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Genetics

Selection for conditional gametogenesis in *Chlamydomonas reinhardi*

(mutant/temperature sensitivity)

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ABSTRACT **A new technique is described for selection of temperature-sensitive mutants affecting gametogenesis in *Chlamydomonas reinhardi*. The first mutant found by this technique is characterized. Cells exhibiting the *gam*-1 phenotype are capable of sexual agglutination, but cannot form zygotes at the restrictive temperature. The mutation, however, has been expressed only in gametes of mating type (−). Cells of mating type (+) which carry this gene are able to engage in normal zygote formation. Temperature shift experiments and antibiotic studies have indicated that this gene is expressed within 6 hr after the onset of gametogenesis in liquid culture, and its product has a lifetime of about 4 hr at 35°.**

Gametogenesis in *Chlamydomonas reinhardi* can be considered a simple developmental change; a vegetative cell, which is unable to mate, is converted to a gamete, which can mate but is unable to grow vegetatively. This change occurs within several hours after vegetative cells are placed in nitrogen-free medium (1), and can be reversed by returning the cells to nitrogen-supplemented growth medium. The cells can be grown axenically in a defined medium. The many chromosomal markers and cytoplasmic mutants available make genetic analysis feasible.

Chlamydomonas reinhardi is heterothallic and isogamous, with mating type controlled by a single nuclear gene. Conditions necessary for efficient gametogenesis were first defined by Sager and Granick (1). Electron micrographic studies have given some indication of the morphological changes involved in gametogenesis and cell fusion (2). These changes include loss of the cell wall followed by flagellar agglutination. The cells of opposite mating types then form a fertilization tubule which shortens until the cells finally fuse laterally. The chemistry of flagellar agglutination is presently being studied (3), mainly with *Chlamydomonas moewusii* rather than with *C. reinhardi*, and it has been suggested that a glucosyl transferase ectoenzyme system is involved in this phenomenon.

Kates (4) has shown that actinomycin D inhibits gamete formation in synchronous liquid cultures, an observation we have confirmed for asynchronous liquid cultures and for cells from agar plates (unpublished data). Jones (5) found an increase in proteolytic activity during gamete formation. Degradation of ribosomes during gametogenesis was shown by Siersma and Chiang (6). Much of the rRNA degraded during this time was then incorporated into DNA.

The results of such studies suggest that gametogenesis in *Chlamydomonas reinhardi* would be an excellent system for the genetic and biochemical analysis of a simple developmental change in a eukaryote. Several classes of gametogenic mutants have previously been reported and described (7–9; and U. W. Goodenough, C. Hwang, and H. Martin, manuscript submitted). However, in order to facilitate genetic analyses, we felt it was necessary to isolate conditional mutants. Using such mutants, the inability to mate could be analyzed at the restrictive temperature, while mating could proceed at the permissive temperature.

This report is concerned with the development of a promising selective procedure for isolation of mutants of *C. reinhardi* which display temperature-sensitive gametogenesis, and with studies of the first such mutant obtained.

Abbreviations: *mt*, the mating type locus; MAM, minimal acetate magnesium medium; SM, streptomycin minimal medium; PD, parental ditype; NPD, non-parental ditype; T, tetratype.

MATERIALS AND METHODS

The mating type(+) (mt^+) parent used in these studies was the wild-type strain, 137c, of *Chlamydomonas reinhardi*. The mt^- parent used, strain NG *sr*2.3.3, which was derived from 137c, was provided by Dr. N. W. Gillham (mt^+ and mt^- represent opposite mating types).

Cells were maintained on solid and liquid media, at 25° or 35°, under constant illumination from daylight fluorescent bulbs, at a light intensity of approximately 500 footcandles (5.4 klx).

Cells were grown in liquid culture in minimal acetate magnesium medium (MAM), which is Surzycki's MM (10) medium [a modification of Sueoka's HSM (11)], plus 0.2% sodium acetate. Cultures were generally maintained on YAc plates, containing MAM without additional magnesium (MA), supplemented with 4 g/liter of yeast extract, and 1.5% agar. Streptomycin minimal medium (SM), used for selection, is MM, again without the additional magnesium, supplemented with 500 μg/ml of streptomycin sulfate. The streptomycin, sterilized by filtration, was added to the medium which had been cooled to 50° or less, just before the plates were poured.

For the temperature gradient studies, a special apparatus was constructed using an aluminum block (70 cm long, and 10 cm × 10 cm in cross section). Channels were drilled at each end of the block and connected to two constant temperature water circulators to produce a thermal gradient. Two rows of 15 holes (1.9 cm in diameter, 8.9 cm deep, and 1.9 cm apart) were drilled along the length of the block, and vertical segments representing ⅓ of the circumference of each hole were exposed to the outside through side openings. Both sides of the block were then covered with plexiglass plates so that the test tubes, containing cell suspensions, could be illuminated, as well as thermally insulated from outside temperature fluctuation, when placed inside the holes. It was found that except for a slight deviation seen in the test tubes at either end, the thermal gradient was nearly

Table 1. Total number of viable zygotes produced in a temperature gradient ($\times 10^{-2}$)

Cross	Temperature						
	27.4°	28.9°	29.2°	29.5°	29.8°	30.4°	31.2°
1. *gam*-1 mt^- × + mt^+	1700 (2200)*	2100	6800	540	320	2.4	1.0 (1000)*
2. *sr2* mt^- × + mt^+	2000 (2800)*			1500			440 (1500)*

To initiate gametogenesis, *gam*-1 (mt^-) and *sr2* (mt^-) cells were each suspended in sterilized tap water. 1.0 ml was then pipetted into each test tube in the gradient, except for the test tubes at 27.4° and 31.2°, where 1.1 ml was added. Wild-type (mt^+) cells were suspended in sterilized tap water, in a 50 ml flask, and were allowed to undergo gametogenesis at 35° overnight. The following morning, 0.1 ml (for dilutions) was taken from each of the four tubes in the gradient containing 1.1 ml to determine the number of cells present prior to mating. These values ($\times 10^{-2}$) are indicated in the table in parentheses. Dilutions were also made from the mt^+ culture, and 9.5×10^5 cells per ml were found to have been present prior to mating. Each mating was then initiated, by adding 1.0 ml of mt^+ gametes to the 1.0 ml of mt^- cells in the test tubes. After 2 hr of mating, samples were removed for zygote counts.
* The number of mt^- cells present prior to mating.

linear. Illumination was provided by two light banks, each holding two cool white fluorescent lamps, with one on either side of the gradient apparatus. The temperature in each of the test tubes in the gradient was read both before and after each experiment.

RESULTS

Induction and selection of mutants

The selection procedure described here takes advantage of the *sr2* mutation, a uniparentally inherited gene for resistance to 500 μg/ml of streptomycin (12).

When wild-type (mt^+) and *sr2* (mt^-) gametes are mated, all of the zygotes show the mt^+ characteristic, sensitivity to streptomycin, within a short time after gamete fusion (13). Exposure to 500 μg/ml of streptomycin kills all unmated wild-type (mt^+) cells and most zygotes. Only those *sr2* cells which have not mated, or cannot mate, survive.

In order to induce mutants, *sr2* (mt^-) cells, grown on YAc medium, were suspended in 30 ml of sterilized tap water. After passage through a Gelman polypropylene filter (10 μm pore size) to eliminate cell clumps, the cells were irradiated with two 15 W Champion G15T8 germicidal ultraviolet lamps for 20 sec at 10 cm to obtain 90–95% killing. Aliquots of 10 ml were added to flasks containing 10 ml of MAM, made up to twice normal concentration, and were maintained in the dark for 16 hr to prevent light-activated repair (14). The cells were then poured into an empty clear flask and illuminated.

The cells were centrifuged in late log phase, washed, and resuspended in twice the original volume of sterilized tap water. Ten milliliters of resuspended cells from each flask were poured into a 50 ml flask, and the flasks were placed in a 35° water-bath shaker overnight to induce gametogenesis. In the morning, the *sr2* (mt^-) cells were mated with an excess of mt^+ cells. After several hours, when large zygote clumps could be seen in the cultures, the clumps were filtered out with a 10 μm filter, and the remaining cells were either plated on SM plates (plate selection), or were centrifuged and resuspended in MAM liquid medium plus 200 μg/ml of streptomycin sulfate (liquid selection). *sr2* (mt^-) cells inoculated into MAM + streptomycin showed little or no growth, but remained viable. However, all wild-type (mt^+) cells, and presumably most of the remaining zygotes, were killed. On day four, the cells were centrifuged, washed, and resuspended in MAM. They were then grown to late log phase, where the selection process could be repeated.

Clones picked from cells inoculated onto SM plates were tested for temperature-sensitive gametogenesis. Each clone was first tested for gametogenesis at 35°. Those that did not produce zygotes after addition of wild-type (mt^+) gametes at 35° were then tested for zygote formation at 25°.

Zygote formation was determined in two ways. The simplest was to observe the formation of large zygote mats, which are usually found floating on the surface of the mating test tube after overnight mating. The second method involves microscopic observations at 100× or 430× magnification. The latter procedure is necessary if mating is poor or if the cells tend to form clumps [many *Chlamydomonas* cells tend to aggregate under adverse conditions (15, 16)]. Zygotes can generally be distinguished as large immobile cells in small to large mats, floating on the surface of the water.

During matings, cells were also observed for sexual agglutination. This process, the first visual sign of mating, involves agglutination of the flagella of large numbers of mt^+ and mt^- gametes, often forming large, highly motile clumps (17).

By means of the above procedures, one temperature-sensitive mutant has been isolated to date. The initial characterization of this mutant follows.

Mutant characterization

The *gam*-1 mutation was obtained from a single selection on streptomycin agar. It is capable of sexual agglutination, but cannot form zygotes at the restrictive temperature. The mutant phenotype appears to be expressed only in gametes of mt^-.

Thermal gradient studies have shown repeatedly that cells carrying this mutation form zygotes efficiently at 29.9° (±0.2°) or below, and cannot form zygotes at 30.8° (±0.2°) or above. Between these two temperatures there is some zygote formation but it is slower and less complete than at lower temperatures. In one attempt to quantify these results, it was found that there was a loss of viable zygotes (those zygotes that germinated on plates following a 1 week maturation period in the dark and subsequent killing of all vegetative cells with a 40 sec exposure to chloroform) in excess of 99% when cells were mated at 31.2° as compared with those mated at 27.4° (see Table 1). In this experiment, zygote clumping was avoided by plating all cultures 1 or 2 hr after mating. Clumping of zygotes occurs when plating is 2 or more hr post-mating. For this calculation, the expected number of cells at 31.2° for *gam*-1 was determined by assuming the same percent loss in viable zygotes seen in the control. It was observed that 72% of the control *sr2* (mt^-)

gametes produced viable zygotes at 27.4°, while only 30% of those gametes present at 31.2° were recovered as zygotes. Therefore, the expected frequency of viable zygotes from *gam*-1 gametes mated at 31.2° would be 30/72, or 42% of the number observed at 27.4°.

Several types of experiments have been done to determine when the mutant gene is first expressed. In the first type, the temperature step-up experiment, vegetative cells from a liquid culture of the mutant are resuspended at 25° in nitrogen-free medium. Cells are obtained from liquid medium either by centrifugation, as described previously, or by collection on Millipore filters with a pore size of 1.2 or 3 μm (for experiments requiring small volumes of cells). Cells are then easily scraped off these filters with a spatula. At specific times during gametogenesis, samples are transferred to 35°. Wild-type (mt^+) gametes are added to each of the samples at 35° after a test mixture of the *gam*-1 (mt^-) cells with wild-type (mt^+) gametes shows high efficiency agglutination. This occurs after 11–12.5 hr of exposure to nitrogen-free medium. After allowing sufficient time to achieve complete mating (12–24 hr), cultures were observed for zygotes. It has been found that mutant cells induced at 25° for 5.5 or fewer hours are incapable of zygote formation. A 6 hr exposure to 25° results in some zygote formation, with increasing numbers of zygotes formed as the length of time at 25° increases.

In a second type of experiment, after cells of *gam*-1 were exposed to gametogenic conditions at 35° overnight, a brief period of time at the permissive temperature was followed by a return to 35°. It has been found that a 45 min exposure of cells to 25° is sufficient to permit some zygote formation (40 min at 25° results in no zygotes), and cells held at 25° for 1.75 hr or longer produce large numbers of zygotes. Wild-type (mt^+) gametes were added to all samples in this experiment shortly after they were returned to 35°. This recovery of zygote forming ability occurs with or without the 1.5 μg/ml of cycloheximide, which has been found to inhibit zygote formation when present during gametogenesis. Chloramphenicol (300 μg/ml) does not affect zygote forming ability either during gametogenesis or in this second type of temperature shift experiment. Addition of the wild-type (mt^+) gamete suspension lowers the concentration of each antibiotic by ⅓ (that is, to 1.0 μg/ml of cycloheximide and 200 μg/ml of chloramphenicol). These results imply that translation is not necessary for recovery of zygote forming ability under these conditions.

To determine how long the temperature-sensitive product(s) remains active at 35°, cells maintained overnight at 35° under gametogenic conditions were placed at 25° for 2, 3, or 4 hr, and were then returned to 35°. Zygote forming ability was lost, in all cases, if the cells were kept at 35° for 4 or more hr before addition of wild-type (mt^+) gametes. These results indicate that the temperature-sensitive product(s) of *gam*-1 loses its activity after 4 hr at 35°.

Genetics

Wild-type (mt^+) cells were first crossed with *gam*-1 (mt^-) (see Table 2, line 1 for tetrad data). The resulting tetrads indicated a Mendelian, rather than a non-Mendelian, mode of inheritance. The data do not deviate significantly from the parental ditype:nonparental ditype (PD:NPD) ratio of 1:1 which is expected with no linkage of *gam*-1 and the mating type locus. The tetrads contained either 0, 1, or 2 products showing the *gam*-1 phenotype, and all of these were mt^-.

Table 2. Tetrad results

Cross	Tetrad type PD*	NPD†	T‡
1. + mt^+ × *gam*-1 mt^-	2	2	14
2. *gam*-1§ mt^+ × + mt^-	4	1	8
3. *gam*-1§ mt^+ × *gam*-1 mt^-	3	0	0

* PD: For Cross 1: both mt^- products show the *gam*-1 phenotype. For Cross 2: no *gam*-1 products are seen. Both *gam*-1 genes are inferred to be in cells also carrying mt^+. For Cross 3: both mt^- products show the *gam*-1 phenotype. Both mt^+ products are also inferred to be carrying the *gam*-1 gene.

† NPD: For Cross 1: no *gam*-1 products are seen. Both *gam*-1 genes are inferred to be in cells also carrying mt^+. For Cross 2: both mt^- products show the *gam*-1 phenotype.

‡ Tetratype (T): These clones were classified on the basis of 3:1 segregation of wild-type:mutant phenotype (all such mutants were mt^-).

§ A mt^+ carrier of the unexpressed *gam*-1 gene.

Therefore, although this mutation segregated independently of the mating type locus, it appeared to be expressed only in gametes of mt^-.

In accordance with this hypothesis was the recovery of mutant individuals in a cross of a known wild-type (mt^-) by a clone of pseudo-wild-type cells of mt^+, into which *gam*-1 had been introduced from the cross of *gam*-1 mt^- × mt^+ (Table 2, line 2). Furthermore, crosses of a mutant (*gam*-1 mt^-) by a carrier (*gam*-1 mt^+) produced tetrads in which all the mt^- cells were mutant, and all the mt^+ cells appeared normal (Table 2, line 3). The progeny of two different tetratype clones were used to obtain the data for Table 2, lines 2 and 3.

DISCUSSION

These results, and those of Goodenough (7–9) suggest that gametogenesis in *Chlamydomonas reinhardi* may be a useful system for studying a simple, eukaryotic, developmental event. We feel that conditional mutations are of value for such studies. Several hundred clones were tested and a large number of mutants were found which could not form zygotes at any temperature, but these were not studied. This report is concerned exclusively with the isolation of clones which display temperature-sensitive gametogenesis.

We have developed a method for the selection of mutants which display temperature-sensitive gametogenesis, and have isolated and studied one such mutant. Several other temperature sensitive isolates appear to produce a thick clear coat around the cells at 35°, preventing agglutination, and, therefore, preventing mating. These clones are not of further interest for this study.

The temperature-sensitive gametogenic mutant, *gam*-1, has some very interesting properties. It appears to be expressed only in gametes of mt^-, and to be unlinked to the mating type locus. This suggests that it may be involved in a structure or function unique to the (−) mating type. Since it can be carried, but not expressed, by the (+) mating type, we believe that *gam*-1 may be an example of a sex-limited trait in *Chlamydomonas*.

Temperature shift experiments show that the temperature-sensitive event occurs before 6 hr after the onset of gametogenesis in cells which were grown in liquid culture. This may represent the presence of a temperature-sensitive

product of the *gam*-1 gene which is synthesized before 6 hr into gametogenesis. If *gam*-1 cells are exposed to 25° long enough to produce the temperature-sensitive product(s), it appears to have a lifetime of about 4 hr at 35°.

Cytoplasmic translation appears to be necessary for zygote formation but not for recovery of zygote forming ability in *gam*-1 cells kept at gametogenic conditions overnight at 35°. A possible explanation for these results is that an inactive protein produced during gametogenesis at 35° is capable of becoming active at 25° due to a configurational change, which can occur as early as 45 min after a shift to the permissive temperature.

Further study of this mutant, as well as isolation of additional temperature-sensitive gametogenic mutants, are necessary. The results reported here, however, suggest that gametogenesis in *Chlamydomonas reinhardi* could provide a useful model system for a detailed genetic and biochemical study of a simple eukaryotic developmental change.

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1. Sager, R. & Granick, S. (1954) *J. Gen. Physiol.* **37**, 729–742.
2. Friedmann, I., Colwin, A. L. & Colwin, L. H. (1968) *J. Cell Sci.* **3**, 115–128.
3. McLean, R. J. & Bosmann, H. B. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 310–313.
4. Kates, J. R. (1965) Ph.D. Dissertation, Princeton University, Princeton, N.J.
5. Jones, R. F. (1970) *Ann. N.Y. Acad. Sci.* **175**, 648–659.
6. Siersma, P. W. & Chiang, K. -S. (1971) *J. Mol. Biol.* **58**, 167–185.
7. Goodenough, U. W. (1974) *J. Cell Biol.* **63**, 116a.
8. Bergman, K., Goodenough, U. W., Goodenough, D. A., Jawitz, J. & Martin, H. (1975) *J. Cell Biol.*, in press.
9. Goodenough, U. W. (1975) *J. Cell Biol.*, in press.
10. Surzycki, S. (1971) in *Methods in Enzymology*, ed. San Pietro, A. (Academic Press, Inc., New York), Vol. XXIII, pp. 67–73.
11. Sueoka, N. (1960) *Proc. Nat. Acad. Sci. USA* **46**, 83–91.
12. Sager, R. (1954) *Proc. Nat. Acad. Sci. USA* **40**, 356–363.
13. Sager, R. & Tsubo, Y. (1961) *Z. Verebungsl.* **92**, 430–438.
14. Gillham, N. W. & Levine, R. P. (1962) *Nature* **194**, 1165–1166.
15. Iwasa, K. & Murakami, S. (1968) *Physiol. Planat.* **21**, 1224–1233.
16. Iwasa, K. & Murakami, S. (1969) *Physiol. Planat.* **22**, 43–50.
17. Wiese, L. (1965) *J. Phycol.* **1**, 46–54.