CYTOPLASMIC INHERITANCE OF CHLORAMPHENICOL RESISTANCE IN *TETRAHYMENA*

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ABSTRACT

Chloramphenicol-resistant mutants of *Tetrahymena pyriformis* were obtained after mutagenesis with nitrosoguanidine at an estimated frequency of 10^{-3} mutants per mutagenized cell. The mutants are still partially sensitive to chloramphenicol and have a lowered growth rate, compared to the wild type strain, in rich medium *without* chloramphenicol. The genetic analysis described here indicates that chloramphenicol resistance is inherited as a cytoplasmic determinant that is not exchanged during conjugation. This represents the first simple cytoplasmic genetic determinant described in this species. A number of arguments favor a mitochondrial localization for this genetic determinant. In addition to the possible utility of such mutants for studies of mitochondrial structure and function in *Tetrahymena*, analogous mutations might serve as cytoplasmic "tags" in other ciliate species where the results of selfing need to be distinguished from those of outcrossing.

IN the course of attempting the isolation of drug-resistant mutants of *Tetrahymena pyriformis*, syngen 1, cells resistant to chloramphenicol were obtained after mutagenesis with nitrosoguanidine. Since chloramphenicol has been shown to specifically inhibit the mitochondrial protein synthesizing system in *Tetrahymena* (MAGER 1960; ALLEN and SUYAMA 1972) and since mitochondria contain DNA that specifies some of the mitochondrial components (SUYAMA 1967; CHI and SUYAMA 1970), a genetic analysis was undertaken to determine the cellular location of the genetic determinant of chloramphenicol resistance.

The genetic analysis described in this paper has revealed that the chloramphenicol resistance marker is inherited cytoplasmically rather than as a nuclear determinant. On the basis of the genetic results and other observations on these mutant strains and by analogy to findings in other organisms, it is suggested that the chloramphenicol resistance marker is located in the mitochondrion.

MATERIALS AND METHODS

Strains: Chloramphenicol-resistant mutants (CA-101, CA-102, and CA-103) were derived from the wild type strain D1968-5 of *Tetrahymena pyriformis*, syngen 1, obtained from DR. DAVID L. NANNEY. This strain belongs to inbred family D (NANNEY 1959) and is homozygous for the *mt^A* allele, which determines the potential to express matings types I, II, III, V and VI (NANNEY, CAUGHEY and TEFANKJIAN 1955). The strain also has a short immaturity period (NANNEY,

personal communication). Strain H9-6 is a temperature-sensitive mutant derived from strain D1968-5 in our laboratory (ORIAS and FLACKS, in preparation). It is homozygous for a recessive nuclear mutation (*ts-2*), which determines a complete inhibition of growth above 37°C. Both strains D1968-5 and H9-6 are sensitive to 250 µg/ml of chloramphenicol. Standard mating type tester strains of syngen I were obtained from DR. NANNY.

Media: (1) *PPY*: 20 g of proteose peptone Difco, 1 g of Bacto yeast extract (Difco), 1 ml of salts solution, in 1000 ml H₂O. (2) *Salts solution*: 10 g of MgSO₄·7H₂O, 5 g of ZnSO₄·7H₂O, 0.5 g of FeSO₄·7H₂O, 0.5 g of CaCl₂·2H₂O, 1000 ml of H₂O. (3) *PPY/P&S*: *PPY* supplemented with streptomycin sulfate and Penicillin G, each at a final concentration of 250 µg/ml. (4) *CAM*: *PPY/P&S* medium containing 250 µg/ml of chloramphenicol. (5) *2% BP*: a 2% (v/v) sterile water suspension 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.

The chloramphenicol was dissolved by agitating for 20 minutes in a rotary shaker at 30°C. Solutions of chloramphenicol, penicillin, and streptomycin were made fresh every day they were needed by adding the antibiotic powder to sterile medium. N-methyl,N'-nitro, N-nitrosoguanidine (MNNG) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/ml and then diluted to the desired concentration in *PPY*. The stock solution was made fresh every time it was needed and used immediately. In control experiments, DMSO alone was ineffective in inducing mutations to chloramphenicol resistance.

Single cell isolation: Single *Tetrahymena* cells (or single conjugating pairs) were isolated by using Pasteur pipettes whose tips had been drawn out over a low flame to approximately 2–3 times the diameter of an average cell. The micropipette was "sterilized" between isolations by dipping into boiling water. The single cells were deposited into isolated drops (approximately 0.03 ml) of culture medium placed on a sterile plastic Petri plate in a regular 6" × 8" array with a sterile Pasteur pipette (Figure 1). The dimensions of this array were designed to match exactly the dimensions of one half of the microtiter plates described below. The Petri plates were incubated in moist chambers (covered plastic "shoe boxes" or refrigerator crispers containing a

FIGURE 1.—Illustration of two methods of culture of *Tetrahymena* cells. The plastic Petri plate contains 48 clones, in drops of approximately 0.03 ml each. Drops of *PPY* or *BP* medium are more than stable enough to withstand routine handling of the plates. Twenty to 100 cells from each of the 48 drops in the Petri plate can be simultaneously transferred to the depression in one half of the microtiter plate (bottom) using a "replicator" (top, right); the depressions contain 0.25 ml of culture medium. Petri and microtiter plates are incubated upright in stacks in a moisture chamber. The units in the bottom scale are inches.

thin layer of water). Such cultures can be kept at room temperature for at least 2 weeks without drying up.

Aside from trying to work quickly, keeping the Petri plates uncovered only while making the 48 single cell isolations and adding penicillin and streptomycin to the medium, no other precautions were taken to prevent contamination of the cultures.

Replication of clones: It was often desired to test the growth of many clones (obtained by single cell isolation as described above) under a variety of conditions. For these purposes, it was found useful to simultaneously "replicate" all the cultures on a Petri plate to several "microtiter plates". This is a 85 mm $\times$ 125 mm rectangular plastic plate, containing 96 depressions arranged in an 8" $\times$ 12" array (Figure 1); the plate was sterilized by irradiation with a germicidal UV lamp.

Replication of cultures from a Petri plate (or from another microtiter plate) to a microtiter plate was accomplished with a device consisting of a hardwood block into which 48 steel rods (45 mm length $\times$ 1.5 mm diameter) were driven (Figure 1). The replicator was sterilized after each use by dipping it into 90% ethanol and passing it quickly over a bunsen burner flame, and then allowing it to cool for 30 seconds before using it again. Microtiter plates were incubated stacked inside moist chambers.

Cell counts: Cell density was measured with a Celloscope cell counter (Particle Data Inc., Elmhurst, Ill.), equipped with a 190 μ orifice. Cells to be counted were diluted with filtered 0.89% NaCl.

Mutagenesis procedure: Cultures grown in PPY/P&S at 30°C to a cell density of 10^5 – 10^6 cells/ml were diluted with an equal volume of PPY/P&S medium containing 20 μ g/ml of MNNG (10 μ g/ml final concentration) and incubated for 6 hours at 30°C on a rotatory shaker. (The number of cells remained constant during this 6-hour exposure to MNNG. Untreated control cultures underwent 1 doubling during the same interval under otherwise identical conditions). Cells were then removed from the mutagen by either dilution with or centrifugation and resuspension in PPY/P&S medium without MNNG (see cross procedure for details of centrifugation). The cultures were incubated at 30°C for 7 days and scored for increased turbidity.

Measurement of mutation frequency: The frequency of chloramphenicol-resistant cells after mutagenesis was estimated as follows. Immediately after washing the mutagen away from the mutagenized cells, 10-, 20-, 50-, 100-, and 200-fold dilutions of the culture were made into CAM medium. Twelve 0.25 ml samples of each dilution were added to separate depressions in a sterile microtiter plate. As controls, some depressions contained wild type and CA-101 cells, respectively. The cell density of the mutagenized culture was determined both prior to and following mutagenesis. The microtiter plate was incubated at 30°C for 5 days and then scored for growth of chloramphenicol resistant clones.

Growth rate determination: Exponential phase cultures of various strains grown in PPY/P&S were diluted into either fresh PPY/P&S or CAM medium in plastic Petri plates. The plates were incubated at either 30° or 41°C, and cell counts were made at appropriate intervals.

Cross procedure: This is a slight modification of the procedure described by S. L. ALLEN (1967). Late exponential phase cultures of strains to be crossed, grown in PPY medium, were separately harvested by centrifugation for 3 minutes at the intermediate setting (635 $\times$ g) in an International clinical centrifuge. The pelleted cells were resuspended in 5 ml of Dryl's solution (DRYL 1959). The procedure was repeated 3 more times, except that the final pellet was resuspended in 10 ml Dryl's solution. To starve the cells in preparation for mating, the suspensions were kept overnight (about 18 hours) at room temperature in sterile plastic Petri plates. Then 5 ml of each suspension was mixed in a plastic Petri plate and kept at room temperature. Within 6 hours after pair formation had begun (or 7–8 hours after mixing), single pairs were isolated to separate drops of 50% PPY/P&S on Petri plates. As the exconjugants of a pair separated (6–12 hours after isolation), they were transferred to separate, adjacent drops of PPY/P&S. These plates were then incubated at 30°C for 3 days. Sets of exconjugant clones in which both exconjugants gave rise to healthy clones were tested for maturity, heat sensitivity, and chloramphenicol resistance, as described below.

Maturity tests: Cells that have undergone normal conjugation become sexually immature

(i.e. incapable of conjugation) for many fissions (NANNEY and CAUGHEY 1953). Cells that are mature immediately after conjugation have usually failed to undergo normal conjugation. In order to eliminate the latter cells from the genetic analysis, maturity tests were performed as follows.

Exconjugant clones were replicated to two microtiter plates containing 2 drops of 2% BP per depression. At the same time, 100-fold dilutions of the parental mating type stocks were made with 10 ml of 2% BP in Petri plates. Both the microtiter and the set of Petri plates were incubated at room temperature. Two days later, the tester stocks were fed (by adding an equal volume of 2% BP to each Petri plate) and then 2 drops of each tester strain were added to each depression of the appropriate microtiter plates. Parental mating types were mixed as controls. Depressions were scored after 18 hours at room temperature for the presence of conjugating pairs.

Temperature sensitivity test: Exconjugant clones were replicated to microtiter plates containing 0.25 ml PPY/P&S per depression. Depressions containing wild type and H9-6 cells were included as controls. These plates were incubated at 41°C for 48 hours and scored for growth as follows. Cultures which had any elongated, transparent, swimming cells were scored as growth-positive; cultures in which all the cells were rounded, opaque and non-motile, resting in the bottom of the plate, were scored as growth-negative (ORIAS and FLACKS, manuscript in preparation). The heat-sensitive phenotype can be unambiguously scored, even if cells grow more slowly at 41°C due to possession of the chloramphenicol resistance marker.

Chloramphenicol resistance test: Exconjugant clones were replicated to microtiter plates containing 0.25 ml of CAM medium per depression, incubated at 30°C for one week and then scored for growth. Resistant and sensitive clones can already be unambiguously distinguished after 3 days. Exposure to chloramphenicol for 5 days results in death of sensitive strains. Wild type and parental chloramphenicol-resistant strains were included in the tests as controls.

Maturation of exconjugant clones: Exconjugants selected for further work were grown to maturity by either of the two following methods. Cells were transferred to a 4" (Wasserman) test tube containing 2 ml of PPY/P&S by using a sterile glass capillary tube (freezing point capillary tube). The culture was incubated without being shaken at 30°C for 48 hours. Four or five serial transfers were made by diluting approximately 0.05 ml (approximately $\frac{1}{2}$ of the content of a capillary tube) of the previous culture into 2 ml of PPY/P&S medium and incubating the culture without shaking it for 2 days at 30°C. In more recent experiments, the cells were incubated in a rotary shaker at 30°C for only 24 hours between transfers. Alternatively, the culture was serially replicated in microtiter plates every 2 days and incubated at 30°C. Five serial transfers were sufficient to yield mature cultures.

Tests of mature clones: An exconjugant clone raised to maturity in mass culture, as described in the previous section, frequently contains cells with different mating types (because of the caryonidal basis of mating type determination in syngen 1; NANNEY and CAUGHEY 1953). To circumvent this problem, a single cell isolation from each exconjugant clone was made in PPY/P&S medium. The cultures thus obtained were tested for mating type by the same method used for maturity tests, except that a clone was separately given the opportunity to mate with cultures of the 5 mating types possible in the inbred family D of syngen 1 (I, II, III, V and VI). Controls to which no mating type testers were added served to identify selfers (NANNEY and CAUGHEY 1955). Mixtures of the testers in all combinations were also run as a further control.

RESULTS

Isolation of the mutants: Mutant strains CA-101, CA-102, and CA-103 were isolated after MNNG mutagenesis of strain D1968-5 by the method previously described. Although the 3 mutants arose in the same mutagenized culture, it is probable that they were of independent origin, in view of the high frequency of appearance of resistant cells described below. The mutant phenotype is stable; reversion to sensitivity has not been observed for any of the mutants after propagation in the absence of chloramphenicol.

Growth rate of chloramphenicol-resistant mutants: Growth curves at 30° and 41°C for the wild type and the chloramphenicol-resistant strain CA-102 grown in PPY/P&S medium in the presence and absence of 250 µg/ml chloramphenicol are shown in Figure 2. The other two resistant strains behaved nearly identically to CA-102 when tested under the same conditions. The growth rate measurements confirm the significantly higher resistance of the mutants to chloramphenicol. However, the mutants are still partially sensitive to the presence of 250 µg/ml of chloramphenicol, as evidenced by a lowered growth rate. In the presence of chloramphenicol, the mutant cells become partially sensitive to high temperature (41°C).

Determination of mutation rate: By determining the frequency of chloramphenicol-resistant mutants prior to and following mutagenesis, it is possible to calculate a rate of mutation to chloramphenicol resistance per mutagenized cell. The results of more than ten control experiments in which a total of at least 10⁷ non-mutagenized cells were screened for chloramphenicol resistance, failed to reveal any resistant cells. From this it can be inferred that the frequency of chloramphenicol-resistant cells prior to mutagenesis is less than 10⁻⁷.

The frequency of chloramphenicol-resistant cells after mutagenesis was determined by several experiments of the type described under MATERIALS AND METHODS. In this analysis it was assumed that one resistant cell is sufficient to yield a healthy culture in the CAM medium and that resistant cells are distributed in the aliquots according to Poisson statistics. If so, then the average number (m) of resistant cells per aliquot is given by the equation

$$P(0) = e^{-m},$$

where $P(0)$ is the fraction of aliquots showing no growth at an appropriate dilution where some aliquots give a healthy culture and the rest do not.

Since no cells were resistant prior to mutagenesis, all the increment can be ascribed to the mutagenic treatment. The induced frequency of mutant cells is 4.6×10^{-3} , or at least a 10⁴-fold increase over the spontaneous mutation frequency.

Inheritance of chloramphenicol resistance: The first experiment consisted of crosses between the various chloramphenicol-resistant strains and the heat-sensitive, chloramphenicol-sensitive strain H9-6. Only pairs that had undergone normal conjugation and nuclear reorganization were included in this genetic analysis. Two criteria were employed to determine whether normal mating had occurred: (1) sexual immaturity of exconjugant clones immediately following conjugation and (2) normal segregation of the nuclear *ts-2* allele of strain H9-6. The results of these crosses are shown in Table 1. Out of 32 pairs whose exconjugants were immature, both exconjugants were heat-insensitive in 31 pairs. (The one exception was a pair from the cross of CA-103 to H9-6, in which both exconjugant clones were heat-sensitive; unfortunately, these clones were lost before they could be investigated further). Upon testing for chloramphenicol resistance, it was found that every set consisted of one sensitive and one resistant exconjugant clone.

Sets of F₁ exconjugant clones derived from six pairs were grown to maturity by

serial subculture in PPY/P&S and tested for mating type. Two sets of exconjugant clones expressing different, nonparental mating types (one set designated F_1 -5A and F_1 -5B, respectively; and the other F_1 -6A and F_1 -6B, respectively) were

FIGURE 2.—Growth rates of wild-type and chloramphenicol-resistant strain CA-102. Key to the symbols: Wild type in the presence ($\bullet$) and absence (O) of $250 \mu\text{g/ml}$ chloramphenicol. Strain CA-102 in the presence ($\blacktriangle$) and absence (Δ) of chloramphenicol. Left, 30°C ; right, 41°C .

TABLE 1

Results of crosses of chloramphenicol-resistant mutants and their progeny

Parents and genotypes	Exconjugant pairs scored	Progeny phenotypes			
		Heat*	Sensitive	Chloramphenicol†	Resistant Sensitive
CA-101 (<i>ts</i> ⁺ / <i>ts</i> ⁺), <i>cam</i> ^r × H9-6 (<i>ts</i> -2/ <i>ts</i> -2, <i>cam</i> ^s)	11	11	0	all	1:1
CA-102 (<i>ts</i> ⁺ / <i>ts</i> ⁺), <i>cam</i> ^r × H9-6 (<i>ts</i> -2/ <i>ts</i> -2, <i>cam</i> ^s)	13	13	0	all	1:1
CA-103 (<i>ts</i> ⁺ / <i>ts</i> ⁺ , <i>cam</i> ^r) × H9-6 (<i>ts</i> -2/ <i>ts</i> -2, <i>cam</i> ^s)	8	7	1	all	1:1
F1-5A (<i>ts</i> -2/ ⁺ , <i>cam</i> ^s) × F1-5B (<i>ts</i> -2/ ⁺ , <i>cam</i> ^r)	12	9	3	all	1:1
F1-6A (<i>ts</i> -2/ ⁺ , <i>cam</i> ^r) × F1-6B (<i>ts</i> -2/ ⁺ , <i>cam</i> ^s)	33	25	8	all	1:1
F1-5A (<i>ts</i> -2/ ⁺ , <i>cam</i> ^s) × H9-6 (<i>ts</i> -2/ <i>ts</i> -2, <i>cam</i> ^s)	18	9	9	all	0:2
F1-5B (<i>ts</i> -2/ ⁺ , <i>cam</i> ^r) × CA-102 (<i>ts</i> ⁺ / <i>ts</i> ⁺ , <i>cam</i> ^r)	7	7	0	all	2:0

* Both exconjugants were alike for the heat sensitivity phenotype in every case.

† 1:1 means that one exconjugant was chloramphenicol-resistant and the other sensitive; 0:2 means that both exconjugants were sensitive; 2:0 means that both exconjugants were resistant.

crossed to one another and pairs were isolated. The F₂ progeny was tested as in the previous crosses. The results from these crosses are also shown in Table 1. Both exconjugant clones were heat-insensitive in 34 pairs, while both exconjugant clones were heat-sensitive in the remaining 11 pairs. The phenotypic identity of both exconjugants of a pair and the 3:1 phenotypic ratio among the sets of exconjugants is the expected behavior of the nuclear marker. In contrast, all 34 pairs behaved identically for the chloramphenicol resistance phenotype: one exconjugant clone of each pair was sensitive and the other was resistant.

Replacement of initially resistant exconjugant clones by sensitive cells has not been observed even after propagation of some F₁ and F₂ clones for 70–100 fissions in the absence of chloramphenicol.

Control crosses (chloramphenicol-resistant × resistant and sensitive × sensitive) were made by backcrossing the appropriate F₁ progeny. The first cross involved a chloramphenicol-sensitive strain homozygous for the *ts*-2 allele (H9-6) and a chloramphenicol-sensitive strain heterozygous for this allele (F₁-5A). In this cross, the heat sensitivity allele segregated in the 1:1 ratio expected from the parental genotypes, 9 pairs being heat-sensitive and 9 being heat-insensitive. Both exconjugants in every case remained chloramphenicol-sensitive. In the second cross, between a chloramphenicol-resistant strain heterozygous for the *ts*-2 allele (F₁-5B) and a chloramphenicol-resistant strain homozygous for the wild type allele (CA-102), all progeny were phenotypically heat-insensitive, as expected from the recessive nature of the *ts*-2 mutation. All pairs of exconjugants from this cross (7 out of 7) remained chloramphenicol-resistant.

DISCUSSION

Cytoplasmic inheritance of chloramphenicol resistance: Normal conjugation in *Tetrahymena* (diagramed in Figure 3) results in the *nuclear* genetic identity of the two exconjugants. This is so because the migratory and stationary haploid nuclei, which participate in reciprocal exchange in each cell, are genetically identical. The phenotypic identity of exconjugants with regards to heat sensitivity is consistent with normal mendelian distribution of nuclear markers in the crosses performed here. On the other hand, the dissimilarity of exconjugants from the same pair with respect to chloramphenicol resistance is inconsistent with the results expected for a nuclear determinant. The possibility that the mutation to chloramphenicol resistance occurred in the macronucleus of the mutagenized cells and that the exconjugants differed because of *macronuclear retention* (S. L. ALLEN 1967) is eliminated by the following arguments: (1) the progeny studied were sexually immature (a diagnostic test of the development of a new macronucleus); (2) the distribution of the heat-sensitive marker was normal among F_1 and F_2 progeny; and (3) the exconjugants of a pair were always alike for the nuclear heat sensitivity marker. Thus chloramphenicol resistance appears to be located on an extranuclear (cytoplasmic) genetic determinant that is not exchanged during conjugation. Although the study of cytoplasmic inheritance has a long and distinguished history with respect to the related ciliate *Paramecium* (SONNEBORN 1947), this appears to be the first established case of simple cytoplasmic inheritance in *Tetrahymena*. Cytoplasmically-inherited mutations to chloramphenicol resistance have previously been reported in yeast (COEN *et al.* 1970) and in *Paramecium aurelia* (ADOUTTE and BEISSON 1972; BEALE, KNOWLES and TAIT 1972).

The apparent lack of exchange of the cytoplasmic determinant for chloramphenicol resistance found in this work may seem surprising in view of the rather efficient exchange of radioactively-labeled protein and RNA observed during conjugation in syngen 1 of *T. pyriformis* by McDONALD (1966). This suggests that the determinants in question are located in structures that are anchored to the membrane system of the cell and/or are too large to pass through the pores in the conjugation plate (ELLIOTT and TREMOR 1958).

In *Paramecium aurelia*, massive cytoplasmic exchange can occur spontaneously and can also be induced (SONNEBORN 1950). This condition allows the exchange of cytoplasmic genetic determinants for chloramphenicol and erythromycin resistance, yielding exconjugants of identical phenotype (BEALE 1969; ADOUTTE and BEISSON 1970 and 1972; BEALE, KNOWLES and TAIT 1972). It is thus possible in *Paramecium* to provide further evidence for the cytoplasmic inheritance of this marker, and furthermore to study the population dynamics in cells containing a mixture of wild-type and drug-resistant cytoplasmic determinants (ADOUTTE and BEISSON 1972; BEALE, KNOWLES and TAIT 1972). Methods to accomplish massive cytoplasmic exchange in *Tetrahymena* have not yet been reported.

The pattern of cytoplasmic inheritance described here could conceivably have

FIGURE 3.—Diagrammatic representation of the conjugation process in *Tetrahymena*. The diagrams are based on the descriptions of NANNY (1953), ELLIOTT and HAYES (1953), and RAY (1956). After pair formation by sexually reactive cells of different mating types (1), the micronucleus of each cell undergoes meiosis (2). Three of the meiotic products disintegrate (3), and the fourth divides mitotically to give the genetically identical "stationary" and "migratory" pronuclei (4). The migratory pronuclei of each cell are transferred to the other cell, where they fuse with the corresponding stationary pronuclei (5). The resulting diploid zygotic nuclei then divide twice (6,7). The two products in the anterior end begin to develop into new macronuclei,

a basis other than the alteration of a cytoplasmic gene. Patches of cortical units arranged with opposite to normal polarity in *Paramecium* (BEISSON and SONNEBORN 1965) and extreme variations in the number of ciliary rows in *Tetrahymena* (NANNEY 1966) are perpetuated in vegetative growth and during conjugation. The "cytoplasmic" pattern of inheritance observed in crosses involving the above traits has been interpreted by postulating (1) the existence of a number of metastable, mutually exclusive structural arrangements of cortical units, possible in the absence of any gene differences, and (2) the strong tendency of the preexisting pattern to perpetuate itself by dictating the pattern to which new units adapt. The concept has analogies to the mechanism of crystal growth. Since the mutations to chloramphenicol resistance described here arose after treatment with a known mutagen, and in view of the plausible molecular basis of their phenotype described below, we do not consider the epigenetic interpretation described above likely to be the correct explanation of our findings.

The ease of isolation of chloramphenicol-resistant mutants and of scoring the phenotype, coupled with the failure of the mutated DNA to be exchanged during conjugation, makes the marker a useful genetic "tag" to trace the cellular origin of exconjugants. These properties could also be useful in facilitating genetic analysis in ciliate species in which selfing as well as outcrossing can occur in the same mating population.

In what cell organelle is the gene for chloramphenicol resistance located? Any extranuclear component postulated to carry the chloramphenicol resistance determinant must satisfy several criteria: (1) it must contain genetic material capable of being expressed; (2) it must remain approximately constant in number through cell division; and (3) its properties must be consistent with the lack of cytoplasmic exchange of this marker.

The experimental approach used in this work cannot, by itself, reveal what structure or organelle contains the determinant for chloramphenicol resistance. However, several arguments suggest the mitochondria as the most likely candidate. (1) Mitochondria of *Tetrahymena*, like those of every other species studied, possess DNA (SUYAMA 1966). This DNA is expressed, as evidenced by the hybridization of mitochondrial ribosomal RNA to this DNA (SUYAMA 1967; CHI and SUYAMA 1970). No other structures or organelles in *Tetrahymena* have been conclusively shown to contain DNA. Evidence for the presence of DNA in basal bodies of *Tetrahymena* is contradictory (RANDALL and DISBREY 1965; FLAVELL and JONES 1971); furthermore, the evidence on the positive side was obtained by using preparations in which contamination with mitochondria could have confused the answer. (2) Mitochondria have a system for protein synthesis that is distinguishable from the cytoplasmic system. In particular, chloramphenicol in-

at which time the old macronuclei are gradually resorbed (8). At the same time, one of the posterior nuclei becomes the new micronucleus, the other one disintegrating (9). By this time, the exconjugants have separated (10). At the first cell division following pair separation the new micronucleus in each exconjugant divides (11), one daughter micronucleus going to each daughter cell (12,13). At this cell division the two new macronuclei in each exconjugant separate, one going to each daughter cell (11-13).

hibits mitochondrial, but not cytoplasmic, protein synthesis in *Tetrahymena* (MAGER 1960; ALLEN and SUYAMA 1972). Thus a simple basis of chloramphenicol resistance could be the genetically determined alteration of a component required for the entry of chloramphenicol into the mitochondria, or the alteration of the mitochondrial component that is the effective target of chloramphenicol. The observation that the smallest dimensions of *Tetrahymena* mitochondria are larger than the pores in the conjugation plate (ELLIOTT and TREMOR 1958; M. L. BATES, personal communication) is consistent with the lack of exchange of the cytoplasmic determinants inferred from our genetic analysis.

A mitochondrial location of the mutations to chloramphenicol resistance would also make the high induced mutation rate per mutagenized cell more readily understandable. If it is assumed (1) that a single altered mitochondrion is sufficient to confer resistance to the cell and (2) that there is an average of about 10^3 mitochondria per cell, then the mutation rate *per mitochondrion* would be about 10^{-7} . This estimate of the average number of mitochondria is based on 2 independent lines of evidence: (1) the estimate of SATO (1960) of 600–800 mitochondria in newly divided cells of strain W of an undetermined syngen of *T. pyriformis* and (2) the observations of apparently at least one mitochondrion per cortical unit (R. D. ALLEN 1967) and approximately 500 cortical units in newly divided cells of syngen 1 (NANNEY 1971).

In *Paramecium*, a mitochondrial location has been suggested for the cytoplasmically inherited mutation to chloramphenicol resistance (ADOUTTE and BEISSON 1972) and demonstrated for erythromycin resistance mutations (BEALE, KNOWLES and TAIT 1972).

Attempts to obtain erythromycin-resistant mutants of *Tetrahymena* have been frustrated by the natural resistance of strain D1968–5 to 1 mg/ml of erythromycin, the highest concentrations of this antibiotic which could be conveniently tested (K. Y. LING, unpublished observations). This observation may be explained by the insensitivity to erythromycin inhibition of radioactive amino acid incorporation into acid-insoluble protein observed in cell-free preparations containing intact mitochondria (CHI and SUYAMA personal communication) and in a mitochondria-free system of *in vitro* protein synthesis dependent on mitochondrial ribosomes and poly-U (ALLEN and SUYAMA 1972); cells of strain ST (of an undetermined syngen of *T. pyriformis*) were the source of mitochondria in these two studies.

If the mutations described here are indeed mitochondrial, they may prove useful for studies of the structure and function of *Tetrahymena* mitochondria. The utility of *Tetrahymena* may be increased if any findings can be generalized to the mitochondria of other animal cells that are experimentally less easily and inexpensively handled.

Lowered growth rate of chloramphenicol-resistant mutants: The chloramphenicol-resistant mutants grow more slowly in the presence of chloramphenicol than in its absence, and this difference is magnified at high growth temperatures (Figure 2). A possible explanation of the partial sensitivity to chloramphenicol of the mutants is that the mutation has merely decreased (rather than completely

eliminating) the accessibility of chloramphenicol to its target or its affinity for it. Alternatively, a second, less sensitive target may become growth-limiting in the presence of chloramphenicol.

A second property of the mutants is their lowered growth rate (as compared to the wild type) in rich medium *in the absence* of chloramphenicol (Figure 2). This effect is probably not due to a deleterious *nuclear* mutation (coincidentally induced with the mutation to resistance), since in exconjugant sets it is regularly coinherited with chloramphenicol resistance. Possible explanations are (1) a second deleterious mutation is coincidentally induced in the same mitochondrion; or (2) the altered component has an essential role in the viability of the cell and this function is impaired as a consequence of the alteration which confers chloramphenicol resistance. This phenomenon appears analogous to the thermosensitivity of certain erythromycin-resistant mutants in *Paramecium aurelia* (ADOUTTE and BEISSON 1970).

The lower growth rate of resistant cells (as compared to the wild type) in nutrient medium without chloramphenicol and the stability of the phenotype of the resistant cells, when taken together, imply the genetic homogeneity of the resistant cells studied. Had the original mutants (or the initially resistant exconjugants in a sensitive $\times$ resistant cross) contained a mixture of determinants for resistance and sensitivity, the growth rate differences are such that it would be reasonable to expect a replacement of the population by sensitive segregants; this has not been observed. Population replacement of one cytoplasmic determinant by another is known to occur upon propagation of *Paramecium* cells containing certain mixtures of genetically different mitochondria (ADOUTTE and BEISSON 1972; BEALE, KNOWLES and TAIT 1972).

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