populations is most striking in the dunes and least evident in the marsh. Considering eight nearest neighbors per individual, the mean proportions of intraclonal contacts are, respectively, for dune, swale, and marsh: 0.29 ± 0.03, 0.18 ± 0.02, and 0.03 ± 0.01. These are significant departures from random expectations, which are, respectively, 0.18 ± 0.02, 0.06 ± 0.01 and < 0.01. The distribution of clones within the dune quadrat reflects local microtopography (*5*). A dune in the southeast third of the quadrat and another in the northwest third are dominated, respectively, by genotype 11, and genotypes 1 and 2. A shallow depression between dunes running northeast to southwest is dominated by genotypes 5 and 6. The swale and marsh quadrats were located in flat, more homogeneous sites. Patchy distributions of clones here could not be related to any microtopographic features or to any range of microenvironmental variables (*5*). Therefore, although environmental heterogeneity cannot be ruled out, chance or historical effects (or both) may play an important part.

The maps of genotype distribution (Fig. 1) also show significant differences in clone size among subpopulations. Large clones, in terms of proportional representation per unit area, predominate in the dune while many rare clones prevail in the marsh (Table 2). This is reflected in a greater genotypic diversity in the marsh than in the dune. Shannon indices of genotypic diversity calculated for dune, swale, and marsh were 2.11, 3.01, and 3.63, respectively. Alternatively, clone size may be viewed as the extent of vegetative spread, which was estimated as the maximum distance between two members of a clone (Table 2). Relatively little difference is seen among subpopulations in the spread of clones, although rare clones may spread further in the swale and in the marsh. In all subpopulations the potential spread of clones is more than 10 m. Some individuals in all populations will produce over 10 m of rhizomes within one season in a common garden, and individual tillers can be followed for more than 10 m in the field in both dune and swale habitats (*5*). The potential exists for individual clones to spread among subpopulations over several years. That this occurs rarely provides evidence that selection among perennating clones promotes local adaptation and restriction to narrow niches.

The foregoing analysis emphasizes the importance of small-scale sampling of clonal populations to resolve differences in the occurrence and pattern of the distribution of genotypes. It also points up the problems in determining genetic structure and estimating gene frequency in vegetatively spreading populations. Estimates of gene frequency and heterozygosity differ, depending on whether one considers individual reproductive units that make up a clone or genetically individual clones (*5*). Similarly, estimates of breeding population size necessitate the identification of genetic individuals within a population.

Thus, the dune subpopulation comprises a small number of common clones. The low genotypic diversity reflects a harsh environment with intense, largely density-dependent selection pressure sieving out a small number of genotypes capable of surviving. These genotypes allocate a greater proportion of available resources to reproduction, incorporating many opportunistic and colonizing characteristics. By contrast, the marsh and swale subpopulations consist of a larger number of interdigitating rare, but spreading, clones. Higher density-dependent regulation has selected genotypes with a greater proportion of biomass devoted to nonreproductive activity. These observations agree with theoretical expectations and observations made on a number of plant species (*6*). In all sites subpopulations are distinct. That interpopulation migration is restricted is supported by independent estimates of gene flow (*5*). As a result, interbreeding populations are small. Selection pressures in different subpopulations are intense. The striking degree of evolutionary divergence among subpopulations is enhanced by a flexible breeding system and a high level of genetic variation among and within populations.

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Osmotic Shock Prevents Nuclear Exchange and Produces Whole-Genome Homozygotes in Conjugating *Tetrahymena*

Abstract. *Exposure of conjugating* Tetrahymena *to a hyperosmotic shock blocks the exchange of gametic nuclei and produces self-fertilized exconjugants that are homozygous for their whole genome. Cells are sensitive to this induction during a brief period after meiosis. The high efficiency of the treatment and the fertility of the progeny make this a useful method for the isolation of induced recessive mutations and enhances the value of* Tetrahymena *as an animal-cell model system in which genetic dissection is practical. The sharp peak of sensitivity is useful in the study of those cellular mechanisms responsible for the independent handling of several functionally distinct nuclei during conjugation.*

The single-celled ciliate *Tetrahymena thermophila* [formerly *T. pyriformis*, syngen 1 (*1*)] is a useful model system for the study of eukaryotic cell and molecular biology, since it can be cultured with ease and speed, crossed at will, and genetically manipulated (*2*). We and others (*3*) have been concerned with developing efficient methods to induce and isolate recessive mutants in order to increase its utility.

Tetrahymena (as a typical ciliate) maintains two functionally distinct nuclei within a common cytoplasm: the somatic (macro) nucleus and the germinal (micro) nucleus. Since the macronucleus is derived from the micronucleus during conjugation, procedures to isolate induced mutations must include a step in which the cells undergo some form of mating in between the step in which they receive mutagen treatment and the step in which phenotypes are selected. The isolation of cells expressing recessive mutations is further complicated by the diploid germinal nucleus; normal crosses of mutagenized cells yield heterozygotes. The problem does not arise in *Paramecium* because it spontaneously undergoes a type of self-fertilization called autogamy (*4*), a process not yet observed in *Tetrahymena*. In autogamy,

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a single cell undergoes meiosis, and two identical copies of a single meiotic product fuse with each other, creating an "instant" whole-genome homozygote.

A partial solution to the problems of generating mutants expressing recessive mutations, called "short-circuit genomic exclusion," has been described in *T. thermophila* (*3*). This process is induced by conjugation to a special strain (for example, the C* strain) having a defective germ line. The progeny are whole-genome homozygotes whose entire genetic information is derived from a single meiotic product of the normal mate. Not more than 5 percent of the progeny, however, develop new somatic nuclei and thereby express their new genetic information. This method has been successfully used to isolate recessive mutants (*3*), but in our experience the mutants so obtained are too frequently sterile.

While crossing normal *T. thermophila* strains we occasionally detected progeny whose phenotype could not have resulted from the cross-fertilization that normally occurs during conjugation. Instead, these progeny appeared to result from self-fertilization within each conjugal mate. A process of this type, called cytogamy, occurs in *Paramecium aurelia* (*4*) and amounts to conjugal mates undergoing autogamy together. We now report a reliable and simple method for inducing cytogamy in up to 50 percent of conjugating *T. thermophila* pairs.

The three major, genetically relevant events of normal conjugation (meiosis, reciprocal fertilization, and differentiation of new germ line and somatic nuclei) have been described (*5*). In normal conjugation the two haploid gametic nuclei within each mate are identical and each fertilization nucleus is derived from one gametic nucleus from each mate. Consequently, the two exconjugants are expected to be genetically identical but not necessarily homozygous, since the population of exconjugant pairs must conform to the standard Mendelian ratios.

Given the appropriate cross, self-fertilization can be distinguished from normal conjugation in two ways: the occurrence of unexpected homozygotes and the phenotypic dissimilarity between exconjugants. Two functional heterokaryons [strains having a micronuclear genotype different from their macronuclear phenotype (*6*)] were extremely useful because various outcomes of conjugation (cross-fertilization, cytogamy, or macronuclear retention) are phenotypically distinguishable (Table 1).

Table 1. Genotypes and phenotypes of strains used to detect self-fertilization.

Strains	Micronuclear genotype*	Macronuclear phenotype	Mating type
CU329	*ChxA2/ChxA2, Mpr⁺/Mpr⁺*	Sensitive to cycloheximide and 6-methylpurine	II
CU324	*Chx⁺/Chx⁺, Mpr/Mpr*	Sensitive to cycloheximide and 6-methylpurine	IV

*Both *Chx* and *Mpr* are dominant mutations conferring resistance to cycloheximide and 6-methylpurine, respectively (*20*). Both antibiotics were used at 15 μg/ml. See (*3*) for the strains construction method. The progeny of normal cross-fertilization should be resistant to both cycloheximide and 6-methylpurine. If the conjugal mates undergo cytogamy, one exconjugant should be resistant only to cycloheximide while the other should be resistant only to 6-methylpurine. Sometimes, for reasons that remain obscure, new macronuclei fail to develop and the old macronucleus remains functional [macronuclear retention (*21*)]; the progeny then continue to express the parental phenotype (sensitivity to both drugs, in this cross), even if their micronuclear genotype has changed.

Our initial crosses of these clones confirmed the occurrence of cytogamy, but its frequency was low and variable from day to day. A clue to the source of variability came in one experiment where a large number of pairs was isolated over a period of several hours, and a jackpot of cytogamies was discovered among a small group of consecutively isolated pairs. Since pairs are transferred from a buffered salts medium to rich proteose peptone growth medium when they are isolated, the time of transfer appeared to be the responsible factor.

To test this idea, an experiment was performed in which pairs were isolated into rich medium at timed intervals throughout a large portion of conjugation time. The results are shown in Fig. 1. A sharp peak of cytogamy (40 percent of the pairs) occurred when pairs were transferred to rich medium 4 3/4 hours after the cells were mixed. The frequency of cytogamy was very low (about 1 percent) on either side of this peak. We conclude that there is a short interval during conjugation when pairs are sensitive to blockage of nuclear transfer. The sharpness of the peak also indicates that a high degree of conjugation synchrony is maintained. Since cytogamy was not induced among pairs that were isolated into rich medium before 4 1/2 hours, we conclude that it was the shock of transfer at the sensitive period that induced the nuclear transfer block. Any inductive effects of a premature shock must have dissipated by the time the pairs entered the sensitive period.

The timing of the sensitivity peak at 30°C has been confirmed four more times with this pair of strains. A repeatable peak was seen also in crosses with other strains, but the exact timing varied slightly depending on the strain used (*7*). Cytological observations (*7*) indicated that at this time the most advanced pairs had completed meiosis and were at the stage of the prezygotic mitotic division that generates the two gametic nuclei. Self-fertilization could also be induced by similar "shocks" in crosses performed at other temperatures, but the timing of peak sensitivity varied with the temperature: 4 hours at 37°C, 4 3/4 hours at 30°C, and 9 hours at room temperature (23°C). Two experiments at 18°C yielded self-fertilizations, but without a very distinct peak; this could be because of poor synchrony in the conjugating population.

What feature of the transfer from buffered salts medium to proteose peptone is important for the induction of cytogamy? Since proteose peptone is an undefined nutrient broth, we tested instead various components of a chemically defined growth medium for *Tetrahymena* (*8*). Conjugating pairs were treated for 1 hour at 30°C, beginning at the peak induction time. High concentrations of glucose (≥ 1 percent) and the amino acid mixture of the defined medium (twice the prescribed concentration) both induced cytogamy, as did treatment with $CaCl_2$ (≥ 20 m*M*). A variety of sugars with different metabolic fates and some amino acids were then tested. If the concentration of the shock medium is expressed as the total osmolarity (Fig. 2), a threshold of induction occurs at around 70 mos*M* (*9*). The simplest interpretation is that hyperosmotic shock is the main cytogamy-inducing factor common to proteose peptone, amino acids, $CaCl_2$, and the various sugars tested, but the possibility that some of the compounds act by a different mechanism cannot be excluded. In order for the frequency of cytogamy to reach its maximum value, conjugating pairs must be exposed to the shock medium (proteose peptone or glucose) for at least 30 minutes (*7*).

Other genetic experiments (*10*) have shown that (i) the induction of cytogamy is not restricted to the strains used here, (ii) the nuclear genetic information of the cytogamous progeny is usually (if not always) derived from a single meiotic product in the cytoplasmic parent of each exconjugant, and (iii) no functional

genetic information is transferred across the cell boundary in cytogamy. The last conclusion has been confirmed by autoradiography (*11*). Although these genetic consequences are all very characteristic of cytogamy as described in *Paramecium*, we do not yet know if the precise sequence of intervening nuclear events is identical in both organisms.

The most important consequence of the ability to induce instant homozygotes is the improvement in the efficiency of recessive mutant isolation. In the absence of added mutagen, homozygotes are not only produced reliably and in high frequency, but most of them are fertile (*10, 12, 13*). For generating mutants by cytogamy, the protocol in Fig. 1 is slightly modified (*14*). A collection of mutants with recessive heat-sensitive phagocytosis has been isolated in our laboratory by using cytogamy (*15*). The use of heterokaryons carrying recently isolated recessive drug-resistance markers (*16*) allows the selection of cytogamous clones (that is, the elimination of normally cross-fertilized progeny as well as nonconjugants), by adding drug after the cells have conjugated. This in turn allows the use of automated equipment to screen efficiently for recessive mutants. Such mutants can now be generated virtually as efficiently as if the organism were haploid (*17*), thus increasing the utility of *Tetrahymena* as an animal-cell model system in which problems of cell and molecular biology can be approached through genetic dissection.

Also of some interest is the nature of

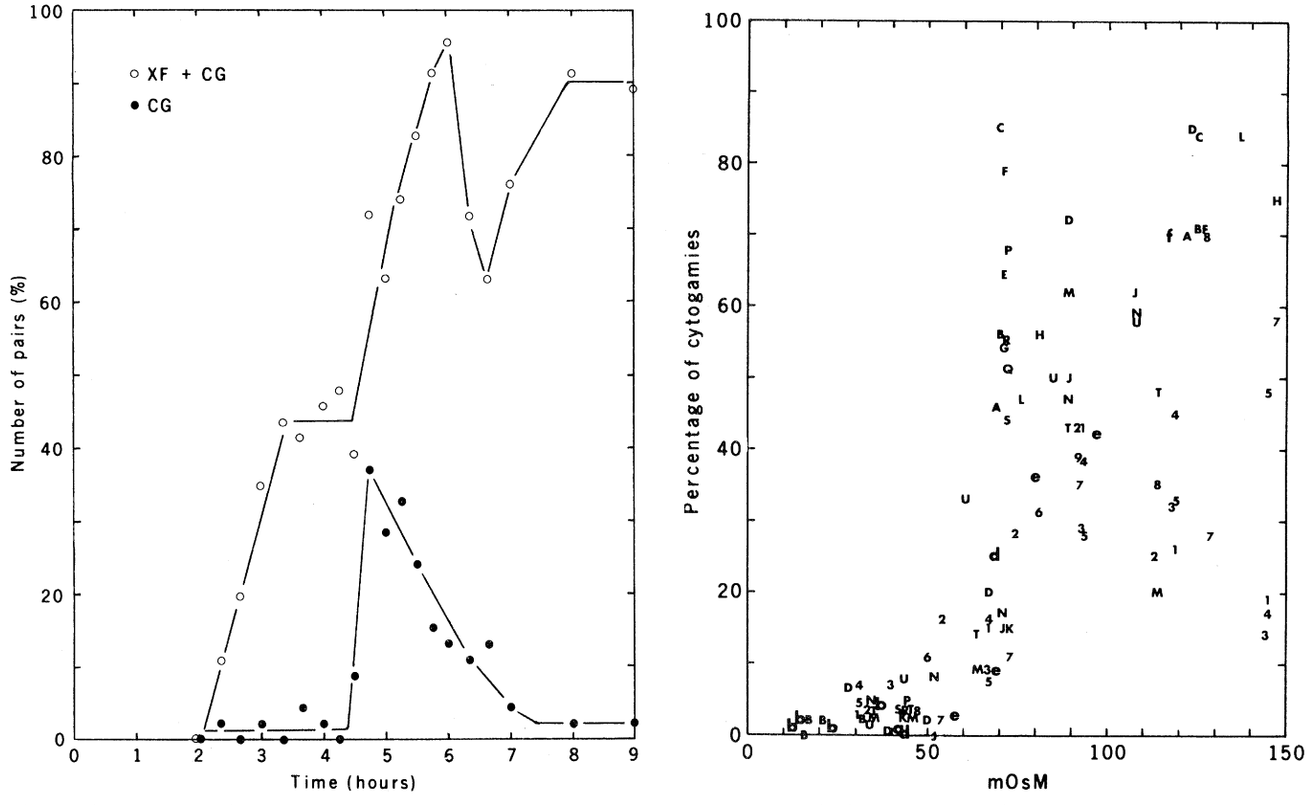

Fig. 1 (left). Induction of cytogamy by 2 percent proteose peptone medium. The abscissa indicates the time (after mixing competent cells) when pairs were isolated into peptone medium; the ordinate indicates the percentage of pairs that developed new macronuclei [cross-fertilization (*XF*) or cytogamy (*CG*)] or specifically cytogamy; 100 percent equals 46 pairs. The difference between each open circle and 100 percent represents the sum of deaths and macronuclear retentions (*22*). The cross procedure was as follows. The two strains were separately grown at 30°C in rich nutrient medium [2 percent proteose peptone supplemented with trace metal salts (*2*)]. The cells were washed and resuspended in Dryl's medium [phosphate-buffered calcium citrate (*23*)] and left overnight at 30°C. The two strains were then mixed at 30°C, each at a final concentration of 1×10^5 to 2×10^5 cells per milliliter. (Pairing under these conditions begins within 1 hour of mixing and lasts for about 12 hours. Routinely, 90 percent or more of the cells end up as pairs.) Forty-six pairs were separately isolated into 0.03-ml drops of rich medium at the various times and incubated at 30°C for three more days; under these conditions, the cells complete conjugation and the exconjugants undergo 10 to 12 binary fissions. These cultures were then replicated to the two antibiotics for phenotypic tests. Cells resistant to one antibiotic were replicated to the other antibiotic and the results were scored 3 days later. See (*2*) for more details. Fig. 2 (right). Dependence of cytogamy induction on osmolarity. The abscissa indicates the osmolarity of the shock medium; the ordinate indicates the percentage of cytogamies among pairs that developed a new macronucleus; each point is based on 46 pairs (100 percent). The protocol was as in Fig. 1, with the following exceptions. In most experiments tris-HCl buffer (10 m*M*, *p*H 7.5) was used as the starvation buffer. Test compounds were added at 4 hours 45 minutes. Thirty or 60 minutes later, the culture was diluted at least tenfold with medium known not to induce cytogamy (tris, Dryl's, or 1 percent peptone). At 7 hours, pairs were isolated into 1 percent proteose peptone. Treatments resulting in fewer than one-third of the pairs developing new macronuclei (predominantly at high osmolarities) were excluded. Osmolarities were measured with a Fiske osmometer (model G-62) for 50 m*M* $CaCl_2$ (148 mos*M*), 2 percent glucose (110 mos*M*), autoclaved 2 percent proteose peptone (100 mos*M*), tris buffer (15 mos*M*), and Dryl's medium (12 mos*M*). Osmolarities were calculated for dilutions and mixtures of these solutions and for solutions of the other sugars. The graph is a composite of many experiments. Each letter or number identifies a group of concentrations of the same compound run on the same day. Different symbols for the same compound means experiments done on different days. Capital letters denote sugars; numbers denote amino acids, peptides, or proteose peptone; and small letters denote $CaCl_2$. Monosaccharides: *A* to *G* = glucose; *H* to *K* = arabinose [transported but not metabolized (*24*)]; *L* = 2-deoxyglucose; *M* = fructose; and *N* = ribose. Disaccharides: *P* to *R* = sucrose [little or no transport (*18*)]; *S* = maltose; and *T* = lactose. Trisaccharide: *U* = raffinose. Amino acids and peptides; *1* and *2* = glycine; *3* = serine; *4* = Gly-Gly; *5* = Gly-Gly-Gly; *6* = amino acid mixture of Holtz's defined medium (*8*); *7* and *8* = proteose peptone medium; *9* = average of 27 experiments with 2 percent proteose peptone (39 ± 5.3 percent). $CaCl_2$, *a*, *b*, and *d* = $CaCl_2$; *e* = 10m*M* $CaCl_2$ supplemented with glucose; *f* = sodium acetate. Average cytogamy frequencies were 2.7 ± 0.04 percent for tris controls (19 experiments) and 1.1 ± 0.01 percent for Dryl's medium controls (seven experiments) (not plotted).

the induction process. How osmotic shock blocks nuclear transfer is far from clear. *Tetrahymena* cells respond to a hyperosmotic shock in two stages (*18*): a quick shrinking lasting a few minutes, followed by a return to normal volume. The second phase (which takes about 1 hour at room temperature) is accompanied by an increase in the intracellular concentration of certain ions and small molecules (mainly amino acids), resulting in an increase in the intracellular osmolarity, so that the cell regains its hyperosmolarity to the medium. The detailed cellular and molecular mechanism involved in this osmoregulation has not been elucidated. Also unknown is the mechanism of reciprocal transfer of migratory gametic nuclei across the partially fused membrane separating the two conjugating cells (*19*). A variety of compounds that affect microtubule assembly also caused cytogamy in *T. thermophila* at concentrations far too low to cause osmotic shock (*7*). This suggests an essential role for microtubules in the mechanism of gametic nuclear transfer.

Conjugation among ciliates is a remarkable developmental program that determines, within a common cytoplasm, the differential location, movement, expression, DNA replication, division, and ultimate fate of two (and later three) distinct types of nuclei. Furthermore, this program possesses enough regulatory potential to bypass some of the major events, for example, nuclear exchange and establishment of new macronuclei, without lethal consequences. The present work suggests the feasibility of exploiting experimental interference and genetic blocks to dissect the interesting cellular mechanisms involved.

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12. After mutagenesis, mutant fertility has varied. Six randomly chosen 2-deoxygalactose- and 2-deoxyglucose-resistant mutants obtained after cytogamy had high fertility (*13*). However, only 40 percent of temperature-sensitive food vacuoleless mutants isolated after cytogamy (*15*) were fertile although it is not excluded that some mutations of this type could also affect fertility as a pleiotropic effect.
13. C. T. Roberts, Jr., and E. Orias, in preparation.
14. A mutagen treatment step (*2*) is inserted just prior to the cross. Because of variability in the preparation of 2 percent proteose peptone medium, a 1-hour treatment with 1.5 percent (83 mos*M*) glucose in conjugation buffer is preferable for the osmotic shock, followed by a tenfold dilution in buffer, as in Fig. 2.
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Obesity Genes: Beneficial Effects in Heterozygous Mice

Abstract. *The mouse mutant genes obese (*ob*) and diabetes (*db*) cause similar obesity-diabetes states in homozygotes. These obesity syndromes are characterized by a more efficient conversion of food to lipid and, once stored, a slower rate of catabolism on fasting. Heterozygous mice, either* ob/+ *or* db/+*, survived a prolonged fast significantly longer than normal homozygotes (+/+); this suggests that the heterozygotes exhibited increased metabolic efficiency, a feature normally associated with both homozygous mutants. The existence of this thriftiness trait, if manifested by heterozygous carriers in wild populations, would lend credence to the thrifty gene concept of diabetes. Beneficial effects of normally deleterious genes may have played a role in the development of diabetes-susceptible human populations, as well as having provided the survival advantage that has allowed both the development and successful establishment of species in desert and other less affluent regions.*

Diabetes has been suggested to be the result of a "thrifty genotype rendered detrimental by progress" (*1*). In undeveloped countries humans foraged for a limited food supply and were subjected to periods of abundance alternating with periods of food deprivation and even famine. Those individuals (thrifty) with a predisposition to diabetes were able to utilize a limited food supply more efficiently and thereby maintained a selective advantage when food was scarce. However, as such countries developed and the food supply increased, or as representatives of such selected cultures moved to more affluent societies and became urbanized, the thrifty genotype became a liability rather than an asset. In situations of affluence, hyperinsulinemia occurred, obesity developed, the insulin synthesizing and secreting capacity of the pancreas was stressed, and diabetes often ensued. Persistence of this thrifty genotype may have provided the survival advantage that has allowed both the successful establishment of species inhabiting warm and arid climates and the persistence of the diabetes genotype in animal and human populations despite strong negative selective pressures.

The factors involved in conferring the thrifty genotype on diabetes-susceptible cultures are probably multiple and cannot be assessed readily in genetically diverse populations. The varying degrees of diabetes susceptibility are probably the results of a variety of deleterious and even beneficial genes acting in conjunction with each other. The chance of getting diabetes by any mechanism would depend on the interaction of several deleterious genes with the entire genome. If the thrifty genotype concept is to have any validity, there should be some selective advantage to the heterozygote populations under reasonably affluent or typical living conditions. However, no metabolic advantage of diabetes-like genes has been demonstrated for heterozygotes living in affluence, and it

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