Uniparental Cytogamy: A Novel Method for Bringing Micronuclear Mutations of *Tetrahymena* into Homozygous Macronuclear Expression With Precocious Sexual Maturity

Eric S. Cole¹ and Peter J. Bruns

Department of Genetics and Development, Cornell University, Ithaca, New York 14853

Manuscript received July 21, 1992

Accepted for publication September 2, 1992

ABSTRACT

A new method of inducing self-fertilization, uniparental cytogamy, yields homozygous germinal and somatic genotypes in the ciliate *Tetrahymena thermophila*. Progeny are highly fertile and show a marked tendency for precocious sexual maturity. This method is highly effective in protocols designed to generate and express nonlethal dominant or recessive mutations.

SEXUALLY competent strains of ciliated protozoa contain two different nuclei: a diploid germinal micronucleus and a highly polyploid somatic macronucleus. Isolation of a new mutation requires that it be present in the micronucleus to be heritable and in the macronucleus to be expressed. Since macronuclei are derived from germinal micronuclei during sexual development, some form of conjugation must be induced between mutagenesis and selection; for isolation of recessive mutations, this mating should be some form of self-mating.

Three methods have been developed that generate whole-genome homozygotes from mutagenized strains of *Tetrahymena thermophila*. Cytogamy, a form of induced self-fertilization in ciliates (Figure 1), is widely employed to bring novel micronuclear mutations into homozygous expression within the macronucleus of *T. thermophila* (SANFORD and ORIAS 1981). This process is brought about by administering a hyperosmotic shock to mating cells at an appropriate stage during conjugation (ORIAS and HAMILTON 1979; ORIAS, HAMILTON and FLACKS 1979). The two members of a pair fail to cross-fertilize, instead, they each self-fertilize and develop macronuclei from the resulting homozygous zygote nuclei. Conventional cytogamy for mutant isolation is complicated by having to select for successful cytogamonts from both non-conjugants and cross-fertilized true exconjugants (Figure 1). This problem has been addressed by using functional heterokaryons: cells with drug-resistance genes in the transcriptionally silent micronucleus, and drug-sensitive alleles of these genes in the transcriptionally active macronucleus (BRUNS and BRUSSARD 1974). Such cells are sensitive to drug treatment unless they undergo a successful mating event which brings the drug resistance phenotype into expression

within a newly constituted macronucleus. Unfortunately, in *Tetrahymena* most available drug markers are dominant, hence, drug selection cannot discriminate between drug-resistant (heterozygous) exconjugants, and drug-resistant (homozygous) cytogamonts. This problem has been partially overcome by the use of the recessive drug marker *2-dgal* (conferring resistance to the drug 2-deoxygalactose, ROBERTS and MORSE 1980). Wild-type cells and cells heterozygous for *2-dgal* show a growth-suppression phenotype when exposed to the drug.

When pairs of cells involving one partner that is a *2-dgal* heterokaryon are subjected to hyperosmotic shock and their progeny are selected with 2-deoxygalactose, most true exconjugants and non-conjugants are effectively outcompeted by the homozygous cytogamonts (Figure 1). Nevertheless, even under the best conditions, about 2% of the drug resistant progeny generated by cytogamy prove to be true exconjugants, heterozygous for the recessive *2-dgal* marker (unpublished observations). These undoubtedly survive because of (a) phenotypic assortment within the macronucleus in which a clone that is heterozygous for *2-dgal* can give rise to homozygous subclones (MERRIAM and BRUNS 1988) and (b) the nonlethal phenotype of 2-deoxygalactose-sensitive cells when exposed to drug (*i.e.*, sensitive cells are quickly overgrown by resistant cells, but they are not necessarily killed). Consequently, cross-fertilized clones heterozygous for a recessive drug-resistance allele can produce subclones that exhibit drug resistance and slip through the drug selection.

A second method for generating homozygotes that does not depend upon the use of recessive drug-markers is genomic exclusion (ALLEN, FILE and KOCH 1967; PRITS 1979). In genomic exclusion matings, a cell line with a defective micronucleus (commonly referred to as a "star strain") is mated to a normal

¹ To whom correspondence should be addressed.

FIGURE 1.—Conventional cytogamy. This figure illustrates the most popular method by which one can induce micronuclear mutations and bring them into homozygous, macronuclear expression. A heterokaryon for the recessive *2-dgal* drug-resistance marker (indicated by shaded nuclei), is mutagenized. This cell is sensitive to 2-deoxygalactose due to its macronuclear genotype, and yet carries nonexpressed copies of a *2-dgal* resistance allele in its transcriptionally silent micronucleus. When mated to a drug-sensitive partner (unshaded nuclei), and exposed to a hyperosmotic shock, three types of progeny are produced. Cells that failed to mate (nonconjugants) possess the parental phenotypes and consequently fail to grow in 2-deoxygalactose. Self-fertilizers (cytogamonts) result in whole-genome homozygotes whose macronuclei express the genes that were previously silent in the micronucleus. Cytogamonts homozygous for *2-dgal* grow well in 2-deoxygalactose. True exconjugants (cross-fertilizers) are heterozygous for *2-dgal*, and consequently grow poorly in 2-deoxygalactose. Drug selection tends to enrich for cytogamonts that are whole-genome homozygotes for *2-dgal*.

drug-resistance heterokaryon as shown in Figure 2. The micronuclear genome of the star strain is discarded early in conjugation, and hence is "excluded" from the genome of the progeny. The nondefective heterokaryon transfers a nucleus unilaterally to the star partner, and both partners usually abort further development. The resulting partners retain their macronuclei and therefore their parental phenotypes. During a second-round mating the two partners complete normal conjugation. To generate clones homozygous for induced mutations, individual "first-round" pairs must be isolated and grown before a "second-round" mating is induced to bring the homozygous micronuclear genome into expression within the macronucleus. Hence this method is not ideally suited

for large-scale mutant selection protocols.

A third method for generating homozygotes is that of "short-circuit genomic exclusion" (BRUNS, BRUSARD and KAVKA 1976). This occurs in a small fraction of first-round genomic exclusion pairs (Figure 2). In short-circuit genomic exclusion, a pronucleus is transferred to the star partner where it is believed to diploidize (presumably by endoreplication) and undergoes postzygotic development culminating in macronuclear anlagen (MA) formation. This occurs most frequently when C*III is the star strain employed (10%), and least frequently with A*III (<1%). Short-circuit genomic exclusion occurs in less than 5% of A*V, B*VI and B*VII pairs (unpublished observations). Unfortunately, there is some question regard-

FIGURE 2.—Genomic exclusion mating. This mating protocol generates whole-genome homozygotes via two consecutive matings involving a “star” cell (a cell possessing a defective micronucleus indicated by a dash). In the first round mating, the heterokaryon passes a pronucleus to its star partner but receives no genetic material in return (in this example, *Pmr*: a dominant paromomycin-resistance marker, is homozygous in the shaded micronuclei). In a small fraction of such matings (<5%) both partners proceed through postzygotic development, and generate homozygous, paromomycin-resistant macronuclei via “short-circuit genomic exclusion.” Most partners, however, abort development shortly after unilateral pronuclear transfer, separate, and can be remated immediately. During a second-round genomic exclusion mating, both partners possess identical, homozygous micronuclei, and can complete normal development resulting in whole-genome homozygotes that express drug resistance.

ing the fertility of short-circuit genomic exclusion progeny (ORIAS, HAMILTON and FLACKS 1979; and J. FRANKEL, personal communication).

Another practical limitation to genetic experimentation with *Tetrahymena* is that, following a successful mating, progeny are incompetent to pair again for 50–80 cell divisions, approximately 2 weeks of culture time (BLEYMAN 1971; ROGERS and KARRER 1985). This has been termed the immaturity interval (NYBERG and BISHOP 1981).

The research reported here describes a novel mating pathway that combines the advantages of conven-

tional cytogamy (use of mass-selection protocols) with those of genomic exclusion (ability to employ dominant drug-resistance markers and produce pure cultures of homozygous progeny). We have named this process “uniparental cytogamy” (UPC). Using appropriately marked heterokaryons, cytogamonts can be selected with 100% efficiency over both non-conjugants and true exconjugants (Figure 3). Cells treated in this fashion yield highly fertile cultures that are pure whole-genome homozygotes. UPC also results in precocious maturity in up to 50% of the progeny, depending upon strain differences. Precocious prog-

FIGURE 3.—UPC. In this protocol, a paromomycin-resistance heterokaryon (shaded nuclei contain *Pmr* alleles), is mated to a "star" partner (just as in first-round genomic exclusion). After 5.75 hr, a hyperosmotic shock is delivered as for conventional cytogamy. Four possible products are shown. Non-conjugants, of course, will exhibit the parental drug-sensitive phenotypes, and be killed by a 4-day exposure to paromomycin. During UPC, the heterokaryon generates a *Pmr*-homozygous macronucleus by self-fertilization. The "star" partner eliminates its old macronucleus, and yet has no MA to replace it with, and consequently, perishes. True exconjugants will be first-round genomic exclusion progeny, retaining their parental (drug-sensitive) macronuclei. As in genomic exclusion, a small fraction of pairs will undergo short-circuit genomic exclusion, resulting in drug-resistant, whole-genome homozygotes. Drug selection will result in the survival of only uniparental cytogamonts and short-circuit genomic exclusion progeny, both whole genome homozygotes.

eny are capable of mating with all six other mating types within 15 cell divisions of the mating event that produces them (the earliest time that we can test them). We have successfully employed UPC, along with nitrosoguanidine mutagenesis, to isolate novel mutations exhibiting (a) resistance to the microtubule-inhibiting drug vinblastine sulfate and (b) alterations in the pattern of organelles in the ciliate cortex.

MATERIALS AND METHODS

Stocks: Heterokaryons carrying the drug markers for cycloheximide resistance (*Chx*), 6-methylpurine resistance (*Mpr*), 2-deoxygalactose resistance (*2-dgal*) or paromomycin resistance (*Pmr*) were of the inbred B strain of *T. thermophila* (see Table 1). Defective "star" strains were of the A strain [A*(III), A*(V)], B strain [B*(VI), B*(VII)] and C strain [C*(III)].

Growth and induction of mating: The growth medium used throughout these experiments was an iron supplemented proteose peptone medium (0.25% proteose pep-

TABLE 1

Heterokaryon Genotypes

Strain	Micronuclear genotype	Macronuclear phenotype	Mating type
1A264	<i>2-dgal/2-dgal</i>	(gal-sens.)	II
1A267	<i>Chx/Chx</i>	(cy-sens.)	III
CU427.2	<i>Chx/Chx</i>	(cy-sens.)	VI
CU428.1	<i>Mpr/Mpr</i>	(mp-sens.)	VII
CU438	<i>Pmr/Pmr</i>	(pm-sens.)	IV

^a gal = 2-deoxygalactose, cy = cycloheximide, mp = 6-methylpurine, pm = paromomycin.

tone, 0.25% Difco yeast extract, 0.5% glucose, 0.33 mM FeCl₃). Stock cultures were maintained by loop transfer and stored at 15°. All experiments were conducted at 30°. Two starvation media were employed: DRYL's (1959) inorganic salts and 10 mM Tris (pH 7.5).

Matings were performed by prestarving cells of uniform mating type at 2.0×10^5 cells/ml for 18 hr in either Dryl's or Tris media. Five-milliliter samples of each mating type

(adjusted to 2×10^5 cells/ml) were then mixed at time = zero.

Cytogamy and environmental shock: A hyperosmotic shock was delivered to mating cells by adding 150 μ l of 20% glucose to 2.0 ml of mating cells (final concentration = 1.4%). After 45 min, the cytogamy mixture was diluted first 1:10 in distilled water and subsequently into growth medium approximately 7–9 hr after the initial mixing. These basic methods were adapted from published procedures for conventional cytogamy (ORIAS, HAMILTON and FLACKS 1979; SANFORD and ORIAS 1981; LESLIE MEEK JENKINS, personal communication). Pairs were isolated for genetic analysis seven to 8 hr after the initial mixing, or diluted and distributed to microtiter plates for drug screening and maturity tests. Samples were taken for 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining at the time of osmotic shock and at 8–9 hr after mixing.

The effects of a secondary environmental shock upon the maturity of UPC progeny were determined in a second set of experiments. Fifty microliters of growth media + 0.2 N CaCl_2 were delivered to microtiter plates already containing 50 μ l of UPC-treated cells in growth media (final CaCl_2 concentration = 0.1 N). These plates were incubated 6–8 days in drug selection medium rather than the typical 4 days (see below).

Drug-resistance assays: Cell lines were tested for the expression of drug markers using the following procedures. Mating cultures (osmotic shock-treated or control cultures) were diluted to 10^{-2} or 10^{-3} in nutrient medium. Fifty-microliter aliquots were delivered to 96 wells in a microtiter plate (approximately 50 and 5 pairs per well for the two dilutions, respectively, assuming 100% pairing). The next day, 50 μ l of nutrient medium supplemented with $2 \times$ drug concentration were delivered to these wells. (For CaCl_2 -treated cultures, 25 μ l of nutrient medium supplemented with $5 \times$ drug concentration were delivered since each well already contained 100 μ l of media.)

Cells were screened for cycloheximide resistance (*Chx*) by exposing them to 25 μ g/ml of cycloheximide (final concentration). Cells were screened for *Mpr* by exposing them to 15 mg/ml of 6-methylpurine. For *Pmr*, cells were exposed to 100 μ g/ml paromomycin. For 2-*dgal* resistance, cells were exposed to 2.5 mg/ml of 2-deoxygalactose. In all drug-resistance assays, media were also supplemented with 250 μ g/ml penicillin, and 250 μ g/ml streptomycin, and survival was assayed after 4 days of incubation at 30° (6–8 days for CaCl_2 -treated cultures).

Microtiter plates carrying dilutions in which approximately 30% of the wells exhibited positive growth in drug-supplemented media were used to determine UPC frequencies using the Poisson formula.

Mutagenesis and mutant selection: Potential drug-resistance mutants: cultures of IA267(III) or CU438(IV) were grown to 2×10^5 cells/ml. To 10 ml of these cultures, nitrosoguanidine was added to a final concentration of 10 μ g/ml. After 3 hr of incubation with agitation at 30°, cells were washed three times in starvation medium and allowed to starve overnight. Mutations were brought into expression by mating the mutagenized stock to A*(V), and treating the mating mixture with an osmotic shock 6 hr after mixing. This "cytogamy" stock was divided into four aliquots, grown 24 hr in nutrient media, and exposed to cycloheximide or paromomycin for 4 days. The cytogamy stock was then used to inoculate growth medium containing 50 μ M vinblastine sulfate (Sigma Chemical Co., St. Louis, Missouri). After 4–6 days, survivors were subcloned into growth medium for further characterization.

Maturity analysis: Microtiter plate cultures were repli-

cated to drug-free nutrient medium after an appropriate time for drug-selection. These were subsequently grown at 30°. After 2 days of growth, they were replicated to seven microtiter plates each containing Tris (starvation) media and one of seven mating-type test strains (I–VII). After 8–12 hr at 30°, mating-type test plates were scored for the appearance of mating pairs. The initial survivors of a 4-day exposure to drugs were considered to be 15 divisions "old." This is approximately the number of divisions required for a single cell pair to reach 1×10^6 cells/ml in 100 μ l of growth medium (maximum cell density for Tetrahymena). Each subsequent replication and 2-day growth interval allowed another seven divisions. Adolescence is defined as the ability of a clone to mate with some but not all of the six (non-self) mating types (ROGERS and KARRER 1985). Maturity is defined as the ability to mate with all six (non-self) mating types. In cases in which a clone gave rise to multiple mating types within a single microtiter well, we also tested for intraclonal pairing by simply replicating these cultures to starvation medium. This provided another assay for maturity.

Microscopy: Nuclear configurations and karyotypes were visualized by staining cell pairs with DAPI (Sigma). Cell samples were fixed in a methanol-acetic acid (3:1) fixative and stained 1:1 with 100 μ g/ml DAPI in fixative (D.CASSIDY-HANLEY, personal communication). Cells were examined with a Zeiss fluorescence microscope.

RESULTS

Analysis of progeny derived from first-round genomic exclusion matings subjected to hyperosmotic shock (UPC): UPC can be induced under a number of different conditions using a variety of star cells as partners. As shall be described, certain conditions and certain star strains produce superior results. Hence, characterization of the cytology of this process will be described for matings performed under optimal conditions and strains; specifically, A*V served as the star-partner and matings were performed at 30° in Dryl's starvation medium unless otherwise stated.

A CU438 heterokaryon bearing the paromomycin resistance marker (*Pmr*) in its micronucleus, and A*V were starved and mated (this would normally constitute a first-round genomic exclusion mating, see Figure 2). Both "parents" express paromomycin sensitive phenotypes. At various times after mixing cells were exposed to a 1.4% concentration of glucose for 45 min. Samples treated at various times were analyzed both for viability and drug resistance of their progeny and for 8 hr nuclear configurations. Clearly, a significant number of such pairs responded to hyperosmotic shock by developing new MA that expressed the drug-resistant phenotype (Figures 4a and 5). The optimum time of treatment for producing drug-resistant progeny appears to be 5.75 hr after mixing (± 15 min), resulting in approximately 25% drug-resistant progeny. The optimum time for MA formation (as revealed by cytology) occurred at 5.25–5.5 hr, resulting in 40–60% of the pairs exhibiting MA. Clearly, more pairs developed MA than survived to produce drug-

FIGURE 4.—Temporal analysis of UPC induction. (These experiments were conducted at 30° in Dryl's medium.) (a) The effect of osmotic shock on exconjugant survival, production of drug-resistant progeny, and MA formation at various times after mixing. Dashed line with box symbols indicates viability of pairs treated at various times. Solid line with box symbols indicates pairs exhibiting some form of MA configuration. X's with solid line indicate percent of progeny exhibiting drug resistance. (b) Induction of unilateral and bilateral MA formation by osmotic shock delivered at various times after mixing. ($n = 100$ for MA measurements. For measurements of drug resistance and viability, values were calculated from the Poisson formula applied to 192 microtiter plate wells as described in MATERIALS AND METHODS.)

resistant progeny. Pair mortality also appeared highest when treated 5–6 hr after mixing.

There appear to be two types of nuclear configurations resulting from osmotic shock (Figures 4b and 5). One, which we have termed UPC, exhibits MA formation in only one partner while the other partner exhibits a single, condensing macronucleus. The second configuration involves MA formation in both partners in what appears to be an induced form of "short-circuit genomic exclusion" (SC), described earlier. UPC configurations are most prevalent when pairs are treated at 5–5.5 hr. SC configurations are most prevalent when pairs are treated at 5.25–5.75 hr (Figure 4b). From these data alone it is not possible to determine to what extent each of the two observed cytological products contributes to the production of viable, drug-resistant progeny.

Pairs of CU438 $\times$ A*V were isolated following hyperosmotic shock. Both exconjugant partners from a given pair were subcloned and assessed for viability and drug resistance. Data from these experiments

FIGURE 5.—Two DAPI-stained pairs of IA267 $\times$ A*V (starved in Dryl's medium) treated with a hyperosmotic shock at 5.75 hr and stained eight hrs after mixing. Upper pair shows the UPC configuration with macronuclear degeneration on both sides (arrows) and MA formation only in the left-hand partner (MA). Lower pair shows bilateral MA formation (induced form of short-circuit genomic exclusion).

appear in Figure 6. It appears that, for a 5.75-hr shock treatment, most drug-resistant progeny (95%) resulted from pairs in which one of the two partners died prior to, and independent of drug selection. The remaining 5% of drug-resistant progeny arose from pairs in which both partners yielded viable, drug-resistant progeny (less than 2% of the total pairing population). From our cytological observations, we would predict that the A* partner of a UPC pair, having destroyed its parental macronucleus without replacing it, should perish. Hence, these may account for at least some (if not most) of the successful progeny. We suspect that the low frequency of drug-resistant progeny derived from UPC-treated pairs in

FIGURE 6.—Analysis of viability and drug sensitivity for progeny of osmotically shocked, first-round genomic exclusion pairs (UPC treatment). CU438 and A*V were mated in Dryl's medium at 30° and subjected to hyperosmotic shock after 5.75 hr. Samples were fed growth medium at 7 hr to prevent second-round genomic exclusion pairing. Pairs were then isolated 12 hr after mixing. (Note: first-round genomic exclusion pairs dissociate approximately 9 hr after mixing at 30°. True conjugants dissociate approximately 12 hr after mixing. Hence, isolation of pairs after 12 hr should result in a sample that is enriched for partners that are completing postzygotic development resulting in MA formation either as a result of UPC or short-circuit genomic exclusion.) pmR indicates a paromomycin-resistance phenotype, pmS indicates a paromomycin-sensitive phenotype.

which both partners survived probably represent short-circuit genomic exclusion progeny (ALLEN, WEREMIUK and PATRICK 1971; BRUNS, BRUSSARD and KAVKA 1976).

Determining the UPC-sensitive stage during conjugation: We examined the nuclear configurations of first round genomic exclusion pairs at various times during conjugation, and correlated these observations with the optimum time for production of osmotic shock-induced drug-resistant progeny (Figure 7). Osmotic shock appears to be most effective when delivered at the same time as the third (gametogenic) nuclear division. Because the osmotic shock persists for 45 min, we cannot say with any certainty that this is the UPC-sensitive stage. However, if this is the

osmotic shock-sensitive step, and if it is blocked by such treatment, then UPC may be the result of a failure in nuclear division rather than the prevention of pronuclear exchange accompanied by a subsequent self-fertilization event that has been hypothesized for conventional cytogamy (see DISCUSSION).

UPC efficiencies for various star lines: As mentioned earlier, UPC can be achieved using a number of different star strains with varying degrees of success. Figure 8a shows the relative efficiencies with which various star-lines produced drug-resistant progeny following hyperosmotic shock delivered at different times. It should be noted that all five star cells produce some drug-resistant progeny even without

FIGURE 8.—The optimum time for osmotic shock-induced cytogamy for (a) first-round genomic exclusion pairs and (b) conventional cytogamy involving two diploid cell lines. Each curve displays data from a single representative experiment conducted at 30° in Dryl's medium. (a) CU438 was mated with each of five star lines (A*III, A*V, B*VI, B*VII and C*III). Peaks represent times at which an osmotic shock produces the greatest number of drug-resistant (predominantly UPC) progeny. Each data point was calculated from the Poisson formula applied to 192 microtiter plate wells as described in MATERIALS AND METHODS. (b) A conventional diploid cross: CU427 × CU428, was subjected to hyperosmotic shock at different times. Ninety-six pairs were isolated and their progeny were screened sequentially for cycloheximide (cy) resistance and 6-methylpurine (6-mp) resistance. When progeny exhibited cy-resistance without 6-mp resistance, they were considered cytogamonts. Clearly the peak for cytogamy was 5 hr after mixing.

hyperosmotic shock due to short-circuit genomic exclusion.

Hyperosmotic shock produced the highest frequency of drug-resistant progeny with A*V and B*VI (25%). B*VII and C*III also produced elevated levels of drug-resistant progeny and A*III showed the poor-

FIGURE 7.—Nuclear dynamics during conjugation of CU438 × A*V starved in either Dryl's starvation medium or 10 mM Tris, correlated with the optimum time for osmotic shock-induced UPC. a-d indicate the percentage of pairs exhibiting specific nuclear configurations at different times after mixing. e shows the frequency of production of drug-resistant progeny (from both UPC and SCGE) for pairs treated to hyperosmotic shock at each time point. Note that the optimum time for induction of drug-resistant progeny (6 hr in the experiment depicted in e) is the same time at which one observes the most pairs exhibiting the third prezygotic nuclear division spindle (b). (n = 100 DAPI-stained pairs for a-d. For determinations of the percentage of drug-resistant progeny, values were calculated from the Poisson formula applied to 192 microtiter plate wells.)

est performance, giving only a low, background level of drug resistance among its progeny. We also tested the amiconucleate strain B3840 and found that it yielded levels as low as that of A*III (data not shown). For A*V and B*VI the optimum time for osmotic shock treatment was 5.75 hr (± 15 min) when starved in Dryl's medium. This is 45 min later than the optimum time for conventional cytogamy (see Figure 8b and ORIAS, HAMILTON and FLACKS 1979).

The effect of starvation medium upon UPC effectiveness: There are pronounced differences in the success of UPC when cells are starved in Tris buffer *vs.* Dryl's inorganic salts. Figure 7e shows the UPC performance of CU438 $\times$ A*V when starved in Tris *vs.* Dryl's medium. Clearly, UPC was most successful when pair formation occurred in Dryl's medium. This was true despite the fact that developmental synchrony (as indicated by the narrowness, and height of the peaks in Figure 7b) was virtually the same for cells starved in Tris or Dryl's medium. The only visible difference (in this experiment) was a delay in developmental events (and consequently UPC peak effectiveness) when cells were starved in Tris *vs.* Dryl's medium. Though values differed, UPC performance was consistently higher for all star strains when tested in Dryl's medium *vs.* Tris.

Maturation of uniparental cytogamonts: Early in our studies, it was noted that an unusually large number of UPC progeny exhibited adolescent or fully mature mating behavior at a very early time after mating. Figure 9a shows the relative frequencies of mature, adolescent and immature progeny from osmotic shock-treated pairs involving A*V and a number of different heterokaryons. For controls, we examined the maturity of progeny from a conventional mating (CU427 $\times$ CU428), and from conventional cytogamonts involving osmotic shock of (CU438 $\times$ CU428) pairs. Twenty-two divisions after mating (in Dryl's medium), successful, drug-selected progeny were tested for maturity and mating type. Though results between experiments were variable, CU438 (our paromomycin heterokaryon), consistently showed the highest incidence of early maturity (50%), yet other strains produced unusually high levels of early maturity and early adolescence as well. When we consider both adolescent and mature progeny ("pair-competent progeny"), we see a considerable range from CU427 (which showed levels of pair competence equal to our control: 19%) to CU428 (79%).

We were concerned that the unusually high frequency of precocious maturity in progeny derived from CU438 could have been due to age-dependent micronuclear deterioration in our stock (see WEINDRUCH and DOERDER 1975). It has been shown that exconjugants derived from parental strains carrying deleterious mutations in their micronuclei can exhibit

FIGURE 9.—Maturity analysis of (a) UPC* progeny from A*V $\times$ various heterokaryon lines, [data from a conventional mating (CU427 $\times$ CU428) are shown as the control. Data from a conventional cytogamy involving the mating: (CU438 $\times$ CU428) were not significantly different from those of the conventional mating: 0% mature, 24% adolescent and 76% immature.] (b) UPC progeny from different derivatives of CU438. (c) [A*V $\times$ CU438] UPC progeny treated to a 0.1 N CaCl₂ shock 12 hr after mixing. Progeny were tested for maturity 21–30 cell divisions after mating by mating to all seven possible mating-type tester strains. Maturity implies an ability to mate with 6/7 of the tester strains. Adolescence indicates an ability to form pairs with at least one mating type tester. Immaturity indicates an inability to pair with any of the seven mating type test strains. *Here the phrase "UPC progeny" is understood to include both UPC and SCGE progeny which could not be distinguished in this experiment.

early maturity (NANNEY and MEYER 1977). We tested this hypothesis in two ways. First, we "rejuvenated" the CU438 cell line by passing it through a first-round genomic exclusion cross and screening for highly fer-

tile, cloned progeny (see WEINDRUCH and DOERDER 1975). We then analyzed the frequency of precocity among UPC progeny derived from the newly regenerated clones (CU438.1a and b). We also tested the fertility of early mature progeny derived from the old, CU438 strain (see fertility results below).

Figure 9b compares the frequencies of mature, adolescent, and immature UPC progeny from CU438 and two, newly derived regenerates; CU438.1a and CU438.1b. Clearly the rejuvenated (highly fertile) lines show as high or higher levels of precocity among their UPC progeny as the original CU438 stock.

Enhancement of precocious maturity by environmental shock: It would be valuable to be able to generate uniparental cytogamonts with a reliably high level of precocity independent of the genetic background of the stocks employed. Toward this end, we attempted to combine UPC with another form of environmental shock. NANNEY and MEYER (1977) demonstrated that exposing mating pairs to either a 37° heat shock or 0.1 N CaCl₂ at 11–12 hr after mixing, frequently resulted in early maturity. (We found that heat shock resulted in an unacceptably high degree of lethality.) We took mating cultures (1A267 × A*V, CU427 × A*V, CU428 × A*V, and CU438 × A*V) and after exposing them to an osmotic shock at 5.75 hr, we exposed them to 0.1 N CaCl₂ continuously starting at 12 hr. At 24 hr after mixing, UPC progeny were selected with the appropriate drug. Successful cytogamy was scored after 4–6 days, and the plates were replicated to growth medium for a maturity analysis. Results of the maturity analysis appear in Figure 9c. Clearly a secondary CaCl₂ treatment results in dramatically elevated levels of precocious maturity in all of the strains tested.

The fertility of UPC and short-circuit genomic exclusion progeny: We conducted fertility tests on the precocious progeny of UPC. We also examined the fertility of progeny derived from short-circuit genomic exclusion matings (see Table 2). Progeny were fertility tested by outcrossing to CU427 and the outcross progeny were tested for both paromomycin resistance and cycloheximide resistance. Fertility was measured as the percentage of pairs yielding progeny expressing both drug markers. Clearly, precocious progeny derived from UPC matings exhibit good fertility (72%). Curiously, short-circuit genomic exclusion progeny, involving identical cell lines, showed very low fertility (12%). It is likely that some proportion of our UPC progeny are actually short-circuit genomic exclusion progeny, and this may account for the few cases of low fertility seen among them (see for example, 5.75-hr UPC clones 9 and 11). It remains to be seen why short-circuit genomic exclusion progeny exhibit such poor fertility (a phenomenon that has been observed previously, ORIAS, HAMILTON and

TABLE 2

Fertility of short-circuit genomic exclusion progeny and precocious mature UPC progeny with and without CaCl₂-treatment all from the cross: CU438 × A*V tested by outcrossing to CU427 and testing drug sensitivities of the outcross progeny

Clones	% cyR/pmR	% cyS or pmS	% Dead
Short-circuit genomic exclusion progeny			
1	13	17	70
2	22	30	48
3	13	9	78
4	0	70	30
Mean fertility = 12%. (N = 23 pairs for each clone tested)			
5.75-hr UPC treatment			
1	96	4	0
2	30	70	0
3	100	0	0
4	96	4	0
5	61	39	0
6	96	0	4
7	78	22	0
8	100	0	0
9	9	39	52
10	100	0	0
11	26	13	61
Mean fertility = 72%. (N = 23 pairs for each clone tested)			
5.75-hr UPC treatment + 12-hr CaCl ₂ treatment.			
1	0	74	26
2	4	31	65
3	0	74	26
4	0	87	13
5	0	70	30
6	4	43	53
7	0	57	43
8	4	65	31
9	0	83	17
Mean fertility = 1.3%. (N = 23 pairs for each clone tested)			

FLACKS 1979; J. FRANKEL, personal communications). One possibility is that short-circuit genomic exclusion results in aneuploid or haploid micronuclear products whereas UPC results in normal diploid micronuclei. The secondary CaCl₂ treatment also produced infertile progeny and hence was abandoned as a method for enhancing precocious maturity.

The ploidy of UPC and short-circuit genomic exclusion progeny: Precocious mature UPC progeny and short-circuit genomic exclusion progeny were grown and mated to A*III. At various time points from 4 to 5 hr after mixing, meiotic chromosome squash preparations were made and examined using DAPI fluorescence. Figure 10 shows three examples of these preparations. In most cases (greater than 90%), UPC progeny displayed the normal, diploid complement of chromosomes during meiosis (Figure 10B). Occasionally, UPC progeny proved to be tetraploid (Figure 10C). Short-circuit genomic exclusion

FIGURE 10.—Karyotype analysis of uniparental cytogametes and short-circuit genomic exclusion progeny. (A) A DAPI-stained example of a short-circuit genomic exclusion progeny mated to A*III and DAPI stained at late anaphase I of meiosis. Note three chromosomes at each pole and two chromosomal fragments midway between. (B) A typical diploid $\times$ A*III showing five chromosomes at each pole (as is seen for most UPC progeny). (C) An apparent

progeny, on the other hand, appeared to have aneuploid or even haploid micronuclei (Figure 10A). We frequently observed such cells exhibiting broken chromosomes during meiosis. It is not clear to us whether short-circuit genomic exclusion results in such aneuploidy, or whether a small fraction of cells in a population already possess damaged micronuclei, and these somehow stimulate the short-circuit genomic exclusion pathway during a star mating.

The effect of mutagenesis upon cytogamy frequency: It has been noted that mutagenesis reduces the efficiency of short-circuit genomic exclusion by an order of magnitude (BRUNS and SANFORD 1978). We compared the UPC success in mutagenized and non-mutagenized mating cultures. Our control mating (CU438 $\times$ A*V in Dryl's medium) produced a peak UPC performance of 12% drug-resistant progeny when treated with a hyperosmotic shock at 5.75 hr. Mutagenized CU438 cells produced a peak of 5% drug-resistant progeny that occurred at 6.5 hr. Hence, mutagenesis can diminish the success rate of UPC and can delay the optimum window of treatment by about 45 min.

UPC as an effective means of bringing novel mutations into homozygous expression: Two cell cultures, IA267 and CU438, were exposed to nitro-soguanidine for 3 hr. These cultures were subsequently starved, mated to A*V, and treated with an osmotic shock at 6 hr. UPC progeny were selected by exposing the UPC cultures to the appropriate drug for 4 days, and the cultures were transferred to fresh growth medium. Samples of these mutagenized stock cultures were exposed to 50 μ M vinblastine sulfate for 6 days, and survivors were cloned and retested for drug resistance. Concentrations of 50 μ M were used because of published reports that this was an effective killing dosage (HAMILTON 1984; HAMILTON, SUHR-JESSEN and ORIAS, 1988).

Cells with vinblastine-resistant phenotypes were found for both mutagenized cell lines. Upon cloning, and subsequent retesting, six of these putative mutants (three from IA267 and three from CU438) grew to high cell density even in 75 μ M vinblastine within 2 days, whereas control cells (from the same stocks), rounded-up and died.

When outcrossed to drug-sensitive partners, the resulting heterozygotes from the IA267 vinblastine-resistant line proved to be recessive in that drug resistance was not observed. Heterozygotes from the CU438 line, on the other hand, exhibited a dominant drug-resistance phenotype. When these outcross prog-

tetraploid UPC product $\times$ A*III showing ten chromosomes at each pole. In all three cases A*III was employed as a partner due to its degenerate micronucleus which allows us to identify unambiguously, the nuclear material of our test strain. Arrows indicate the star cell micronuclear material.

eny were subsequently brought to homozygous expression by genomic exclusion, approximately one half expressed the drug-resistance phenotype suggesting that a single genetic locus conferred resistance. We suspect that in both cases, the three mutants isolated represent multiple isolates from clonal progeny of the same mutational event. Nevertheless, it appears that two types of vinblastine resistant mutants have been isolated.

In another experiment, CU438 cells were mutagenized, and mated in a first-round, genomic exclusion mating to A*III to generate homozygous, mutant heterokaryons. Two thousand of these first-round pairs were isolated, grown to high cell density, and mated in microtiter plate cultures to prestarved B*VI. An osmotic shock was delivered at 6 hr, and UPC progeny (or second-round progeny from offspring from the original mating) were selected using paromomycin. Among these progeny (which were generated for use in a screen for conjugation mutants), clones with unusual morphologies were analyzed for cortical pattern defects by silver staining (see NELSEN and DEBAULT 1978). One such clone, CU452, exhibited supernumerary cortical organelles associated with the oral apparatus and contractile vacuole pores, and an elongate body profile. This morphology was reminiscent of the temperature-sensitive *mpD* mutation isolated in the FRANKEL laboratory (FRANKEL *et al.* 1984), yet complements this mutation (L. M. JENKINS and J. FRANKEL, personal communication). Our mutant proved to be fertile, and outcross progeny were easily obtained. The *mpD*-like phenotype assorted as a single-gene, recessive mutation, that we have named *lcd* for "lengthened cortical domains."

DISCUSSION

A comparison of UPC with other methods of generating whole-genome homozygotes: Numerous techniques have been developed for bringing novel micronuclear mutations into homozygous expression within the macronucleus of *T. thermophila* [see ORIAS and BRUNS (1976) for review]. Each of these techniques has limitations. Genomic exclusion (Figure 2), produces highly fertile progeny, yet "first-round" pairs must be individually isolated, grown and remated to generate homozygous progeny from the "second-round" mating. Hence, it is a labor-intensive procedure that is not amenable to mass-selection protocols.

Short-circuit genomic exclusion (Figure 2) bypasses the need for pair isolation by producing whole-genome homozygotes in a single mating episode. Although this particular pathway is rare (only 5–10% of first-round pairs exhibit this form of mating using C*III), such progeny can be selected for by using drug-resistance markers and functional heterokaryons

(see BRUNS, BRUSSARD and KAVKA 1976). Unfortunately, such progeny have a pronounced tendency toward infertility, probably resulting from the possession of aneuploid micronuclei.

Cytogamy (Figure 1) has proven to be the best method for mass-induction of whole-genome homozygotes from mutagenized cell lines. The major limitation here is that one is forced to employ a recessive (*2-dgal*) drug-resistance marker and a nonlethal screening procedure to enrich for homozygotes (the result of cytogamy) over growth-suppressed non-conjugants, and heterozygotes (the result of cross-fertilization). Hence this technique cannot make use of the growing number of more effective dominant drug-resistance markers and lethal drug screens, and it becomes important to test progeny from conventional cytogamy for heterozygosity.

UPC produces whole-genome homozygotes within a single pairing episode. Progeny can be selected by using any of the currently available dominant drug-resistance markers. UPC progeny are highly fertile predominantly diploid cell lines showing a strain-specific tendency toward precocious maturity.

The optimum conditions for inducing UPC are the following: (1) star strains A*V and B*VI are the most reliable UPC partners, (2) Dryl's inorganic salt starvation medium is superior to Tris for inducing UPC, (3) a hyperosmotic shock (1.4% glucose final concentration) is most effective at producing drug-resistant progeny when delivered at 5.75 hr (± 0.25 hr) after the mixing of prestarved partners when incubated at 30°. We did not explore variations in the osmolarity of the "shock" treatment, relying upon published values for conventional cytogamy (ORIAS, HAMILTON and FLACKS 1979; SANFORD and ORIAS 1981). Preliminary experiments suggested that reducing the duration of the osmotic shock from 45 min to 30 or 15 min decreased the frequency of drug-resistant progeny (unpublished observations). We did not attempt to increase the duration of treatment. Precocious maturity was observed most frequently with CU438.1 and CU428.1, and least frequently with IA267 and CU427.2.

We have indicated that a 5.75-hr osmotic shock treatment produces the highest frequency of drug-resistant progeny from a heterokaryon $\times$ "star" mating. Furthermore, the progeny from such matings are highly fertile and result predominantly from pairs in which one partner has died. We have assumed that these represent UPC pairs in which the "star" partner has perished due to an inability to construct a MA. Paradoxically, our cytology suggests that a 5.75 hr osmotic shock treatment produces more short-circuit genomic exclusion (SCGE) nuclear configurations than UPC nuclear configurations (Figure 4b). Furthermore, the percentage of total drug-resistant prog-

eny from pairs treated at 5.75 hr (progeny produced primarily from single-parent survivors) appears to exceed the percentage of UPC configurations as detected by cytological observations (compare Figure 4, a and b). To account for these observations, we suggest that the osmotic shock-induced form of SCGE (a) may produce an alternative (fertile) type of progeny that is fundamentally different from spontaneous (infertile) SCGE progeny, and (b) such pairs frequently produce only single surviving exconjugants. Consequently they could be misidentified as UPC progeny. Clearly a detailed analysis will be required to determine the relative contribution of induced SCGE progeny to the drug-resistant progeny generated by this procedure. What is required is a method of assessing the nuclear configuration of osmotic shock-treated pairs and determining (for the exact same pairs) the viability of their exconjugant progeny. This could be done by examining the nuclear configurations of living, isolated pairs with Nomarski optics microscopy, and culturing their exconjugant progeny.

Triggering the postzygotic developmental program in genomic exclusion pairs: When a normal diploid cell is mated to a "star" partner: one lacking a functional micronucleus, two developmental fates are observed. The most common fate, genomic exclusion, occurs in approximately 90% of pairs using the A* lines, and involves unilateral pronuclear transfer (from the diploid partner to the star partner), endoreplication of the pronuclear material to restore diploidy, and macronuclear retention (ALLEN 1967b; ALLEN, FILE and KOCH 1967; DOERDER and SHABATURA 1980). In short, these cells abort the postzygotic developmental program. Alternatively, 10% of pairs involving an A* partner complete postzygotic development resulting in MA formation in both partners. Most of these pairs die (DOERDER and SHABATURA 1980) but the few survivors, known as short-circuit genomic exclusion progeny (see BRUNS, BRUSSARD and KAVKA 1976), exhibit poor fertility associated with micronuclear aneuploidy. It has recently been observed that star partners can, on occasion, produce a pronucleus that participates in nuclear exchange (E. MARLO NELSEN, personal communication). Such a pronucleus would of necessity be aneuploid (since it is derived from a "star cell's" micronuclear fragment). If the star cell's aneuploid pronucleus successfully fused with the diploid partner's pronucleus, the resulting synkaryon would exhibit exactly the kind of aneuploidy that we have observed cytologically for short-circuit genomic exclusion progeny (E. MARLO NELSEN, personal communication).

We have demonstrated that a hyperosmotic shock delivered at or slightly before the time of the third prezygotic nuclear division, produces a third type of progeny, the uniparental cytogamont. This treatment

creates conditions that trigger MA formation in one of the two partners in a "star" mating. This occurs in 30% of pairs when treated under optimal conditions, and may result either from blocked nuclear transfer and self-fertilization, or from a failure of the third prezygotic mitosis within the diploid partner. In either case, the postzygotic developmental program is triggered in a way that produces predominantly fertile, diploid offspring.

These results raise an old question: "what are the conditions that are necessary and sufficient to stimulate postzygotic development leading to MA formation?" Early studies based on the use of the anti-microtubule drugs vinblastine and nocodazole, narrowed the field of possible "triggering events" down to those events occurring sometime at or after the third prezygotic division, and prior to nuclear exchange (HAMILTON 1984; KACZANOWSKI *et al.* 1991). Pronuclear exchange itself was ruled out because of the success of conventional cytogamy which prevents nuclear exchange and yet generates MA. Pronuclear fusion has also been effectively eliminated as a necessary condition for the completion of postzygotic development by blocking this stage with vinblastine or nocodazole and yet observing MA formation (HAMILTON, SUHR-JESSEN and ORIAS 1988; KACZANOWSKI *et al.* 1991). Consequently, we must focus either on the association of two migratory pronuclei (one from each partner) with the exchange junction, or the third prezygotic division itself as potential "trigger events" or checkpoints regulating entry into the postzygotic developmental pathway. A third, and simplistic possibility is that any conditions that produce a diploid nucleus (actually tetraploid, see below) at the exchange junction at the appropriate time during development may provoke postzygotic development.

Meiotic products and gametic pronuclei have both been shown to possess a 2C complement of DNA due to synthesis immediately following anaphase of the second meiotic division and the third prezygotic division respectively. This results in the production of a 4C zygotic nucleus after fertilization (DOERDER and DEBAULT 1975; and DOERDER and SHABATURA 1980). Osmotic shock could provoke postzygotic nuclear development by generating a 4C nucleus at the exchange junction at the appropriate stage in development by blocking the third prezygotic nuclear division without blocking DNA replication. Evidence supporting this "nuclear ploidy model" comes from the observation that the peak of responsiveness to osmotic shock-induced UPC coincides with the peak appearance of pairs displaying the third, prezygotic division spindle. Furthermore, the frequency of occurrence of UPC progeny is similar to the frequency of pairs at this stage at the time when the shock is delivered. This hypothesis predicts that any treatment resulting in the

production of a 4C nucleus at the exchange junction (at the appropriate time) should trigger postzygotic development, even in first-round genomic exclusion matings. Experiments are underway in our laboratory to test this hypothesis.

UPC and precocious maturity: One of the practical limitations to the efficiency of genetic experimentation with *Tetrahymena* is that following a successful mating, progeny are incompetent to pair again for 50–80 cell divisions, approximately 1–2 weeks depending upon culture conditions (BLEYMAN 1971; ROGERS and KARRER 1985). This has been termed the immaturity interval (NYBERG and BISHOP 1981). Attempts have been made to circumvent this immaturity interval by searching for mutations that confer an early maturity phenotype. Most such attempts have resulted in the isolation of dominant “early mature” mutations (BLEYMAN and SIMON 1967), which also exhibit a recessive lethal phenotype (they are viable only as heterozygotes). Such mutations are of limited usefulness. Only one search has been successful in producing a non-lethal early mature mutant (E. ORIAS, personal communication). This *Prc* (precocious) mutation exhibits an early onset of “adolescence,” *i.e.*, the ability to pair with a fully mature partner, but not with another “adolescent.”

Normal exconjugants can be provoked to exhibit early maturity by subjecting mating pairs to some form of environmental trauma during or shortly after mating. NANNEY and MEYER (1977) proposed that this response of exconjugant cells to stress (induced either environmentally or due to an unhealthy phenotype) may represent an adaptive strategy that allows individuals with maladaptive combinations of alleles in their newly reconstituted macronuclear genome to immediately resample from the local gene pool. There are two ways in which UPC-induced precocity could be due to such a “stress response.” First, the osmotic shock itself could provide sufficient environmental stress to provoke precocious maturity. This would constitute a nongenic source of environmental stress. Second, if a heterokaryon possessed chromosomal abnormalities (or deleterious alleles) in the micronuclear genome, these could be expressed in a newly constituted (homozygous) macronucleus and result in exconjugant trauma as the cell attempts to survive with a deleterious macronuclear genotype.

The fact that three out of four different heterokaryons exhibited elevated levels of precocious maturity following UPC, suggests that it is the osmotic shock, and not the genotype of the heterokaryon that provokes precocious exconjugant maturity. We tested whether CU438 (in particular) resulted in early maturity as a result of a defective micronuclear genome in two ways; by “rejuvenating” the CU438 heterokaryon through a first-round genomic exclusion mating

and screening for highly fertile progeny, and assessing the level of precocious maturity among the UPC progeny of these rejuvenated lines, and by testing the fertility of precocious UPC progeny. Results from both experiments suggested that the heterokaryon genome was fertile, and that UPC-induced precocity was due to the osmotic shock procedure rather than to preexisting micronuclear damage in the heterokaryon.

These results raise the question of whether diploid heterokaryons would produce precocious progeny following conventional cytogamy which also employs osmotic shock, or if it was the combination of osmotic shock applied to genomic-exclusion matings (or perhaps to A*V matings specifically) that provide the conditions necessary to provoke this phenomenon. Further work will be required to answer these questions.

The fact that some heterokaryons exhibited higher levels of early maturity than others suggests that there is also genic variation between heterokaryons causing some to exhibit early maturity more readily in response to osmotic shock. Attempts to increase precocious maturity among UPC progeny by administering a second environmental shock such as calcium chloride (NANNEY and MEYER 1977) at 12 hr resulted in profoundly diminished fertility, and hence such procedures were abandoned. Nevertheless, the elevated frequency of precocious maturity among UPC progeny should be useful in accelerating genetic analysis, at least for work with specific strains.

Prospects: We have demonstrated that delivering a hyperosmotic shock to first-round genomic exclusion pairs under proper conditions can be an effective method for creating whole-genome homozygotes from drug-resistance heterokaryons. These progeny are viable and fertile, and some strains exhibit a marked tendency toward precocious sexual maturity. We have also demonstrated that this technique is useful in generating new, homozygous mutants. During our first, trial runs, we produced several vinblastine-resistant mutants, and a nonconditional mutation affecting cortical patterning reminiscent of *mpD* (see FRANKEL *et al.* 1984). We are currently employing this procedure in a screen for mutations that affect the conjugation pathway itself, and our preliminary findings have been extremely promising. We present this as a simplified, superior method for generating whole-genome homozygous mutants.

This work was funded in part by the U.S. Department of Agriculture Cooperative State Research Service (Hatch project 186414). Funds were awarded through the Cornell University Agricultural Experiment Station. The authors gratefully acknowledge JOSEPH FRANKEL, E. MARLO NELSEN and JACEK GAERTIG as well as LESLIE MEEK JENKINS and KATHLEEN STUART for careful criticism of the manuscript. The authors would especially like to thank JACEK GAERTIG for sharing his insights into the possible mechanism behind

cytogamy, and E. MARLO NELSEN for his insights regarding short-circuit genomic exclusion.

LITERATURE CITED

- ALLEN, S. L., 1967a Genomic exclusion: a rapid means for inducing homozygous diploid lines in *Tetrahymena pyriformis*, syngen 1. *Science* **155**: 575-577.
- ALLEN, S. L., 1967b Cytogenetics of genomic exclusion in *Tetrahymena*. *Genetics* **55**: 797-822.
- ALLEN, S. L., S. K. FILE and S. L. KOCH, 1967 Genomic exclusion in *Tetrahymena*. *Genetics* **55**: 823-837.
- ALLEN, S. L., S. L. WEREMIUK and C. A. PATRICK, 1971 Is there selective mating in *Tetrahymena* during genomic exclusion? *J. Protozool.* **18**: 515-517.
- BLEYMAN, L. K., 1971 pp. 67-91 in *Developmental Aspects of the Cell Cycle*, edited by I. CAMERON, G. PADILLA and D. ZIMMERMAN. Academic Press, New York.
- BLEYMAN, L. K., and E. M. SIMON, 1967 Genetic control of maturity in *Tetrahymena pyriformis*. *Genet. Res.* **10**: 319-321.
- BRUNS, P. J., and T. B. BRUSSARD, 1974 Positive selection for mating with functional heterokaryons in *Tetrahymena pyriformis*. *Genetics* **78**: 831-841.
- BRUNS, P. J., T. B. BRUSSARD and A. B. KAVKA, 1976 Isolation of homozygous mutants after induced self-fertilization in *Tetrahymena*. *Proc. Natl. Acad. Sci. USA* **73**: 3243-3247.
- BRUNS, P. J., and Y. M. SANFORD, 1978 Mass isolation and fertility testing of temperature-sensitive mutants in *Tetrahymena*. *Proc. Natl. Acad. Sci. USA* **75**: 3355-3358.
- DOERDER, F. P., and L. E. DEBAULT, 1975 Cytofluorimetric analysis of nuclear DNA during meiosis, fertilization, and macronuclear development in the ciliate *Tetrahymena pyriformis*, syngen 1. *J. Cell Sci.* **17**: 471-493.
- DOERDER, F. P., and S. K. SHABATURA, 1980 Genomic exclusion in *Tetrahymena thermophila*: a cytogenetic and cytofluorometric study. *Dev. Genet.* **1**: 205-218.
- DRYL, S., 1959 Antigenic transformation in *Paramecium aurelia* after homologous antiserum treatment during autogamy and conjugation. *J. Protozool.* **6** (Suppl.): 25.
- FRANKEL, J., E. M. NELSEN, J. BAKOWSKA and L. M. JENKINS, 1984 Mutational analysis of patterning of oral structures in *Tetrahymena*. II. A graded basis for the individuality of intracellular structural arrays. *J. Embryol. Exp. Morphol.* **82**: 67-95.
- HAMILTON, E. P., 1984 Dissection of fertilization and development in *Tetrahymena* using antimicrotubule drugs. Ph.D. Thesis, University of California at Santa Barbara.
- HAMILTON, E. P., P. B. SUHR-JESSEN and E. ORIAS, 1988 Pronuclear fusion failure: an alternative conjugation pathway in *Tetrahymena thermophila* induced with vinblastine. *Genetics* **118**: 627-636.
- KACZANOWSKI, A., M. RAMEL, J. KACZANOWSKA and D. WHEATLEY, 1991 Macronuclear differentiation in conjugating pairs of *Tetrahymena* treated with the anti-microtubule drug nocodazole. *Exp. Cell Res.* **195**: 330-337.
- MERRIAM, E. V., and P. J. BRUNS, 1988 Phenotypic assortment in *Tetrahymena thermophila*: assortment kinetics of antibiotic-resistance markers, tsA, death, and the highly amplified rDNA locus. *Genetics* **120**: 389-395.
- NANNEY, D., and E. B. MEYER, 1977 Traumatic induction of early maturity in *Tetrahymena*. *Genetics* **86**: 103-112.
- NELSEN, E. M., and L. E. DEBAULT, 1978 Transformation in *Tetrahymena pyriformis*-description of an inducible phenotype. *J. Protozool.* **25**: 113-119.
- NYBERG, D., and P. BISHOP, 1981 The immaturity interval in *Tetrahymena*: genetic and environmental sources of variation. *Dev. Genet.* **2**: 159-170.
- ORIAS, E., and P. J. BRUNS, 1976 Induction and isolation of mutants in *Tetrahymena*. *Methods Cell Biol.* **13**: 247-282.
- ORIAS, E., and E. P. HAMILTON, 1979 Cytogamy: an inducible, alternate pathway of conjugation in *Tetrahymena thermophila*. *Genetics* **91**: 657-671.
- ORIAS, E., E. P. HAMILTON and M. FLACKS, 1979 Osmotic shock prevents nuclear exchange and produces whole-genome homozygotes in conjugating *Tetrahymena*. *Science* **203**: 660-663.
- PITTS, R. A., 1979 Age-associated micronuclear defects of *Tetrahymena thermophila*: genetic and cytogenetic studies. Ph.D. Thesis, University of Pittsburgh.
- ROBERTS, C. T., and D. E. MORSE, 1980 Galactokinase-deficient mutants of *Tetrahymena thermophila*: selection and characterization. *Mol. Gen. Genet.* **180**: 129-134.
- ROGERS, M. B., and K. M. KARRER, 1985 Adolescence in *Tetrahymena thermophila*. *Proc. Natl. Acad. Sci. USA* **82**: 436-439.
- SANFORD, Y. M., and E. ORIAS, 1981 Phenylketonuric *Tetrahymena*: phenylalanine hydroxylase mutants and other tyrosine auxotrophs. *Proc. Natl. Acad. Sci. USA* **78**: 7614-7618.
- WEINDRUCH, R. H., and F. P. DOERDER, 1975 Age-dependent micronuclear deterioration in *Tetrahymena pyriformis*; syngen 1. *Mech. Ageing Dev.* **4**: 263-279.

Communicating editor: S. L. ALLEN