

# USE OF GENOMIC EXCLUSION TO ISOLATE HEAT-SENSITIVE MUTANTS IN TETRAHYMENA

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## ABSTRACT

We have used the abnormal form of conjugation known as "genomic exclusion" to isolate a collection of heat-sensitive mutants of *Tetrahymena pyriformis*, syngen 1. Growth at room temperature in bacterized medium and no growth at 40°C in the same medium was the criterion used for the isolation. The mutant strains were tested for growth in pure (axenic) culture in proteose peptone medium; of the 31 strains which grew normally at room temperature and not at 40°C in that medium, 21 also failed to grow at 37°C. Preliminary results of complementation tests suggest that most, if not all, the mutations are recessive and that a variety of genes was affected. A detailed genetic analysis was performed on one mutant (H9). The results are all consistent with the idea that the heat-sensitive phenotype of this mutant is determined by a single recessive mutation, designated *ts-2*. Heterozygotes *ts-2/+* yield heat-sensitive segregants during vegetative growth; we interpret this finding as another example of *allelic exclusion*, a phenomenon universally encountered among heterozygotes in syngen 1 of *T. pyriformis*. Our results are discussed in the context of some questions of current interest in Tetrahymena genetics.

THE statistical difficulties in isolating recessive mutants of Tetrahymena have been an important factor preventing the full utilization of this organism as a model system in which to perform "genetic dissection" of various biological processes of general interest in animal cell biology. Some of its great advantages are, on the biochemical side, the ability to grow in pure (axenic) culture to high densities ( $10^6$  cells/ml) with fast doubling time (up to two hours) in essentially unlimited volumes of liquid culture. Advantages on the genetic side include its ability to undergo conjugation under controlled conditions, with normal Mendelian transmission of genetic information, and the facility to construct strains with any combination of available markers.

ALLEN (1967) suggested the use of genomic exclusion to isolate recessive mutants. Genomic exclusion is an abnormal form of conjugation induced by the C\* strain (ALLEN 1967); this strain does not contribute any known genetic information to its sexual progeny. The genetic material of the progeny is instead all derived from the diploidization of the lone surviving product of normal meiosis of the micronucleus of the normal mate of the C\* strain. However a second round of mating is required before the new genotype is expressed, because the progeny of

the first conjugation still retain their old (pre-conjugation) macronucleus.

The object of this study was to implement the use of genomic exclusion to isolate recessive mutants. We chose to look for heat-sensitive mutants because they would be expected to have a high aggregate frequency of occurrence, assuming that (1) every essential gene is potentially capable of mutating to one of probably many different heat-sensitive alleles and (2) there probably are thousands of essential genes in *Tetrahymena*. In this paper we report the isolation of many heat-sensitive mutations which, from preliminary observations, appear to be recessive and to have affected a variety of genes. A detailed study of the genetics of one of the mutants is also reported here.

#### MATERIALS AND METHODS

*Strains:* All the strains used belong to the biological species designated *Tetrahymena pyriformis*, syngen 1. The C\* strain (ALLEN 1967) was obtained from DR. SALLY L. ALLEN. Cells of this strain are unable to contribute genetic material to the next sexual generation; during conjugation, they and their normal mates undergo a type of nuclear reorganization called "genomic exclusion" (ALLEN 1967) (See Figure 2 and DISCUSSION section). Strain D1968-5 (ROBERTS and ORIAS 1973), obtained from DR. DAVID L. NANNEY, is a wild-type strain which belongs to inbred family D. Standard mating type testers of syngen 1 were also obtained from DR. D. L. NANNEY. Strain CA10 is a chloramphenicol-resistant mutant derived in our laboratory from strain D1968-5 after nitrosoguanidine mutagenesis. The mutation is transmitted as a cytoplasmic determinant which is not exchanged during conjugation, and thus behaves identically to other chloramphenicol-resistant mutations similarly obtained (ROBERTS and ORIAS 1973).

*Culture media and methods:* (1) *PPY*: 20 g of proteose peptone Difco, 1 g of Bacto yeast extract (Difco), 1 ml of salts solution, in 1000 ml H<sub>2</sub>O. (2) *Salts solution*: 10 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 g of ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g of FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g of CaCl<sub>2</sub>·2H<sub>2</sub>O, 1000 ml of H<sub>2</sub>O. (3) *PPY + PS*: *PPY* medium supplemented with streptomycin sulfate and Penicillin G, each at a final concentration of 250 µg/ml. (4) *PPY-agar*: *PPY* medium supplemented with 1.5% (w/v) Difco Bacto-agar. (5) *Tryptone-agar*: 10 gm of Difco Bacto-tryptone, 5 gm of NaCl, 15 gm of Bacto-agar in 1000 ml of H<sub>2</sub>O. (6) *1% and 2% BP*: a 1% or 2%, respectively, (v/v) suspension in sterile water, of a stationary phase culture of *Aerobacter aerogenes*; the culture was grown by incubating 50 ml of *PPY* medium in a 250 ml Erlenmeyer flask and aerating by shaking overnight at 37°C. (7) *Dryl's solution* (DRYL 1959); 0.5 gm of NA Citrate, 0.14 gm of NaH<sub>2</sub>PO<sub>4</sub>, 0.14 gm of Na<sub>2</sub>HPO<sub>4</sub> and 0.17 gm of CaCl<sub>2</sub> in 1000 ml of H<sub>2</sub>O; in order to avoid formation of a stubborn precipitate, the CaCl<sub>2</sub> was autoclaved separately from the rest and the two sterile solutions were mixed while still warm.

With few exceptions, the cells were grown axenically in proteose peptone (*PPY*) media. Bacterized peptone (*BP*) media were used only when large numbers of clones were to be individually mated (mating type tests, second-round mating of mutagenized clones undergoing exclusion). Methods for single cell isolation, simultaneous replication of clones to various growth conditions, cell counts and tests for growth in 250 µg/ml of chloramphenicol have been previously described (ROBERTS and ORIAS 1973; see this paper also for more details on some of the methods described here and their rationale).

*Maintenance of stocks:* Stocks are maintained axenically in loosely capped 6" test tubes containing 5 ml of *PPY* medium at 18°C. The stocks were transferred every six months by inoculating about 0.1 ml of the most recent culture into fresh medium. Unfortunately, stocks kept without sexual reorganization for extended periods of time lose their potential to breed sexually (NANNEY and ALLEN, cited in NANNEY 1957). The preferable method of storage under liquid nitrogen (SIMON 1972) is now being implemented.

Stationary phase cultures of frequently used strains were prepared once a week by inoculating 50 ml of *PPY* medium in a 250 ml Erlenmeyer flask with 1 ml of a prior stationary phase

culture. The cultures were kept at room temperature for two to three days and then transferred to 18°C.

*Sterility tests:* Spot tests for bacterial contamination were performed by transferring with a capillary tube a sample of 0.01–0.02 ml of culture to a petri plate containing PPY- or tryptone-agar medium. The plates were incubated at the same temperature as the source culture for two to four days.

#### *Standard Axenic Cross*

All crosses, except those involving the C\* strain, were performed axenically by the following slight modification of the method of ALLEN (1967).

a.) Growth of the culture in PPY medium: Five ml of a stationary culture of each strain were added to each of two separate sterile petri plates containing 5 ml of PPY medium, and were incubated at 30°C for six hours. Under these conditions the population doubles one or two times.

b.) Starvation in Dryl's solution: After incubation, the cells were spun for two minutes (from turn-on to turn-off) at the intermediate setting of an IEC clinical centrifuge (about 600 × g) and were resuspended with an equal volume of sterile Dryl's solution. The procedure was repeated two more times. The final suspension (5–10 mls) of each strain was transferred to separate sterile petri plates and kept overnight at room temperature (about 22°C) for 16–20 hours.

c.) Mixing the cells: Approximately equal number of cells were mixed the next day. Depending on the volumes to be mixed, the mixtures were made in sterile petri plates or microtiter plates (plastic plates containing 96 × 0.2 ml depressions, sterilized by UV irradiation; ROBERTS and ORIAS 1973). Incubation was at 30°C or room temperature. Pairing normally began within one-half to one hour after mixing the cells. If exconjugants were to be separated, the cells were mixed at about 3 p.m., pairs were isolated at about 9 p.m. and exconjugants separated the next morning; otherwise, the mixture was made at about 9 a.m. and pairs were isolated in the afternoon.

d.) Isolation of pairs: Single pairs were isolated in single drops (about 0.03 ml) of 50% PPY + PS medium. The drops are arranged in a 6 × 8 array whose dimensions are designed to match the dimensions of one-half of a microtiter plate (ROBERTS and ORIAS 1973). If exconjugants were to be separated, the pairs were first isolated into columns 1 and 4; the two empty adjacent columns were saved for the transfer of the two exconjugants produced in each drop of each of the original columns. The plates were incubated for at least three days at 30°C after the isolation of exconjugants.

e.) Maturity tests: The clones which had grown in the petri plate drops were replicated to two microtiter plates containing, in each depression, two drops of 2% BP medium. The device used for the replication, a block of wood supporting 48 steel rods, was previously described (ROBERTS and ORIAS 1973). At the same time as the cross progeny were replicated, cultures of two testers of different mating types were fed, by inoculating separate petri plates containing 10 ml of 2% BP medium with 0.1 ml of a stationary culture of each of the tester strains grown in PPY medium. The plates were incubated at room temperature.

Two days later the depressions were checked for the presence or absence of cells, the testers were fed again (by adding an equal volume of 2% BP medium) and two drops of tester suspension were added to each depression containing a clone to be tested. Each of the originally prepared identical plates received one of two different testers. The mixtures were incubated at room temperature and were scored the next day for the presence or absence of pairs. Progeny which were mature were considered non-conjugant (NANNEY 1956; ALLEN 1967) and disregarded in the genetic analysis.

f.) Heat sensitivity tests: These tests were performed in connection with the genetic analysis of mutant H9, to be described later. The three-day-old clones in petri plates obtained in step (d) were tested for heat sensitivity by replication to a microtiter plate containing about 0.2 ml of PPY + PS medium per depression. The plates were incubated at 40°C for three days and were then scored. Depressions which had any healthy cells (elongated, transparent, actively-swimming) were scored as having phenotypically wild-type cells. Depressions containing only dark,

spherical, non-motile cells sitting at the bottom ("marbles") were scored as having cells with the H9 mutant phenotype. This is a very characteristic and easily-recognized phenotype which we have also observed with several heat-sensitive mutants.

g.) Raising progeny to maturity: Serial subcultures of the progeny were made as follows in order to allow enough cell divisions to produce mature cells. The drop of each desired progeny still present in the original petri plate (after the maturity test had been completed) was drawn into a sterile capillary tube (standard freezing-point depression capillary tube with both ends open) and the contents gently blown into a 4" (Wasserman) tube containing 1 ml of PPY medium. Every day, about 0.02 ml (about one-fifth the volume of a capillary tube) was transferred to a new 4" tube containing 1 ml of PPY medium. Twelve serial transfers (and possibly less) are sufficient to yield mature clones.

An alternative method was used successfully to raise selected backcross and  $F_2$  progeny to maturity. Using sterile capillaries, cells of the desired clones were transferred to depressions or a microtiter plate containing about 0.2 ml of PPY medium. After incubation at 30°C for 2 days, the contents of the depressions were replicated to a new plate containing 6 serial replications. This method has the advantage that 48 clones can be subcultured in the same operation and is particularly useful when many clones must be raised to maturity.

Since, in syngen 1 the asexual progeny of an exconjugant regularly may contain subclones of different mating type (NANNEY and CAUGHEY 1953), the mature culture obtained by either of the methods described above must be cloned before it is crossed again. This was accomplished by doing a few single cell isolations into drops of PPY + PS in a sterile plastic petri plate and incubating at 30°C for three days.

Mating type tests were performed by separately crossing samples of the mature clones to cultures of the five possible mating types of inbred family D (NANNEY 1959), using the method described above for maturity tests.

*Crosses to the C\* Strain:* The standard axenic cross procedure was used through the separation of exconjugants. Three days later, two cells from each exconjugant culture were transferred to an adjacent drop of 1% BP, and incubated at room temperature. After these cells gave rise to clones of about 100 cells, they starved and pairs formed (two to three days later); a single pair was then isolated into an adjacent drop of PPY medium, and the plate was incubated at room temperature for four to five days. At that time all the cultures derived from each pair (exconjugants of round I and progeny of round II pairs) were tested for maturity and heat sensitivity.

*Isolation of heat-sensitive mutants:* The following procedure was designed to exploit the phenomenon of genomic exclusion to isolate heat-sensitive mutants. (Its rationale is considered in the DISCUSSION).

a.) Mutagenesis: Cultures of strains C\* and D1968-5 were grown as described in step (a) of the standard axenic cross, with one exception: the growth medium for strain D1968-5 contained N-methyl N'-nitro N-nitrosoguanidine (NG) at a concentration of 10  $\mu\text{g}/\text{ml}$ . The length of NG exposure was six hours. The NG was first dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20  $\mu\text{g}/\text{ml}$ . The NG solutions were freshly made immediately before use each time.

b.) Round I cross and isolation of round I pairs: the cells were starved and crossed according to steps (b) and (c) of the standard axenic cross. Cells were mixed in the morning and pairs were isolated in the afternoon into drops (about 0.03 ml) of 1% BP medium arranged in a regular array. Only drops in alternate columns were used. The plates were incubated at room temperature.

c.) Isolation of round II pairs: Three days later, the progeny of each pair had grown to a small clone (about 100 cells) and starved; most of the drops had conjugating pairs. Drops of 1% BP medium were added to the vacant columns, and one pair was isolated from each drop to its adjacent drop. The plates were incubated at room temperature.

d.) Test of the round II progeny for heat sensitivity: After four days of growth at room temperature, the plates were replicated to petri plates containing drops of 2% BP medium (in the early work) or microtiter plates containing about 0.2 ml of 2% BP medium (in the last experiments). The plates were incubated at 40°C and scored after three days.

e.) Further tests of suspects: Clones which had grown at room temperature but failed to

grow at 40°C were transferred (using a capillary tube) from the petri plate to a 6" test tube containing 5 ml of 2% BP; the tube was incubated at room temperature for two to three days. Suspects were retested by inoculating a 0.1 ml sample from the test-tube culture into two separate 6" test tubes containing 5 ml of 2% BP; one tube was incubated at room temperature and the other at 40°C. Results were scored after two days. A similarly-treated culture of strain D1968-5 was incubated as a control with each batch of tests.

f.) Determination of the mating type of mutants: Six single cell isolations were made from the test-tube culture of each mutant prepared for the previous test; the cells were isolated into a drop of 2% BP. Serial single cell transfers into fresh 2% BP medium were made three times a week, in order to raise the mutant clones to maturity. After the eighth serial culture had grown up, it was transferred with a capillary tube to a 6" test tube containing 5 ml of 2% BP, and incubated at room temperature. Three days later, an equal volume of 2% BP was added to the tube. Mixtures of each clone and each of the mating type testers were separately made by mixing in a depression of microtiter plate three drops of the culture to be tested with three drops of a 50-fold dilution with 2% BP of a stationary PPY culture of the tester. Depressions containing (1) the culture to be tested alone; (2) mixtures of all the mating type testers in all combinations and (3) mating type testers alone were also included as controls. The results were scored the next day and, in some cases, again a day later. Subclones of different mating type were obtained for many of the mutants. One mutant which had a normal growth rate at room temperature remained "immature" (incapable of mating) even after as many as 16 single cell transfers; it has not been studied further.

g.) Establishment of axenic cultures: Six single cell isolations were made from the tube containing the mature cultures into separate drops of PPY + PS medium in a petri plate. After 15 minutes of incubation at room temperature, the cells were recovered with a micropipette and transferred to a fresh drop of PPY + PS medium. This operation was repeated once more. The plate was incubated at room temperature for three days. Cultures which were free of bacterial turbidity were transferred with a capillary tube to a 4" tube containing 2 ml of PPY-PS medium. A sample of the tube contents was tested for sterility on a tryptone plate incubated at room temperature for four days. One PPY axenic culture of each mutant subclone was saved and stored at 18°C in 5 ml of PPY medium in a 6" tube.

h.) Improvements of the method: Our methods for handling *Tetrahymena* cells axenically in routine genetic work have evolved considerably from the time when mutants were first being isolated. Now we would isolate the round II pairs (step d) directly into drops of PPY + PS medium and then (a) perform the growth tests, (b) do further single cell isolation, (c) raise cells to maturity, and (d) test for mating type, all according to the methods described in the previous section (*Standard Axenic Cross*) and/or in a previous manuscript (ROBERTS and ORIAS 1973). *Growth tests of the mutants at various temperatures:* One subclone of each mutant was tested for growth at 40°C, 37°C and room temperature, using the following procedure. The 18°C stock culture was used to inoculate 2 ml of PPY + PS medium in a 6" test tube at an initial concentration of  $10^3$  cells/ml (starter culture). This culture was incubated at room temperature until the next day. The cell concentration of the starter culture was then determined and a dilution made with PPY + PS medium, to give a final concentration of  $5 \times 10^2$  cells/ml. The diluted culture was split into three samples of 2 ml each in separate 4" test tubes, and these were incubated at 40°C, 37°C and room temperature, respectively. Once a day, a 0.1 ml sample of each culture was diluted with 9.9 ml of filtered saline and counted. A culture of strain D1968-5 was included as control with each batch of mutant strains tested. Tests for sterility were performed on the 18°C stock culture, on the starter culture and on the test cultures.

A set of growth tests was performed for mutant H9 at 37°C, taking more frequent samples and inoculating from an exponentially-growing culture. The details of the protocol used are given under Figure 1.

## RESULTS

*Isolation of heat-sensitive mutants:* Using the methods described above, a col-

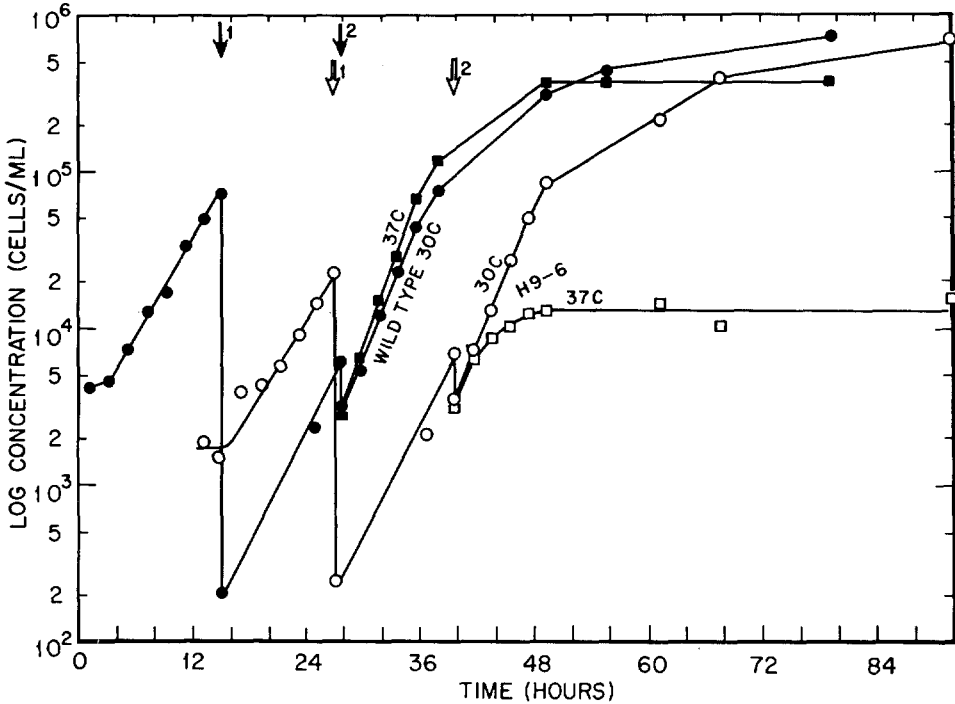


FIGURE 1.—A temperature shift experiment with H9-6 and the wild-type strain. Open figures: strain H9-6; filled figures: strain D1968-5. Both strains were run concurrently, but the results with H9-6 have been plotted with a 12-hour lag merely to facilitate the display. The protocol used for each strain was as follows: A sterile plastic petri plate containing 25 ml of PPY + PS medium (supplemented with 250  $\mu\text{g}/\text{ml}$  each of penicillin and streptomycin sulfate) was inoculated with a 0.25 ml sample of a stationary phase culture grown in the same medium (minus antibiotics). The cultures were grown at 30°C. Twelve hours later a large dilution ( $\geq 100$ -fold) of each culture was made into 25 ml of the same medium (1st arrow in each curve) and incubation was continued at 30°C for another 12 hours. A two-fold dilution of each culture was then made, and each culture was split into two halves (second arrow in each curve); one half continued to be incubated at 30°C (circles) and the second half was shifted to 37°C (squares). The cultures were kept in stationary plastic Petri plates throughout. Counts were made with a Celloscope cell counter (Particle Data, Inc., Elmhurst, Ill.) using a 2 ml metering siphon and with the cells suspended in 0.89% NaCl. A test for bacterial contamination at the end of the experiment was negative.

lection of 31 heat-sensitive mutants was obtained from among 18,000 round I pairs isolated in the course of this study. Subclones of different mating types were obtained for many of the mutants.

*Growth tests at various temperatures:* The 31 mutant strains have been tested for their ability to grow in PPY medium at various temperatures, by the method described in a previous section. All the mutant strains cease growth when an early stationary phase culture grown at 25°C is subcultured at 40°C. At 37°C, 21 of the mutants cease growth while the remaining 10 attain yields comparable to those of the parental wild-type strain.

The growth properties at 30°C and 37°C in PPY medium have been studied more carefully for mutant H9 (Figure 1). The mutant grows with the same doubling time as the wild-type parental strain at 30°C. At 37°C, the mutant population completely stops growing after undergoing 2 doublings at a steadily-decreasing rate.

If H9-6 cells are kept at 40°C for one day, most of them are killed (i.e. they cease to be clone-formers upon shifting the temperature back to room temperature), but a small fraction (less than  $10^{-2}$ ) survives. No cells (less than  $2 \times 10^{-6}$ ) survive exposure to 40°C for three days.

*Genetics of mutant H9:* H9 is the first mutant selected for further study. We sought answers to the following questions. Is the mutant phenotype caused by a single mutation? Is the mutation recessive? Is the mutant phenotype under macronuclear control?

a.) Crosses of H9-6 to a homozygous wild-type strain. The results of two crosses to strain CA10 are shown in Table 1 (crosses #1 and #2). Strain CA10 is resistant to 250 µg/ml of chloramphenicol, but is otherwise wild type. Exconjugants were separated and tested as described under the *Standard axenic cross* procedure.

As shown in Table 1, all the progeny which underwent conjugation were phenotypically wild-type (heat-insensitive). Thus in a "young" heterozygote, the mutant allele is recessive. For 46 pairs, both exconjugant clones were tested for chloramphenicol resistance; in each case one exconjugant clone was sensitive and the other was resistant. These results are consistent with the finding that chloramphenicol resistance is determined by a cytoplasmically-inherited mutation (ROBERTS and ORIAS 1973).

b.) Cross of H9-6 to H9-2: H9-2 and H9-6 are two subclones of mutant H9, derived respectively from two cells isolated from the original round II drop in the mutant isolation procedure. These two strains have been crossed and tested in mass culture containing the progeny of at least 1000 pairs; no heat-insensitive progeny have been obtained.

TABLE 1

*Phenotypic ratios among crosses of H9-6 or its progeny*

Cross No.	Strains	+	Progeny phenotype		
			—	x	NC
1	H9-6 × CA10	21	0	4	5
2	H9-6 × CA10	34	0	10	4
3	NC1-19-28 × H9-6	13	14	12	9
4	NC1-19-68 × H9-6	9	10	17	12
5	NC1-19-28 × CA10	39	0	8	1
6	NC1-19-68 × CA10	34	0	10	4
7	NC1-19-28 × NC1-19-68	13	6	24	5

Key to symbols—+: heat insensitive; —: heat sensitive; X: pair failed to give rise to progeny; NC: non-conjugant (the progeny from these pairs were sexually mature as early as they could be tested). The strains are described in the text.

c.) Cross of H9-6 to the C\* strain: Exconjugants from 24 round I pairs were isolated, of which 4 sets died without giving rise to a culture. In 19 of the remaining sets, one exconjugant was heat-sensitive and of the same mating type as H9-6 and the other was heat-insensitive and of the same mating type as the C\* strain. In the exceptional set, both exconjugants were heat-sensitive, one was immature and the other gave a weak positive test for maturity (few pairs observed). It seems unlikely that the exceptional pair was a round II pair because it was isolated only seven hours after mixing the H9-6 and C\* cultures, and the mixture had been incubated at room temperature. No further work was done with the exceptional pair.

Viable round II progeny were obtained from the crosses of three sets of round I exconjugants. All three cultures were heat-sensitive and immature. These results are consistent with the idea that strain H9-6 is homozygous for a nuclear mutation determining heat sensitivity. Furthermore, the round I exconjugants derived from the C\* strain are inferred to have a homozygous mutant genotype, even though they are phenotypically heat-insensitive. We conclude that the pertinent gene is expressed in the macronucleus.

d.) Backcrosses of F<sub>1</sub> strains to H9-6: Strains NC1-19-28 and NC1-19-68 are two F<sub>1</sub> progeny of a cross of H9-6 and CA10. Each of these progeny was raised to maturity and back-crossed to the H9-6 parent; the results are shown in Table 1 (Crosses #3 and #4). These backcrosses yielded a 1:1 ratio of phenotypically wild-type (heat-insensitive) to mutant (heat-sensitive) progeny. These are the results to be expected if strain H9-6 is homozygous for a single recessive mutation determining heat sensitivity, and the F<sub>1</sub> strains are heterozygous.

e.) Backcrosses of F<sub>1</sub> strains to CA10: Strains NC1-19-28 and NC1-19-68 were crossed to strain CA10. The results are shown in Table 1 (crosses #5 and #6). All the progeny had the wild-type (heat-insensitive) phenotype, as expected from the cross of a heterozygous to the homozygous dominant parent.

f.) F<sub>1</sub> × F<sub>1</sub> cross: Strains NC1-19-28 and NC1-19-68 were crossed to one another (cross #, Table 1). The phenotype ratio obtained (13 wild to 6 mutant) does not differ significantly from the 3:1 ratio expected (14.75 wild to 4.25 mutant) for the cross between two heterozygotes.

g.) Test crosses of F<sub>2</sub> and backcross progeny: Phenotypically wild-type progeny of crosses 3 and 7 (Table 1) were individually test-crossed to strain H9-6 in order to determine the relative frequency of heterozygotes and homozygotes among them. The results are shown in Table 2.

All ten of the phenotypically wild progeny of the H9-6 backcross (F<sub>1</sub> progeny × H9-6) gave results expected of heterozygotes. A grand total of 61 wild to 61 mutant progeny were collectively obtained in these test crosses. The ratio coincides with the expected 1:1 ratio.

Eight phenotypically wild-type F<sub>2</sub> progeny were test-crossed. Of these, six behaved as heterozygotes and two behaved as wild-type homozygous (Table 2). The ratio of six heterozygotes to two homozygotes is not significantly different from the expected 2:1 ratio (5.3 heterozygotes to 2.7 homozygotes). If the results of the test crosses of clones which behaved as heterozygotes are pooled, then 62



TABLE 2

*Phenotypic ratios among test crosses of F<sub>2</sub> and backcross progeny to strain H9-6*

Progeny of cross #3	Viable conjugants			X	NC	Classification
	+	—	U			
31	6	6	0	11	25	het
33	11	5	0	14	18	het
34	4	4	1	3	36	het
36	10	9	1	15	13	het
37	5	2	0	11	30	het
38	2	8	0	12	26	het
42	5	4	11	16	2	het
43	0	1	0	7	4	het
44	10	13	0	6	19	het
45	8	9	1	4	26	het
Progeny of cross #7						
13	10	0	0	14	15	hom
14	12	13	0	16	7	het
15	3	3	3	12	27	het
16	6	5	0	19	18	het
17	18	12	1	8	9	het
18	12	16	0	6	14	het
22	11	12	0	10	15	het
24	16	0	0	20	12	hom

Key to symbols—U: undetermined (viable progeny which were lost before the phenotypic test was complete); hom; homozygote; het: heterozygote, See Table 1 for other symbols and cross numbers.

of their progeny were wild-type and 61 were heat-sensitive, a ratio not significantly different from the 1:1 ratio expected.

Thus all of the results described are in agreement with the simple hypothesis that H9-6 is homozygous for a recessive nuclear mutation which determines its heat sensitivity. The mutant allele is designated *ts-2*; the name *ts-1* is reserved for a heat-sensitive mutation previously isolated by McCoy (1973).

*Stable heat-sensitive segregants of ts-2/+ heterozygotes:* As stated above, the *ts-2/+* heterozygotes are phenotypically wild-type (heat-insensitive) when tested at the age of 12–15 fissions. Upon subculturing of heterozygous clones, some of the subclones initiated at the age of 50–70 fissions are found to be heat-sensitive. Furthermore, further subculturing of those subclones yields exclusively heat-sensitive vegetative descendants. The vegetative segregation of such subclones is assumed to represent another example of “allelic exclusion”, a phenomenon which, with one exception, has been detected in heterozygotes for all the allelic differences previously described in Tetrahymena (see pertinent section of the DISCUSSION).

The following observations have been made concerning the heat-sensitive segregants.

a.) They have only been observed in heterozygous clones; heat-sensitive segregants have not been observed upon subculturing homozygotes.

b.) Heat-sensitive segregants were most often detected without deliberate ef-

fort, simply in the course of raising heterozygous progeny to maturity. In some cases, we deliberately set out to isolate them from a heterozygote, always successfully.

c.) The micronucleus (germ line) of the one heat-sensitive segregant tested is still heterozygous, capable of transmitting both alleles (*ts-s* and *ts-2<sup>+</sup>*) with equal frequency. This is shown by crosses #3, #4 and #7 of Table 1, since strain NC1-19-28 is a stable heat-sensitive segregant of a heterozygous clone which expressed the wild type-phenotype when tested at the age of about 13 fissions.

d.) Heat-sensitive segregants are not detected at frequencies larger than 2% (the limit of the tests used) until after 50 fissions in heterozygous clones propagated in mass culture.

e.) Spontaneous vegetative "reversion" of a heat-sensitive segregant to the wild-type phenotype has not been detected with certainty. The frequency must be less than  $10^{-6}$ /cell division.

f.) The rate of appearance of heat-sensitive segregants has not yet been measured, but unsystematic observations indicate that its magnitude must be of the same order found in the other reported cases (see review in ALLEN 1971; CARLSON 1971; GRASS 1972; DOERDER 1972).

#### DISCUSSION

*Genomic exclusion as the basis for mutant isolation:* The present work implements ALLEN's suggestion (1967) that genomic exclusion ought to provide a useful basis for the isolation of recessive mutants in Tetrahymena. Figure 2 describes ALLEN's interpretation of the cytogenetic details of genomic exclusion induced by the C\* strain. This strain is unable to contribute genetic information to the next sexual generation. The genetic and cytogenetic evidence of ALLEN indicates that the micronucleus of each "exconjugant" originates from a diploidization of a single product of meiosis of the normal mate. Thus the micronuclei of both "exconjugants" of a pair are expected to be homozygous for all their genetic complement and, furthermore, genetically identical to one another.

Thus if a recessive mutation was induced in the normal mate, in one half of the cases both exconjugants of the cross to the C\* strain ought to be homozygous for the mutant allele. Unfortunately, the new macronuclei, derived from the same diploidized nucleus, do not become functional; instead, the old macronuclei are retained. Since the cell only expresses the genetic information of the macronucleus, the exconjugants still express the old (wild-type) phenotype. Fortunately, the two exconjugants are still mature, express their old mating types, and furthermore can undergo a normal conjugation when crossed again. Thus, if vegetative descendants of two exconjugants of the first round of mating can be crossed to one another, both the micronucleus and macronucleus of the progeny will derive from the same homozygous diploid fusion nucleus and should therefore express the mutant phenotype. The results reported here confirm those expectations.

The method as used here requires eight days from mutagenesis to setting up tests of the homozygous second-round progeny. It still has the disadvantage that two serial single pair isolations had to be done for every clone screened. The

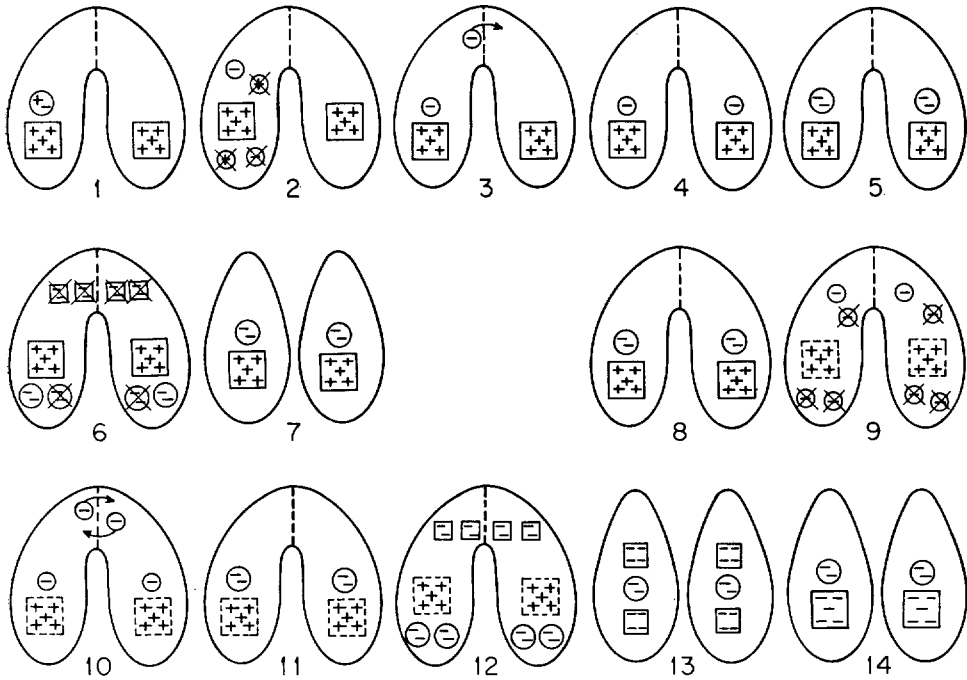


FIGURE 2.—Diagrammatic representation tracing a newly induced micronuclear mutation through the stages in genomic exclusion. Modified from ALLEN (1967).

Key to Symbols.—Square: macronucleus; circle: micronucleus or prezygotic nuclei derived from it; +: wild-type allele; —: mutant allele. The number of symbols in micronuclei (or its prezygote products) and their size represent their ploidy. Only 5 (of the estimated 45) functional allele copies present in the macronucleus are shown.

Description of the Stages—Stages 1–7 represent round I; 8–14, round II. The C\* cell is shown on the right throughout round I. (1.) A conjugating pair formed between a C\* cell and a phenotypically wild-type cell having a recently-induced micronuclear mutation to heat sensitivity (—). (2.) The normal cell has undergone meiosis; only one of the haploid products survives, carrying a “—” allele in this case. (A “+” allele will survive for the other half of the pairs, an event of no interest here). Meiotic division cannot be completed in the C\* strain. (3.) The lone meiotic survivor divides mitotically. (4.) One daughter (migratory nucleus) has been extruded into the C\* mate, the other (stationary nucleus) remains in the normal cell. (5.) The haploid nucleus of each cell diploidizes; the mechanism of this step has not been elucidated. (6.) The diploid nucleus undergoes two divisions. Three of the four products disintegrate; the other becomes the new micronucleus. (7.) The two cells separate, becoming the round I exconjugants; these are still mature and phenotypically wild-type (heat insensitive) because the old macronucleus has been retained. (8.) Cells derived from each of the round I exconjugants made, initiating round II. This is now a normal round of conjugation. (9.) Micronucleus undergoes meiosis; only one product survives. (10.) The lone survivor divides once mitotically. (11.) The haploid migratory nucleus of each cell is extruded into its mate and fuses with the resident haploid stationary nucleus, forming the diploid fusion nucleus. (12.) The fusion nucleus divides twice. The two anterior products increase their DNA content and become new macronuclei; one of the posterior products becomes the new micronucleus and the other distintegrates. (13.) The cells separate, becoming the round II exconjugants. (14.) At the first binary fission of each exconjugant the micronucleus divides and one copy goes to each daughter cell. The macronuclei do not divide but are segregated, one to each daughter. The round II exconjugants are sexually immature and express the mutant phenotype.

necessity to isolate individual round I pairs follows from the requirement that descendants from the *same* round I pair mate in the second round in order to yield cells homozygous for a rare mutation; in mass culture, the round I exconjugants would nearly always mate with genetically wild-type cells, yielding only heterozygotes. The necessity for the isolation of round II pairs would be removed by having a method of positive selection for the mutants. The necessity for at least one single pair isolation per clone screened makes the method still impractical for the isolation of mutants whose frequency is less than  $10^{-4}$ . A heat-sensitive mutant of *syn*gen 1 has been isolated by DR. PETER BRUNS (personal communication) using a similar approach; the genetic basis for the mutant phenotype has not been determined.

In spite of these difficulties, this method appears to have some practical advantages over the "selfer" method previously used by McCoy (1973). In the selfer method, a clone capable of selfing (i.e. mating among its members; NANNY and CAUGHEY 1955) is mutagenized and selfing is then allowed under conditions where cells of the same mutant subclone have a high probability of mating with one another. In practice, the selfer method requires the same number of single cell or pair isolations (per clone screened) as the "genomic exclusion" method (one or two, depending on whether selective pressure is or is not available, respectively, to isolate the desired mutants). However, only one-fourth of the isolations will yield a recessive homozygote, as opposed to one-half in the genomic exclusion method. Furthermore, a selfer clone requires more maintenance care to be sure that its members are still capable of selfing with the high frequency required. A very promising method based on the phenomenon of allelic exclusion in heterozygotes (see below) may improve the efficiency of isolations of many recessive mutants (CARLSON 1971; DOERDER 1972).

The genomic exclusion method may be still capable of improvement. It was noted that the round I progeny already contain a diploid macronucleus. Unfortunately, the new genotype is not expressed; the new macronuclear anlage do not develop and instead the old macronucleus, expressing the wild-type phenotype, is regenerated. It would seem possible that under favorable experimental circumstances, the development of the new macronuclear anlage could occur successfully. The exceptional round I progeny obtained in the cross of H9-6 to the C\* strain and other preliminary observations in our laboratory suggest that such an event can occur spontaneously. Unfortunately, not enough is known about the mechanism controlling the antagonistic interaction of the new and old macronucleus during conjugation to guide an intelligent search for the conditions which promote the development of new macronuclei after round I. Preliminary attempts to inhibit macronuclear retention by UV irradiation of the C\* cells prior to conjugation have been unsuccessful (JANE NELSON, unpublished observations); resumption of growth of the exconjugant derived from the irradiated cell is delayed for a period of time (which depends on the UV dose) but when growth is resumed the phenotype of the clone makes it clear that macronuclear retention was not prevented.

Complementation tests were performed in order to determine if the "genomic

exclusion" method had yielded mutations in a variety of genes. Two mutants to be tested were crossed to one another, and their progeny (which ought to be "trans" double heterozygotes) were tested in mass culture for their ability to grow at 40°C. Some twenty mutants have been so tested in most of the pairwise combinations (ORIAS and FLACKS, unpublished results). Most of the combinations give a positive result, suggesting that a variety of genes were probably affected by the various mutations. However, it is still not known if, and to what extent, genetic recombination occurs in the macronucleus. Therefore we cannot rule out the possibility that some of the cases of apparent complementation are due to the formation of a recombinant wild-type allele from two different mutant alleles of the *same* gene. The positive results obtained also imply that the mutations possessed by all of the mutants tested are recessive. A more complete report of this work will be published, pending further investigation.

*Genetics of the ts-2 mutation:* All of the results obtained are consistent with the idea that the heat-sensitive phenotype of the H9 strain is due to a single recessive nuclear mutation. An experimental approach limited to performing crosses and counting progeny cannot by itself exclude the possibility that two or more mutations determine the mutant phenotype. If so, however, the multiple mutations must be closely linked in order to account for our experimental results. We suppose that the *ts-2* mutation was induced by the nitrosoguanidine, but have no way of proving it; we do know that under identical conditions NG can induce mutations to chloramphenicol resistance in a cytoplasmic (probably mitochondrial) genetic determinant in the same strain (ROBERTS and ORIAS 1973). Another nuclear mutation to heat sensitivity was previously obtained by WYNNE McCoy (1973) using the selfer method following NG mutagenesis.

The *ts-2* mutation follows the hereto universal ciliate rule that only macronuclear genetic information is expressed during vegetative life. We conclude this from the findings that (a) "heterokaryons" containing a *ts-2/ts-2* micronucleus and a *ts+* macronucleus (Round I progeny of H9-6 × C\*) have the wild-type (heat-insensitive) phenotype and (b) phenotypically heat-sensitive subclones of heterozygotes still possess the *ts-2+* allele in the micronucleus.

The dominance of the *ts+* allele (and the recessiveness of the *ts-2* allele) in young heterozygotes deserves a brief comment. Extensive cytological, biochemical and genetic evidence indicates that the *Tetrahymena* macronucleus contains many functional copies (approximately 45) of most (if not all) the genetic information (NANNEY 1964; WOODARD, KANESHIRO and GOROVSKY 1972; NILSSON 1970; ALLEN and GIBSON 1972). For the purposes of dominance in gene expression, the *Tetrahymena* heterozygote is thus more analogous to a bacterial host infected with many phage particles, some wild-type and some mutant for a given gene, than to a somatic cell of a higher eukaryotic organism with a diploid complement of chromosomes. In all probability, then, the macronucleus of a young heterozygote contains many copies of both alleles of the *ts-2* gene, all active in gene expression. The dominance of the wild-type allele then merely indicates that in the young heterozygous cells there are enough copies of the *ts+* allele to synthesize its specified product in sufficient quantity to maintain the viability of the

cells and to allow subsequent reproduction upon raising the temperature to 40°C. We know nothing concerning the nature of the product specified by the *ts-2*<sup>+</sup> allele beyond the obvious inference that it is essential for growth at 40°C. The *ts-2* mutation could have affected one of many genes whose products are essential for growth at any temperature, such that the altered product is functional at temperatures up to 30°C, but denatures in the 30–37°C range. Alternatively, the *ts-2* mutation could have inactivated or deleted the essential product of one of possibly many genes which are expressed only at high temperatures. [The gene which determines the presence of the T antigen is an example of a gene in *Tetrahymena* which is only expressed at high temperatures (PHILLIPS 1967a), although its product may not be essential for viability at those temperatures (DOERDER 1972)]. We have not yet made an effort to determine what component or even what major process is *primarily* affected by the *ts-2* mutation because of the large number of possibilities and the difficulties of disentangling primary from secondary consequences of the genetic alteration.

*Allele exclusion in ts-2/+ heterozygotes:* Vegetative growth of *Tetrahymena* heterozygotes eventually yields sublines which express only one of the two alleles originally present in the heterozygote; this state is faithfully perpetuated during further vegetative growth of the clone. This functional loss is restricted to the macronucleus; the micronucleus still contains both alleles, capable of being transmitted in equal frequency to the sexual progeny through conjugation. This phenomenon has been called “allelic exclusion” (NANNEY 1964) (no relationship to genomic exclusion) and with one exception has been reported in all the studies of inheritance of Mendelian genes in syngen 1 of *T. pyriformis* [H antigen: NANNEY and DUBERT 1960; T antigen: PHILLIPS 1967b; esterase-1 and -2: ALLEN 1965; acid phosphatase-1: ALLEN, MISCH and MORRISON 1963; St antigen: GRASS 1972; caffeine resistance (*caf-1* and *caf-2*) and allyl alcohol resistance: CARLSON 1971; serotype regulatory mutations: DOERDER 1972]. The only exception is the *ts-1* mutation studied by McCoy (1973).

The *ts-2/+* heterozygotes yield heat-sensitive segregants and thus appear to be no exception to this rule, with one reservation: though we have no reason to doubt it, we do not yet have any way of convincingly demonstrating that the heat-sensitive phenotype of the segregants is determined by the *ts-2* mutation. We assume this to be the case only from the circumstantial evidence that (a) the segregants appear reliably in *ts-2/+* heterozygotes and are not seen in wild-type homozygotes, and (b) the segregant cells after three days at 40°C have identical appearance to the *ts-2/ts-2* homozygotes under the same conditions.

Two hypotheses have been proposed to explain the appearance of functionally homozygous segregants. One hypothesis (ALLEN and NANNEY 1958; SCHENSTED 1958) ascribes it to the random assortment of 45 segregating units (“subnuclei”), each hereditarily determined to express one of the two alleles. The other hypothesis (ALLEN and GIBSON, cited in ALLEN 1971) ascribes it to the occasional loss of one of two “master” copies (one of each allele) which determine the genetic composition of 45 non-replicating “slave” copies. In a separate study of *ts-2/+* heterozygotes (ORIAS and FLACKS, manuscript in preparation) we contribute evidence

which rules out any simple version of the master-slave hypothesis, and which is entirely consistent with the hypothesis of random assortment of many replicating units.

The segregating units have been proposed to be diploid (ALLEN and NANNEY 1958) or haploid (ALLEN and GIBSON 1972; NILSSON 1970; WOODARD, KANE-SHIRO and GOROVSKY 1972). To explain allelic exclusion the diploid proposal requires the additional postulate that only one of the two alternative alleles of a heterozygous segregating unit is expressed, and that this functional state is maintained among the lineal descendants of that segregating unit. This hypothesis implies intricate regulatory mechanisms to insure that the  $ts^+$  allele is active and the  $ts-2$  allele is repressed in some of the segregating units of a young heterozygote, while the  $ts-2$  allele is active and the  $ts^+$  is repressed in the remainder. The problem becomes more apparent by considering that there are no nuclear membranes to compartmentalize and isolate segregating units from one another; thus a seemingly essential component to assure the coexistence of the antagonistic biochemical interactions required to maintain the diverse hereditary differentiations is missing.

Our work raises two additional difficulties with the hypothesis of mutual repression of alternative alleles in diploid segregating units. The existence of complex regulatory systems mediating mutual repression is plausible in the case of heterozygotes for *naturally occurring* alternative alleles, such as in the mating type, serotype and isozyme genes previously studied; the existence of such regulatory mechanisms may have been of adaptive value in the evolutionary history of the species (NANNEY 1964; PHILLIPS 1967b). Indeed, complex and ill-understood regulatory systems must govern the nuclear differentiation which leads to the expression of only one mating type out of the many genetically possible for a given cell (NANNEY and CAUGHEY 1953; NANNEY 1956). By contrast, the pre-existence of such mechanisms in the case of heterozygotes for the *laboratory-induced*  $ts-2$  mutation is difficult to defend, particularly since the  $ts-2$  allele is selectively neutral under the best conditions and lethal under the worst. More importantly, the  $ts-2$  allele was never allowed to be expressed in the heterozygous state before it was isolated. Thus one cannot argue that there was any selective pressure to coincidentally isolate the handful of mutations seemingly required to create a reliable regulatory system of mutual repression in the heterozygote.

A second difficulty stems from the extremely high stability of the differentiated state. Allelic-excluded segregants have never been seen to "revert" in any of the syngen 1 systems previously studied, but because of the lack of selective pressure, the sample of cells which could be examined was necessarily restricted. The excellent selective pressure for heat-insensitive revertants provided by the  $ts-2$  allele has allowed us to determine that the frequency of reversion of heat-sensitive segregants to the wild-type phenotype is less than  $10^{-6}$ . It seems unlikely to us that cellular regulatory systems based on mutual repression could work with such a high degree of reliability.

If one accepts the hypothesis that the segregating units are haploid, the existence of very stable heat-sensitive segregants can be readily explained, since the

macronucleus would contain, with regard to the *ts-2* gene, copies of the mutant allele exclusively. Such a macronucleus would be genetically identical to that of a *ts-2/ts-2* homozygote. In that case, the vegetative reversion of a heat-sensitive segregant of a *ts-2/+* heterozygote would simply require a mutation in one of the macronuclear copies of *ts-2* allele; the rate of such an event ought to be as low as that of a vegetative reversion of *ts-2* homozygote. Objections against a simple model of haploid segregating units have been raised by NANNY (1964) and ALLEN (1971) on the basis of a number of observations therein reviewed. However, these observations cannot yet be used to discriminate between the haploid and diploid model since then their explanation on *either* model requires additional unproven assumptions. The arguments derived from our findings obviously cannot settle the question of the ploidy of the segregating units; they merely provide more constraints for any detailed diploid model.

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