Mass isolation and fertility testing of temperature-sensitive mutants in *Tetrahymena*  
(mutagenesis/short-circuit genomic exclusion)

PETER J. BRUNS AND YVONNE M. SANFORD

Section of Botany, Genetics and Development, Cornell University, Ithaca, New York 14853

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ABSTRACT \ A set of 239 heat-sensitive (38°) and 16 cold-sensitive (18°) conditional mutants of *Tetrahymena* has been generated by combining techniques to manipulate large numbers of clones with a method for the selection of self-fertilized cells after mutagen treatment. A simple technique is presented for determining the fertility of individual clones; 179 of the clones in this set (71%) are fertile. The fertile conditional mutants have already been shown to have lesions in a number of diverse functions, including nucleic acid metabolism, mobility, cell cycle, and cortical pattern.

The ciliated protozoan *Tetrahymena thermophila* (formerly *T. pyriformis*, syngen 1; see ref. 1) has been successfully used for various physiological, developmental, and genetic studies (see refs. 2 and 3 for reviews). The scope of genetic studies has been limited, however, because the number of available induced mutations has been small compared to many other organisms (including some other protozoa). Recent technological advances have expanded the opportunities to isolate many new independently induced mutations. One has been the adoption of 96-well microtiter plates and a host of matching accessories to facilitate manipulations with large numbers of clones (4, 5), including growing, washing into fresh reagents, and mass matings.

Another development has been the establishment of techniques whereby the two nuclei (the actively transcribed somatic macronucleus, and the silent germinal micronucleus) can be manipulated independently (6). This approach allows the creation of strains with positively selectable dominant mutations in the micronucleus (such as for drug resistance) but with macronuclei expressing the recessive phenotype (for example, sensitivity). Because new macronuclei containing the resistance mutations of the micronucleus can only develop by conjugation, addition of the drug provides a simple but extremely effective selection for cells that have completed conjugation. This approach has been used to select cells that have performed various kinds of self-mating after mutagenesis and have thereby become homozygous and capable of expressing new mutations (4, 7).

We report here the isolation of 253 independent temperature-sensitive mutants acquired by these new techniques. Moreover, because it has been observed by ourselves and others that strains isolated after induced mutagenesis frequently are sterile, a method is presented here that allows identification of the fertile clones among the mutants.

MATERIALS AND METHODS

Strains. All stocks were derived from inbred strain B-1868 except C*, which is a vegetative derivative of inbred strain C (8). All cells were grown in 1% proteose peptone (Difco). Following the previously suggested notation (6), micronuclear genotype is indicated by a three-letter code with a capital first letter if the mutation is dominant and a superscript + for wild type. Macronuclear expressed phenotype (including mating type if needed) is indicated by appropriate abbreviations in parenthesis.

The two unlinked dominant mutations ChzA2 and Mpr confer, when expressed, resistance to cycloheximide (cy) at 25 μg/ml and 6-methylpurine (6-MePur) at 15 μg/ml, respectively (5, 9).

Mutagen Treatment. A fresh solution (2 mg/ml) of 3-N-methyl-N'-nitroso-guanidine was prepared in 95% ethanol, and a sufficient amount was added to a logarithmic phase culture to produce a final concentration of 10 μg/ml. After 3 hr, the cells were washed three times in fresh peptone and allowed to recover overnight in peptone. After this mutagen treatment, growth does not resume for about 12–14 hr; there must have been little, if any, vegetative duplication of induced mutations during the recovery period.

Matings. By using described methods (10), starvation conditions necessary for mating were obtained by separately washing cells of different mating types twice in 10 mM Tris-HCl (pH 7.4) and then resuspending the separate cultures in 10 mM Tris to achieve a final cell concentration of 10⁶ cells per ml. These cultures were prestarved for 6 hr at 30°, mixed together, and allowed to mate for 6 hr.

Microtiter Methods. All microtiter plates were sterilized by exposure to ultraviolet light for 24 hr. Media, drug solutions, and cells were usually distributed to microtiter plates by using a multichannel dispenser (Cooke Laboratory Products, Alexandria, VA); if only a small number of wells needed filling, a Cooke permanent pipette (0.025 ml, 0.05 ml) was used. All plates were then scored with either a test reading mirror (Cooke) or a dissecting microscope. A metal-pronged replicator (4) was used to replicate clones into fresh microtiter plates.

For mating, clones were replicated to V-bottom microtiter plates (0.1 ml of peptone per well) and allowed to grow for 2 days at 30°. The plates were then centrifuged for 2 min at 900–1000 Xg in an International centrifuge equipped to hold the plates (Cooke centrifuge plate carriers). The supernate was removed by using a custom-made multichannel aspirator consisting of 96 small tubes (arrayed in the microtiter pattern) opening into a Lucite box to which a vacuum is applied. Each plate was then washed once in 10 mM Tris (centrifuging and aspirating as above), and a prestarved strain with appropriate markers was added in the final distribution of starvation medium. Cells were allowed to mate for 48 hr at 30°.

Drug Testing. An equal volume of drug dissolved in peptone was added to give a final concentration of 25 μg of cy and 15 μg of 6-MePur per ml.

Abbreviations: cy, cycloheximide; 6-MePur, 6-methylpurine.
RESULTS

As described (8, 11), crosses between normal strains and strain C* do not follow the normal course of events of conjugation. C* has a defective micronucleus which disappears in an early phase of conjugation. The other conjugant's micronuclear genome still undergoes meiosis, mitosis of one of the resulting haploid products to produce a stationary and a migratory pronucleus, and transfer of the migratory pronucleus to the C* conjugant. Because C* offers no pronucleus in return, the two conjugants have a haploid germinal nucleus, which becomes diploid (and therefore homozygous) by a DNA synthesis in the absence of nuclear division (F. P. Doerder, personal communication). Although most pairs then fail to complete the development of a new macronucleus from the new homozygous micronucleus, about 5% of the pairs apparently do, with the exconjugants expressing genes from the parental micronuclei. This process has been termed short-circuit genomic exclusion (7).

To facilitate selection for conjugants that have developed new macronuclei, cells homozygous for the dominant mutation Mpr in the micronucleus, but with macronucleus expressing the wild-type recessive allele (and therefore sensitivity), were manipulated as described in Fig. 1. Strains of this type have been termed heterokaryons (6) because, although the micro- and macronuclei always have different structure and function, in these strains they also have different genotypes. They were exposed to mutagen, mated to C*, diluted to various concentrations in peptone, and distributed to sterile microtiter plates. An equal volume of double strength 6-MePur in peptone was subsequently added, and the plates were scored for frequency of wells with live cells (only cells that had completed short-circuit genomic exclusion and constructed new macronuclei would become resistant to the drug and survive). Although high frequencies of somatic resistance mutations have been found by B. C. Byrne (personal communication), they never occurred without longer growth between mutagen treatment and drug addition; the resistant cells selected in this study must have been progeny with new macronuclei.

Although it was impossible to determine whether a well containing resistant cells was inoculated with one or more than one resistant cell, the wells with no resistant cells were assumed to be the zero class of a Poisson distribution and were used to calculate the average inoculum of resistant cells per well. Poisson statistics predict that the zero class equals e^{-x}, in which x is the average inoculum and e is the base of the system of natural logarithms. Table 1 presents the data from four experiments of this type and indicates that, with this mutagen treatment, about 0.4% of the heterokaryons successfully completed short-circuit genomic exclusion. This frequency confirms previous observations (4, 7), and is about 0.5% that obtained in the absence of mutagen treatment. The last column in the table lists the expected number of wells inoculated with a single

Table 1. Cloning of potential mutants by inoculating microtiter plates with dilutions of short-circuit genomic exclusion mating mixtures

| Exp. | No. heterokaryons/well | No. wells inoculated | Freq. of wells with no 6-MePur-resistant cells | Average no. short-circuit/well | Freq. of short-circuit genomic exclusion | Calculated no. monoclones
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<tbody>
<tr>
<td>1</td>
<td>66</td>
<td>960</td>
<td>0.74</td>
<td>0.30</td>
<td>0.30/66 = 0.0045</td>
<td>214</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>960</td>
<td>0.65</td>
<td>0.43</td>
<td>0.43/100 = 0.0043</td>
<td>269</td>
</tr>
<tr>
<td>3</td>
<td>150</td>
<td>960</td>
<td>0.53</td>
<td>0.63</td>
<td>0.63/150 = 0.0042</td>
<td>323</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
<td>960</td>
<td>0.44</td>
<td>0.82</td>
<td>0.82/200 = 0.0041</td>
<td>347</td>
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<td>Total 1153</td>
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* Calculated by assuming that frequency of wells with no resistant cells was the zero class in a Poisson distribution.
† See text.
maturity by serially transferring microtiter cultures with a replica plate (5) and then assigned to one of the following categories: (i) fertile, (ii) sterile but able to pair (C*-like), and (iii) sterile and unable to pair. The approach is based on the use of heterokaryons homozygous in the micronucleus for a dominant mutation (ChxA2; see ref. 5) that confers resistance to cy but with a macronuclear expressing sensitivity. To ensure mating with all clones, ChxA2-bearing cy-sensitive heterokaryons of two different mating types (II and IV) were used in separate replicate crosses, because the mating types of the mutants were unknown. Three control wells were inoculated to ensure accuracy of the procedure: one contained C* (cy-sensitive, 6-MePur-sensitive, III), another Mpr/Mpr (6-MePur-sensitive, IV), and the last ChxA2/ ChxA2 (cy-sensitive, II). Fig. 2 presents the protocol for the test, and Fig. 3 indicates the genotype and phenotype of parents and progeny for each category, as explained below.

Use of V-bottom microtiter plates made it possible to test the large number of clones generated in this study. All operations (culturing the mutant clones, mating with the cy functional heterokaryons, and testing progeny for drug resistances) were performed sequentially in the same plate. If the unknown clone is fertile (category 1), cross fertilization will occur and new macronuclei expressing the dominant phenotype from each parent will result; the well will contain cells resistant to both drugs. If the unknown is C*-like (category 2), the test yields progeny that are cy-resistant and 6-MePur-sensitive because only genes from the non-C*-like parent are retained in the progeny of such crosses. If the unknown clone is unable to pair or to survive pairing (category 3), only unmated parental cells (some resistant to 6-MePur but none resistant to cy) result. These three categories are easily identified if the first drug added is the one to which the heterokaryon confers resistance—in this case, cy. The results, presented in Table 3, indicate that 71% of the clones are fertile.

**DISCUSSION**

A combination of several genetic and cell culture techniques has implemented the isolation and fertility testing of large numbers of temperature-sensitive mutants in *Tetrahymena*. Heterokaryons, short-circuit genomic exclusion, cloning by diluting cell suspensions, and extensive use of the microtiter system have all been used. The 90% decrease in yield of short-circuit genomic exclusion progeny (0.4% versus 4–5%) after mutagen treatment is in agreement with previous studies (4, 7). Orias and Bruns (4) reported the same yield of viable homozygotes after mutagenesis and a self-mating. Because that study did not use short-circuit genomic exclusion, we conclude that its use does not impose an added degree of inviability. Although a certain, and not trivial, amount of infertility is found among the selected clones, the technique outlined here provides a direct means for screening against clones whose genotype cannot be sexually inherited and therefore may not be experimentally useful. It should be noted that clones with low fertility (<1% normal conjugants) could appear as fertile clones in the mass test. Although many of the clones that tested as fertile yielded viable progeny in subsequent crosses, one of them gave no viable progeny when it was crossed to wild type and individual pairs

<table>
<thead>
<tr>
<th>Mutant clone</th>
<th>No. observed</th>
<th>Total no. tested</th>
<th>Frequency</th>
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<tr>
<td>Fertile</td>
<td>179</td>
<td>253</td>
<td>0.71</td>
</tr>
<tr>
<td>Sterile (C*-like)</td>
<td>68</td>
<td>253</td>
<td>0.27</td>
</tr>
<tr>
<td>Sterile (nonmater)</td>
<td>6</td>
<td>253</td>
<td>0.02</td>
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were isolated. However, mass crosses and selection for double resistant progeny (as in the fertility test presented here) succeeded in yielding progeny of the mutant clone that were fertile (J. Frankel and L. M. Jenkins, personal communication). Part of the infertility probably reflected micronuclear genetic erosion found in most, if not all, clones of Tetrahymena, whether or not exposed to a mutagen (see ref. 12, pp. 84–85, for a review of genetic death in Tetrahymena). Because the clonal deterioration is a function of growth after the conjugation that originated the sexual generation, the set of mutants is now stored frozen in liquid nitrogen (see refs. 13 and 14 for this technique).

The work presented in this report has made available a large number of new mutant clones in Tetrahymena. Hallberg et al. (15) have studied patterns of RNA and protein accumulation at permissive (30°C) and non-permissive (38°C) temperatures with these mutant strains in exponential growth. Conditional mutants in both RNA and RNA-protein accumulation were identified. Further analysis of the RNA accumulation mutants revealed six clones defective in ribosomal RNA synthesis and/or processing. Other work in our laboratory (unpublished data) has demonstrated two clones with altered swimming ability. Although normal at 30°C, both lose the ability to swim at 38°C; upon return to 30°C, normal swimming quickly returns. Preliminary genetic analyses indicate recessive mutations at two complementing loci. Several other isolates manifest cell cycle blocks that appear similar to those in Tetrahymena mutants isolated by other means (J. Frankel, personal communication; refs. 16 and 17). A recessive mutation has been recovered that causes a major alteration in cortical pattern (J. Frankel and L. M. Jenkins, personal communication). Further genetic analyses of several of the mutations mentioned above indicated recessive mutations showing normal patterns of inheritance. We have not yet analyzed enough mutants to determine if the manipulations presented here yield a bias in the type of mutant phenotype or mutant allele that is recovered.

We conclude that this set of mutants includes many extremely interesting clones, defective in a wide variety of functions. More importantly, the techniques and strategies presented here allow the continued isolation of many new fertile mutant cell lines in Tetrahymena.

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3. Elliott, A. M. (1973) Biology of Tetrahymena. (Dowden, Hutchinson, & Ross, Stroudsberg, PA.)