery of nuclear streptomycin-resistant mutants (*str-50*) from the same cultures showed striking departures from Poisson expectation.

In many cases, cultures previously treated with MMS showed frequencies of nuclear mutants two- or four-fold less than predicted by the relative increases in viable cell number, after allowing one generation for the segregation of a mutational heteroduplex. However, in a few tubes with the largest numbers of nuclear mutants, the number of mutants was approximately that predicted from the relative increases in viable cell number and, hence, probably resulted from the proliferation of single preexisting spontaneous mutants introduced into these tubes prior to growth. Among the control cultures, an equivalent frequency of tubes yielded numbers of mutants comparable to the relative increases in viable cell number. However, no cultures showed the two- to four-fold reduction in mutant frequency discussed above for cultures previously treated with MMS. Reconstruction growth-rate experiments failed to show reduced growth, relative to wild-type cells, by the nuclear mutants recovered after MMS mutagenesis.

Such mutants may therefore grow more slowly shortly after induction but, with time, approach wild-type growth rates.

How can we account for the absence of fluctuation in the frequency of chloroplast mutations to streptomycin resistance? It is likely that some fluctuation tubes initially inoculated with MMS-treated cultures received cells with such mutations prior to growth. The initial induced level of both nuclear and chloroplast mutants was comparable, and several tubes showed fluctuation in the number of nuclear mutations. Furthermore, calculations based on both the initial incidence of cells with chloroplast mutations and the total number of viable cells distributed to the fluctuation tubes make it highly unlikely that chance prevented all tubes from receiving a cell with a chloroplast resistance mutation.

As with nuclear mutants, chloroplast streptomycin-resistant mutants failed to show any growth disadvantage in competition with an excess of wild-type cells. Similar experiments by GILLHAM and LEVINE (1962) involving three different spontaneous chloroplast streptomycin-resistant mutants and mutant/wild-type starting ratios that more closely mimicked those in the fluctuation experiments also revealed no consistent growth-rate differences between mutant and wild-type cells. Both sets of experiments, however, fail to eliminate the possibility that cells grow very slowly immediately after the occurrence of a chloroplast mutation, but later exhibit shorter doubling times. Evidence for such a growth lag in many, if not all, induced nuclear mutants was discussed above. Nevertheless, there is no compelling reason to suspect that such reduced growth would be more severe for cells with a chloroplast mutation than for cells with a nuclear mutation.

It is unlikely that a streptomycin-sensitive repair system removed chloroplast mutations preferentially from cells grown in streptomycin-free liquid medium. The cultures plated immediately after mutagenesis grew for about 24 hr (about three doublings) prior to streptomycin exposure, so that they too should have had ample opportunity for their mutations to be removed.

We have reason to think that cell lines with only one or a few mutant chloroplast alleles per cell were routinely detected despite an excess of wild-type alleles since, using the same selection procedure, induced chloroplast mutants were readily recovered with plating directly after mutagenesis. We can not eliminate the possibility, however, that only a small fraction of the total cells with one or a few mutant chloroplast genomes actually give rise to mutant colonies. If so, the possible early clonal transmission of these mutations could go undetected and hence explain the lack of fluctuations in the recovery of chloroplast mutants.

We suggest that the absence of fluctuations in the frequency of chloroplast mutants in these experiments results from the manner in which chloroplast chromosomes are partitioned during vegetative cell division. As shown in Figure 1, we propose that their segregation is usually nonrandom and reductional (using the terminology of BIRKY 1978) so that copies of the mutant allele tend to be transmitted to the same chloroplast (and daughter cell) for several cell generations. This mode of chromosome partition is identical to that proposed for the segregation of mutant nuclei in multinucleate bacteria (RYAN and WAINWRIGHT

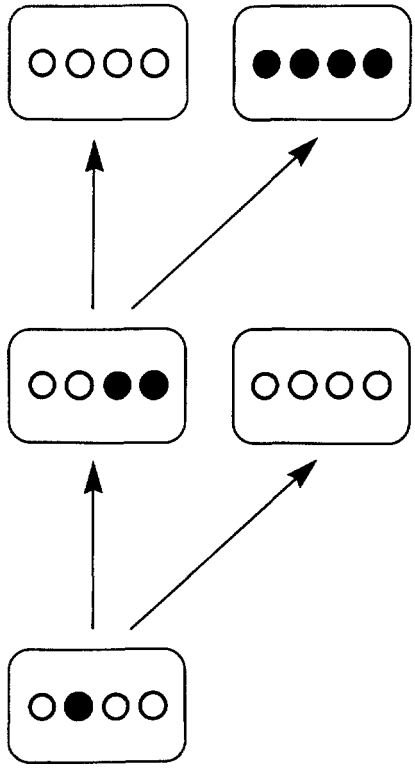

FIGURE 1.—Proposed segregation of chloroplast mutations during vegetative cell division of *C. reinhardtii*. Wild-type chromosomes are represented by white circles and mutant chromosomes by black circles. The single chloroplast of *C. reinhardtii* has 60 to 100 chromosomes, so that 6 to 7 divisions would be required before the segregation of a cell homoplasmic for a chloroplast mutation.

1954; RYAN, FRIED and SCHWARTZ 1954). With such a model, segregation of the mutant allele to more than one daughter cell would usually be delayed until cells become homoplasmic, or nearly so, for this mutation. If all mutant and wild-type alleles replicate once with each chloroplast (or cell) doubling and if the partition of chloroplast chromosomes is numerically equal, then the length of this delay in cell doublings ($N$) would be related to the average number of chromosomes per chloroplast ($C$) according to the equation:

$$2^N = C, \text{ or } N = \log C/\log 2.$$

If one assumes that the average chloroplast chromosome multiplicity of *C. reinhardtii* under the conditions employed (about 100 copies) is representative of individual cells, then this hypothesis predicts a lag of seven culture doublings plus one doubling for the segregation of a mutational heteroduplex before a single mutation would proliferate among daughter cells.

The predictions of the model as proposed might not change appreciably even if chloroplast chromosomes replicate randomly, as recently shown for the mito-

chondrial chromosomes in cultured mouse cells (BOGENHAGEN and CLAYTON 1977). A lag in mutant proliferation would still be expected; however, there would be greater variance in the duration of this lag. We expect that this increased variance would not be dramatic in a genome with many segregating units (see review by BIRKY 1978). In this connection, one study on the replication of *C. reinhardtii* chloroplast DNA with nitrogen transfer techniques (CHIANG and SUEOKA 1967) gave results that are clearly incompatible with such random replication. Other workers, however, have been unable to confirm these results (LEE and JONES 1973). A numerically unequal partition of chloroplast chromosomes could also increase the variance in the duration of the segregation lag predicted by the proposed model. Our data do not support this possibility since some tubes would have been expected to show increased numbers of chloroplast mutants.

Departures from a reductional segregation of chloroplast genes, as proposed, would increase both the number of cells containing at least one mutant genome and the average number of generations needed for cells to become homozygous. If only one mutant chloroplast genome is usually sufficient for the detection of a chloroplast streptomycin-resistant colony, then frequent disruptions of this kind would predict fluctuations in the recovery of these mutants that, again, we failed to observe. For the same reasons, a random segregation or nonrandom equational segregation of chloroplast alleles would predict an even greater fluctuation of chloroplast mutants.

Biparental zygotes of *C. reinhardtii* (i.e., those that transmit chloroplast genes from both parents) have been utilized to study the origin of cells homoplasmic for one allele or the other during meiotic germination and during subsequent mitotic divisions. These studies have led to several models on the mechanism of chloroplast gene segregation in this alga. Two of these models (SAGER and RAMANIS 1968; GILLHAM, BOYNTON and LEE 1974; see further discussion by SAGER 1977 and GILLHAM 1978) share the proposal that chloroplast gene copies frequently segregate in an equational fashion; hence, for the reasons discussed above, they are not supported by our results. With these models, the origin of homoplasmic segregants requires recombination events that disrupt the equational segregation. The model of SAGER and RAMANIS, moreover, proposes that the chloroplast genome is functionally diploid. In the case of such a small number of copies, no matter what the mode of segregation, one would predict that chloroplast mutant numbers should show clonal growth in fluctuation experiments. A third model of chloroplast gene segregation in this alga proposes that cells homoplasmic for one or the other parental allele arise by the random drift of gene frequencies through repeated rounds of genome pairing and gene conversion (BIRKY 1978, and in preparation). This model may be consistent with our results if it includes the reservation that chloroplast mutations not lost by gene conversion usually segregate in a reductional fashion. Without such random gene drift, the reductional segregation scheme predicts a lag of about eight culture doublings before the clonal growth of chloroplast mutants; with random gene drift plus reductional segregation, some cell lineages should show clonal

growth sooner and others later. These predictions may be sufficiently different to be tested in fluctuation experiments.

Only the recent model of VANWINKLE-SWIFT (1978, 1980) proposes that chloroplast alleles from sexually generated heteroplasmic cells of *C. reinhardtii* segregate by a largely nonrandom, reductional scheme; hence, it is directly supported by our results. In this model, the units of segregation in heteroplasmic zygotes and their heteroplasmic progeny are clusters of chloroplast DNA molecules (nucleoids). With few exceptions, these nucleoids are individually homoplasmic for genomes derived from one parent or the other. Segregation of cells homoplasmic for one type of nucleoid is thought to be aided by an incomplete mixing of nucleoids in the zygote and by a tendency for daughter nucleoids to remain in the same daughter chloroplast because of their proposed spacial proximity. We would suggest that initially the units of segregation in a heteroplasmic cell generated by mutation are individual chloroplast DNA molecules and that mutant daughter molecules tend to remain in one daughter nucleoid until a homoplasmic nucleoid is generated. Thereafter, the reductional segregation of nucleoids could typically occur if daughter nucleoids tend to remain in the same daughter chloroplast. Thus, the overall pattern of mutant gene transmission would usually be nonrandom and reductional. Frequent disruptions of this reductional segregation at the level of single genes or nucleoids would predict important proliferations of chloroplast mutants in fluctuation experiments. Once again, we failed to observe this.

In previous investigations on the origin of chloroplast streptomycin-resistant mutants in nonmutagenized cultures of *C. reinhardtii*, SAGER (1962) used both the respreading technique (NEWCOMBE 1949) and the fluctuation test. She found no evidence for the clonal propagation of these mutants before plating on antibiotic medium. GILLHAM and LEVINE (1962) also using nonmutagenized cultures and the fluctuation test found similar results, although some minor departures from Poisson expectation were reported. SAGER suggested that the absence of chloroplast mutant fluctuations was best explained by the hypothesis that these mutations were induced by streptomycin and did not precede action of the antibiotic. GILLHAM and LEVINE considered this hypothesis as well, but they also considered another, that of intracellular selection. In its original form, this hypothesis proposed that each cell contains a number of particles capable of mutation from sensitivity to resistance, and in the absence of antibiotic there is strong selection against the resistant particle so that it would not be passed on clonally. After plating on streptomycin medium, this hypothesis proposed that the direction of selection is reversed to favor the replication and clonal transmission of the resistant particle. As discussed recently by GILLHAM (1978), the intracellular selection hypothesis now seems unlikely in view of our current knowledge that the mutated particle is almost certainly chloroplast DNA and that mutations for resistance confer resistance to chloroplast ribosomes. The induction hypothesis now seems unnecessary since the MMS-induced chloroplast mutations examined in this study fail to be transmitted clonally prior to plating on selection medium.

Recent studies on the origin of chloroplast spectinomycin-resistant mutants in *C. reinhardtii*, using fluctuation experiments, also found no evidence for the clonal propagation of cells, preceding action of the antibiotic, that could form resistant colonies (WURTZ *et al.* 1979). In these experiments, the medium in the fluctuation tubes contained the thymidine analog, 5-fluorodeoxyuridine (FdUrd). It was shown in the same study that FdUrd was an effective mutagen of chloroplast genes, but almost exclusively on cells entering the stationary phase of growth. Hence, as the authors point out, the distribution of these mutants would be expected to be nonclonal since most of the mutations would have arisen in cells that had no further opportunity to divide before plating.

In only one study, other than that reported here, was there good evidence that cells with chloroplast mutations were introduced into the fluctuation tubes prior to growth (GILLHAM 1965). In this study, MNNG-treated cells, previously taken from logarithmic growth, were distributed to fluctuation tubes and cultured for three doublings in one experiment and for six to seven doublings in another. In the latter experiment, where convincing fluctuations were detected, four of 23 culture tubes yielded about 50 to 100 chloroplast streptomycin-resistant mutants (after correcting for fractional platings). If these fluctuations resulted from the clonal growth of single mutant cells, then such cells transmitted the mutation to most or all daughter cells in each of the first six to seven culture doublings.

It is unlikely that the different results in the experiments of GILLHAM and those reported here are a function of the different mutagens employed. Studies with MNNG-induced chloroplast mutations to streptomycin resistance by LEE (1971) failed to detect evidence for the clonal propagation of these mutants during the first few divisions following mutagenesis, while nuclear streptomycin-resistant mutants in the same cultures showed the expected proliferation. The results of GILLHAM might be reconciled with those of LEE (1971) and those reported here if more chloroplast mutants were induced in the experiment of GILLHAM and if a minority of these mutants typically show clonal propagation. Such an hypothesis is testable since it predicts that, after MNNG or MMS mutagenesis, tubes showing fluctuations in chloroplast mutants should appear, but at frequencies significantly lower than those of tubes showing nuclear mutant fluctuations.

Studies on the origin of mitochondrial mutations in yeast have involved techniques similar to those employed for studies on chloroplast mutations in *C. reinhardtii*. BIRKY (1973), using nonmutagenized cultures together with respreading techniques, found little evidence for the clonal propagation of cells capable of forming mitochondrial-based, erythromycin- or chloramphenicol-resistant colonies preceding action of the antibiotic. This author favored an intracellular selection hypothesis similar to that of GILLHAM and LEVINE (1962), as discussed earlier. DUJON *et al.* (1976), using the fluctuation test and nonmutagenized cultures under nonselective conditions, found evidence for only minor clonal proliferation of cells that were capable of forming the same mutant colonies. These authors also favored the importance of intracellular selection in the origin of fully resistant cells on antibiotic medium; however, they suggested that the

limited variation in the recovery of cells with these mutations might be associated with the need for a critical accumulation of the resistance mutations. In the absence of selection, this buildup might occur only rarely even without evoking strong selection against mitochondrial harboring such mutations. Finally, PUTRAMENT *et al.* (1976) found that cells capable of forming manganese-induced, mitochondrial-coded erythromycin-resistant colonies increased only 1.5- to 2.2-fold under nonselective conditions, while the entire cell population increased five- to six-fold. These results were again discussed in terms of intracellular selection.

Although intracellular selection of resistant mitochondria may well be involved with the formation of yeast cell lines homoplasmic for mitochondrial mutations, as shown directly in Paramecium (ADOUTTE and BEISSON 1972), we suggest that the limited proliferation or lag in proliferation of yeast cells with newly arisen mitochondrial mutations may result at least in part from the nonrandom segregation of mitochondrial mutations, as suggested here for the chloroplast mutations of *C. reinhardtii*. Mitochondrial DNA molecules in yeast cells are typically clustered into aggregates termed chondriolites (WILLIAMSON *et al.* 1977). Although the size of chondriolites is subject to variation, it has been estimated that log-phase cells typically contain four to five mitochondrial DNA molecules per chondriolite. It is possible that copies of a single mutation arising in one chondriolite and not lost by gene conversion (see BIRKY 1978)) may tend to segregate to the same daughter chondriolite, hence to the same daughter mitochondrion for the first few cell divisions following the origin of the mutation. In the absence of extensive cytoplasmic mixing, daughter chondriolites might also tend to segregate together, although recent experiments on the vegetative segregation of mitochondrial genetic markers from heteroplasmic zygotes are consistent with the random segregation of genetic units that are likely to be chondriolites (BIRKY *et al.* 1978).

The segregation lag hypothesis, as proposed here for the chloroplast mutations of *C. reinhardtii*, predicts that the proliferation of cells capable of forming chloroplast mutant colonies on streptomycin medium should begin after about eight culture doublings. To detect or rule out such a proliferation above the typical background level of these mutants that appear on the selection plates, we suggest that four additional culture doublings are needed. Also, in order to detect a possible minority of chloroplast mutants that might show earlier clonal growth, more initial mutants should be employed than was the case in this study. Such experiments will require significantly more platings than used here; for this and other technical reasons, they are impractical at this time.

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## LITERATURE CITED

ADOUTTE, A. and J. BEISSON, 1972 Evolution of mixed populations of genetically different mitochondria in *Paramecium aurelia*. Nature **235**: 393–396.

BEHN, W. and R. G. HERRMANN, 1977 Circular molecules of the β-satellite DNA of *Chlamydomonas reinhardii*. Molec. Gen. Genet. **157**: 25–30.

BIRKY, C. W., JR., 1973 On the origin of mitochondrial mutants: Evidence for intracellular selection of mitochondria in the origin of antibiotic-resistant cells in yeast. Genetics **74**: 421–432. ——, 1978 Transmission genetics in mitochondria and chloroplasts, Ann Rev. Genet. **12**: 471–512.

BIRKY, W. C. JR., R. L. STRAUSBERG, P. PERLMAN and J. L. FORSTER, 1978 Vegetative segregation of mitochondria in yeast: estimating parameters using a random model. Molec. Gen. Genet. **158**: 251–261.

BOGENHAGEN, D. and D. A. CLAYTON, 1977 Mouse L cell mitochondrial DNA molecules are selected randomly for replication throughout the cell cycle. Cell **11**: 719–727.

CHIANG, K. S. and N. SUEOKA, 1967 Replication of chlorplast DNA in *Chlamydomonas reinhardi* during vegetative cell cycle: its mode and regulation. Proc. Natl. Acad. Sci. U.S. **57**: 1506–1513.

DUJON, B., M. BOLOTIN-FUKUHARA, D. COEN, J. DEUTSCH, P. NETTER, P. P. SLONIMSKI and L. WEILL, 1976 XI. Mutations at the mitochondrial locus ω affecting the recombination of mitochondrial genes in *Saccharomyces cerevisiae*. Molec. Gen. Genet. **143**: 131–165.

GILLHAM, N. W., 1965 Induction of chromosomal and nonchromosomal mutations in *Chlamydomonas reinhardi* with N-methyl-N′-nitro-N-nitrosoguanidine. Genetics **52**: 529–537. ——, 1969 Uniparental inheritance in *Chlamydomonas*. Am. Naturalist **103**: 355–388. ——, 1978 *Organelle Heredity*, Raven Press, New York.

GILLHAM, N. W., J. E. BOYNTON and R. W. LEE, 1974 Segregation and recombination of non-Mendelian genes in Chlamydomonas. XIII. International Congress of Genetics Symposia. Genetics **78**: 439–457.

GILLHAM, N. W. and R. P. LEVINE, 1962 Studies on the origin of streptomycin resistant mutants in *Chlamydomonas reinhardi*. Genetics **47**: 1463–1474.

HARRIS, E. H., J. E. BOYNTON, N. W. GILLHAM, C. L. TINGLE and S. B. FOX, 1977 Mapping of chloroplast genes involved in chloroplast ribosome biogenesis in *Chlamydomonas reinhardtii*. Molec. Gen. Genet. **155**: 249–265.

HAUGHN, G. W. and R. W. LEE, 1978 Genetic evidence for multiple copies of the chloroplast genome in *Chlamydomonas reinhardtii*. Can. J. Genet. Cytol. **20**: 445 (abstract).

HAWKS, B. G. and R. W. LEE, 1976 Methyl methanesulfonate mutagenesis of synchronized *Chlamydomonas*. Mutation Res **73**: 221–228.

HOWELL, S. H., P. HEIZMANN and S. GELVIN 1977 Properties of the mRNA and localization of the gene coding for the large subunit of ribulose bisphosphate carboxylase in *Chlamydomonas reinhardi*. pp. 1–24. In: *Acides nucléiques et synthèse des protéines chez les végétaux*. Edited by L. BOGORAD and J. H. WEIL. Centre National de la Recherche Scientifique, Paris.

JONES, R. F., 1962 Extracellular mucilage of the red alga *Porphyridium cruentum*. J. Cell. Physiol. **60**: 61–64.

KIRK, J. T. O. and R. A. E. TILNEY-BASSETT, 1978 *The Plastids: Their chemistry, structure, growth and inheritance*, 2nd edition. Elsevier/North-Holland Biochemical Press, Amsterdam.

LEE, R. W., 1971 Studies on nitrosoguanidine mutagenesis in *Chlamydomonas reinhardtii*. Ph.D. Thesis, State Univ. of New York at Stony Brook.

LEE, R. W., N. W. GILLHAM, K. P. VAN WINKLE and J. E. BOYNTON, 1973 Molec. Gen. Genet. **121**: 109–116.

LEE, R. W. and R. F. JONES, 1973 Induction of Mendelian and non-Mendelian streptomycin resistant mutants during the synchronous cell cycle of *Chlamydomonas reinhardtii*, Molec. Gen. Genet. **121**: 99–108.

LEE, R. W. and J. A. SAPP, 1978 Nuclear mutation increases streptomycin and spectinomycin sensitivity in Chlamydomonas. Genetics **88**: 643–650.

LEVINE, R. P. and W. T. EBERSOLD, 1958 The relation of calcium and magnesium to crossing over in *Chlamydomonas reinhardi*. Z. Vererbungsl. **89**: 631–635.

LURIA, S. E. and DELBRÜCK, M., 1943 Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**: 491–511.

NEWCOMBE, H. B., 1949 Origin of bacterial variants. Nature **164**: 150–151.

PUTRAMENT, A., R. POLAKOWSKA, H. BARANOWSKA and A. EJCHART, 1976 On homozygotization of mitochondrial mutations in *Saccharomyces cerevisiae*. pp. 415–517. In: *Genetics and biogenesis of chloroplasts and mitochondria*. Edited by TH. BÜCHER *et al.* North Holland, Amsterdam.

ROCHAIX, J. D., 1977 Restriction enzyme analysis of the chloroplast DNA of *Chlamydomonas reinhardii* and construction of chloroplast DNA-plasmid hybrids. pp. 77–83. In: *Acides nucléiques et synthèse des protéines chez les végétaux*. Edited by BOGORAD and J. H. WEIL. Centre National de la Recherche Scientifique, Paris.

RYAN, F. J., P. FRIED and M. SCHWARTZ, 1954 Nuclear segregation and the growth of clones of bacterial mutants induced by ultraviolet light. J. Gen. Microbiol. **11**: 380–393.

RYAN, F. J. and L. K. WAINWRIGHT, 1954 Nuclear segregation and the growth of clones of spontaneous mutants of bacteria. J. Gen. Microbiol. **11**: 364–379.

SAGER, R., 1954 Mendelian and non-Mendelian inheritance of streptomycin resistance in *Chlamydomonas reinhardi*. Proc. Natl. Acad. Sci. U.S. **40**: 456–363. ——, 1962 Streptomycin as a mutagen for nonchromosomal genes. Proc. Nat. Acad. Sci. U.S. **48**: 2018–2026. ——, 1977 Genetic analysis of chloroplast DNA in *Chlamydomonas*. Advan. Genet. **19**: 287–340.

SAGER, R. and G. E. PALADE, 1954 Chloroplast structure in green and yellow strains of *Chlamydomas*. Exp. Cell. Res. **7**: 584–588.

SAGER, R. and Z. RAMANIS, 1968 The pattern of segregation of cytoplasmic genes in *Chlamydomonas*. Proc. Natl. Acad. Sci. U.S. **61**: 324–331.

SAGER, R. and Y. TSUBO, 1961 Genetic analysis of streptomycin resistance and dependence in *Chlamydomonas*. Z. Verbungsl. **92**: 430–438.

SUEOKA, N., K. S. CHIANG and J. R. KATES, 1967 Deoxyribonucleic acid replication in meiosis of *Chlamydomonas reinhardi*. I. Isotopic transfer experiments with a strain with eight zoospores. J. Molec. Biol. **25**: 47–66.

VANWINKLE-SWIFT, K. P., 1978 A model for the vegetative segregation of multiple copies of chloroplast genes in *Chlamydomonas*. Genetics **88**: s103. ——, 1980 A model for the rapid segregation of multiple chloroplast genomes in *Chlamydomonas*: assumptions and predictions of the model. Current Genetics 1 (in press).

WHITEWAY, M. S. and R. W. LEE, 1977 Chloroplast DNA content increases with nuclear ploidy in *Chlamydomonas*. Molec. Gen. Genet. **157**: 11–15.

WILLIAMSON, D. H., L. H. JOHNSTON, K. M. V. RICHMOND and J. C. GAME, 1977 Mitochondrial DNA and the heritable unit of the yeast mitochondrial genome: a review. pp. 1–24. In: *Mitochondria 1977, Genetics and biogenesis of Mitochondria*. Edited by W. BANDLOW *et al.* Walter de Gruyter, Berlin.

WURTZ, E. A., B. B. SEARS, D. K. RABERT, H. S. SHEPHERD, N. W. GILLHAM and J. E. BOYNTON, 1979 A specific increase in chloroplast gene mutations following growth of *Chlamydomonas* in 5-fluorodeoxyuridine. Molec. Gen. Genet. **17**: 235–242.

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