MUTATIONS AFFECTING CELL DIVISION IN *TETRAHYMENA PYRIFORMIS*. I. SELECTION AND GENETIC ANALYSIS[1]

JOSEPH FRANKEL, LESLIE M. JENKINS, F. PAUL DOERDER[2] AND E. MARLO NELSEN

Department of Zoology, University of Iowa, Iowa City, Iowa 52242

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ABSTRACT

Fourteen nitrosoguanidine-induced mutations that bring about temperature-sensitive morphological abnormalities resulting from a specific effect on cell division have been isolated as heterozygous phenotypic assortants in *Tetrahymena pyriformis* syngen 1. Genetic analysis revealed all to be single-gene recessives. Detailed analysis of the kinetics of assortment for one of the mutated alleles revealed a rate (0.0104 pure lines per fission) consistent with that previously observed at other loci in this organism. The mutations fall into six complementation groups (*mo1*, *mo2*, *mo3*, *mo6*, *mo8*, and *mo12*). Homozygotes of *mo2* are unconditionally expressed, while all alleles of *mo1*, *mo6*, *mo8*, and *mo12* are heat sensitive for division arrest. At the *mo3* locus two alleles are heat sensitive, one is primarily cold sensitive, while two are sensitive to both heat and cold. Two out of three combinations of different *mo3* alleles show conventional Mendelian segregation of conditions of expression. Different alleles of *mo1*, *mo3*, *mo8*, and *mo12* also manifest differences in penetrance at the restrictive temperature. Despite these differences involving expression, the abnormal phenotypes themselves are locus-specific and distinctive; in the one case (*mo1*^{a} and *mo1*^{b}) in which two alleles manifest somewhat different phenotypes, the F_1 between them is intermediate. One additional recessive mutation (*fat1*) brings about a nonconditional lengthening of the cell cycle, with some arrest of cell division at the restrictive temperature. These findings demonstrate that selection of heterozygotes undergoing phenotypic assortment can be an effective method for obtaining substantial numbers of a desired class of temperature-sensitive mutations in *T. pyriformis*.

THE study of temperature-sensitive mutations affecting specific steps of the cell division cycle offers a potentially powerful approach toward characterizing these steps and analyzing their interrelationship (HARTWELL *et al.* 1974). A satisfactory application of this mode of study demands an easily grown cellular system in which mutations that bring about blockage in specific cell cycle stages can readily be screened and in which genetic loci can be delineated by segregation and complementation analysis. No animal cell system fulfills all of these criteria as ideally as does the budding yeast (*Saccharomyces cerevisiae*) studied by HARTWELL and his collaborators (HARTWELL *et al.* 1973). However, at least two

[1] A preliminary report of this investigation has been published in the proceedings of a conference (FRANKEL *et al.* 1975).

[2] Present address: Department of Life Sciences, University of Pittsburgh, Pittsburgh, Pa. 15260.

species-groups of ciliated protozoans, the *Tetrahymena pyriformis* and "*Paramecium aurelia*" complexes, do meet the basic genetic requirements for relatively easy selection as well as genetic analysis of recessive mutations. "*P. aurelia*" provides instantaneous homozygosity and total inbreeding through autogamy (SONNEBORN 1947), while *T. pyriformis*, though not capable of autogamy, manifests an enigmatic but useful phenomenon known as macronuclear or phenotypic assortment (ALLEN and NANNEY 1958; NANNEY 1964) that limits expression to only one allele at virtually every known locus (see ALLEN and GIBSON 1973; SONNEBORN 1974). In macronuclear assortment, either allele at a genetic locus may be expressed by the cell, with the choice bearing no relation to conventional dominance, while the micronucleus remains totally unaffected by the process and can transmit alleles regardless of which allele happens to be expressed (NANNEY and DUBERT 1960). In effect, the same cells become functionally homozygous (or haploid) with respect to expression but remain heterozygous (diploid) with respect to transmission. CARLSON (1971) was the first to adapt this feature as the basis for a mutant screening protocol. This same basic method was carried further by DOERDER (1973) in a successful search for serotype mutations and by DOERDER *et al.* (1975) in the identification of a mutation affecting cell shape. We here apply this method for the screening and analysis of conditionally expressed mutations affecting cell division.

This report will consider the isolation and genetics of mutations at six loci affecting specific stages of cell division, and a mutation at another locus that has nonspecific effects on the cell cycle. A more detailed consideration of ways in which these mutations have been applied to characterize the developmental processes of this cell will appear elsewhere (FRANKEL, JENKINS and DEBAULT 1976; FRANKEL *et al.* 1977).

MATERIALS AND METHODS

Strains

Inbred strains of *Tetrahymena pyriformis*, syngen 1, were used in this study. Strains B and D, both in the 18th generation of inbreeding, were obtained from DR. DAVID L. NANNEY (for derivation and genotypes of these strains, see Tables 1 and 2 in ALLEN and GIBSON 1973, and Table 1 in BORDEN *et al.* 1973). Strain A* (pronounced A-star) is a derivative of strain A (WEINDRUCH and DOERDER 1975).

Media

1) Stock culture media:
   a. 1% proteose peptone (Difco)
   b. 1% proteose peptone + 0.1% Bacto yeast extract (Difco).
   c. 0.3% Bacto tryptone (Difco) + 0.5% dextrin (Difco) + vitamins + salts (TDVS medium) (see FRANKEL 1965 for detailed formula).
2) Media for studies of growth and development:
   a. 2% proteose peptone + 0.5% Bacto yeast extract (PPY medium).
   b. 0.3% Bacto tryptone + 0.5% glucose + vitamins + salts (TGVS medium). This is identical to the TDVS medium mentioned above, except that glucose is substituted for dextrin.
3) Medium for crosses and short-term maintenance of stocks undergoing genetic analysis: bacterized peptone (a 24-hour culture of *Enterobacter aerogenes* in 1% proteose peptone diluted 1/70 with distilled water prior to use).

4) Medium for the initial scoring of phenotypes and transfer of cells from bacterized to axenic cultures: either the PPY or TGVS medium supplemented with 1.4 g/l penicillin G (Pfizer) and 2.2 g/l streptomycin sulfate (Pfizer) (to be referred to as pen-strep PPY or TGVS).

Maintenance of stocks

Long-term stock cultures were maintained at 18° and 20° in 1% proteose peptone, either without or (later) with 0.1% yeast extract, in 5 ml aliquots in 20 × 150 mm culture tubes, with transfer every second week. Stocks undergoing current experimentation were maintained at 28°, initially in the TDVS medium and later in PPY medium, in 5 ml aliquots in 20 × 150 mm culture tubes kept at a slant, with transfer every day or every second day.

Procedures for making crosses

The basic methods of *Tetrahymena* genetics introduced by NANNEY (e.g., NANNEY and CAUGHEY 1955; NANNEY, CAUGHEY and TEFANKJIAN 1955) were followed with only minor modification. Procedures involving crosses were performed using bacterized peptone medium. Lines to be crossed were maintained in 13 × 100 mm culture tubes with feeding a day or two before the cross was made. Mating mixtures were made in the center well of three-spot depression slides ("spot plates", Corning no. 7223), with parental controls in the two lateral wells. Within twelve hours of formation of the first pair, a drop of bacterized peptone was added to induce separation of pairs not yet firmly united. Three hours later, pairs were individually isolated into drops of bacterized peptone. Typically some pairs failed to separate. while others separated and failed to divide or divided at most only two or three times; such pairs were considered "dead". The remainder of the pairs (synclones) were fed periodically and allowed to produce thriving cultures of several hundred cells, at which time one-half of the culture was withdrawn and tubed (with a small portion often removed for testing of phenotype, described below), while the remainder was immediately tested for true conjugation by addition of a sexually reactive tester of a nonparental mating type (cf. NANNEY, CAUGHEY and TEFANKJIAN 1955). Since true conjugation involving development of a new macronucleus results in sexual immaturity, all lines that mated at this time, with the exception of occasionally-appearing and distinguishable early-mature lines (BLEYMAN 1971), probably were descended from pairs that had not formed a new macronucleus. Such "nonconjugants" were thus not included among the progeny of a cross in tabulations such as those in Tables 1 and 2, but were considered in crosses in which anomalous genetic ratios were observed, since such anomalies might be caused by selective gametic death or abortion of macronuclear development.

If progeny of a cross were to be used in further crosses, then single cells (generally three in number) were isolated from each chosen synclone, and returned to depression slide culture. Each such clone was serially transferred by single cell isolations made every second day; sexual maturity was generally attained after four such transfers, 50–60 fissions after the previous conjugation. At this time the clones were tubed, fed, and the mating type of each clone ascertained by mixing small samples of each with approximately equal samples of "tester" clones representing the seven standard mating types. This test was performed in 96-well microtiter plates (Cooke Engineering Co.), and included controls of the unmixed clones to be tested, unmixed tester clones, and tester clones mixed with each other. Where possible, lines with infrequent mating types (NANNEY 1959) were kept for further crosses.

Certain crosses utilized "genomic exclusion" (ALLEN 1967). This involves a sequence of two successive crosses to a sterile ("star") strain with a defective micronucleus. The end result is to bring about recovery of genes only from the fertile partner, all in a homozygous state, with a 1:1 ratio of homozygotes for the two alleles at any originally heterozygous locus. Genomic exclusion crosses were performed according to the procedure of ALLEN (1967), using an A* (mt III and V) line (WEINDRUCH and DOERDER 1975) in place of ALLEN's C* as the sterile, defective partner.

The kinetics of phenotypic assortment were determined by methods similar to those of NANNEY and DUBERT (1960) and DOERDER (1973).

Mutations and screening

Mutations were obtained and selected following the method originally devised by CARLSON (1971) and further elaborated by DOERDER (1973). In outline, the procedure involves four basic

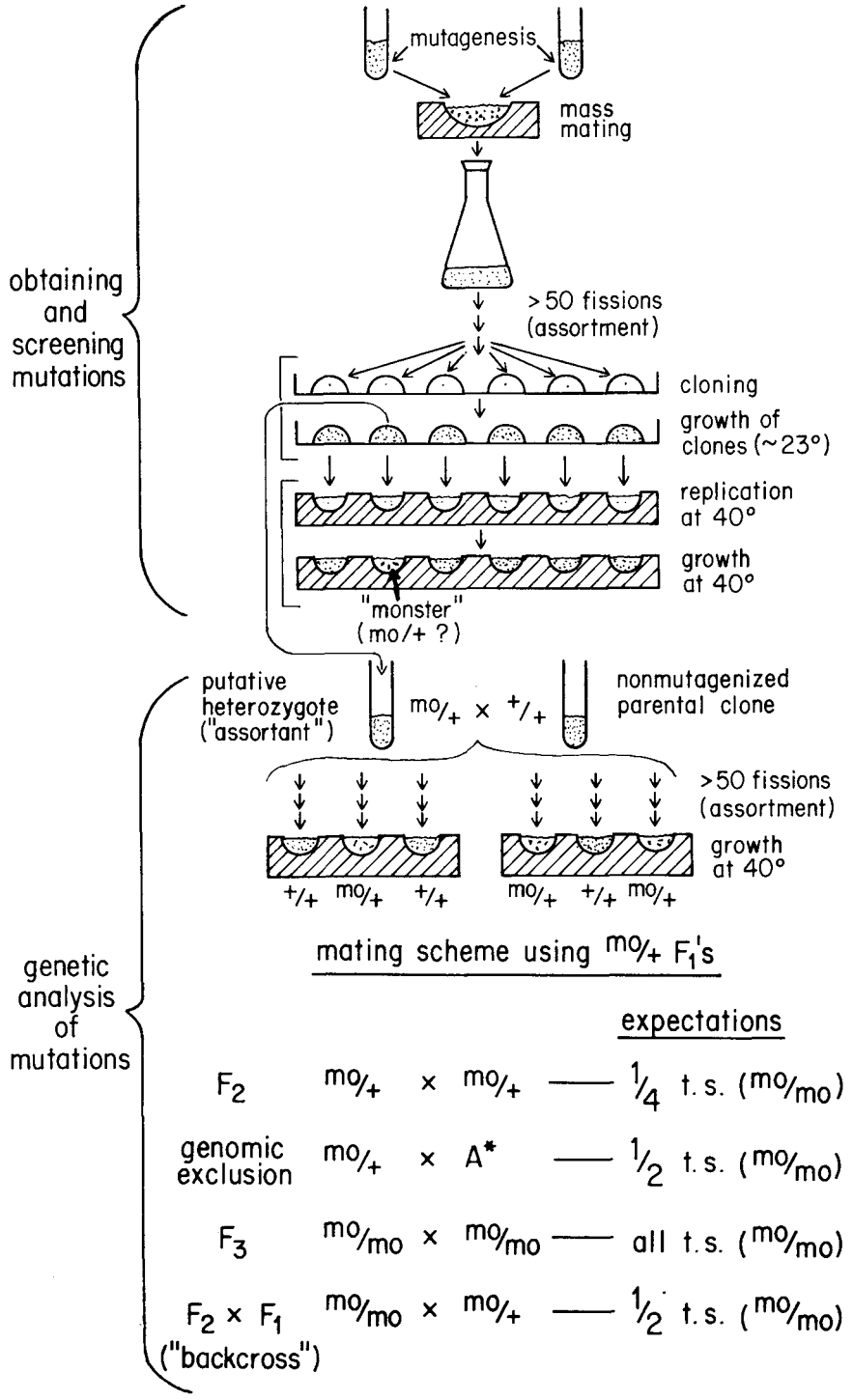

FIGURE 1.—Procedure for obtaining, screening, and analyzing morphologically abnormal mutants in *T. pyriformis*, syngen 1. For explanation, see the text.

steps (Figure 1, top): (1) Exposure of cells of two mating types to nitrosoguanidine; (2) a cross of these two mutagenized lines to bring micronuclear mutations into the macronucleus (generated anew at conjugation); (3) propagation of exconjugants for a sufficient number of cell generations to allow recessive alleles to come to expression as a consequence of macronuclear assortment; (4) isolation of single cells, formation of clones, subculturing, and scoring of subcultures at 39.5°. This procedure was executed as follows: cells of two different mating types (first D 1868 III and V; later B 1868 VI and VII) were separately grown under axenic conditions to mid-exponential phase in 10 ml of TGVS medium in two 20 × 150 mm culture tubes. N-methyl-N′-nitro-N-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wisconsin) was then added to each at a final concentration of 10 μg per ml. The two cultures were incubated with the mutagen while being shaken at room temperature for 4 hours in TGVS medium. The mutagenized cultures were then washed four times with glass distilled water to remove the mutagen and the cells resuspended in 20 to 30 ml of bacterized peptone. Each of these large cultures was subdivided into five 13 × 100 mm tubes and fed further with bacterized peptone. After 24 hours of growth, samples of cultures of the two mating types were crossed in depression slides. Following effective pairing, the entire mating mixture was transferred to 50 ml of bacterized peptone in a 250 ml flask. The cultures were then fed every second day by pouring off most of the used medium and adding fresh bacterized peptone. This procedure was continued for about two weeks to allow sufficient time for recessive mutated alleles in those cells which had succeeded in conjugating to come to expression through the process of macronuclear assortment.

The procedure of isolation and subculturing (replication) of clones for screening closely follows that described in detail by ROBERTS and ORIAS (1973) and is illustrated in Figure 1. Single cells were isolated, one per drop, into small drops of TGVS medium in siliconized plastic petri plates, the drops being arranged in a rectangular pattern of 48 drops per plate, corresponding to the positions of wells of a microtiter plate (see Figure 1 of ROBERTS and ORIAS 1973). The petri plates were incubated in moist chambers (refrigerator crispers containing a thin layer of water) kept at room temperature (about 23°) for two days. The clones of a few hundred cells that grew up in each drop were then subcultured (replicated) into pen-strep TGVS medium contained in 48 matching depressions of a microtiter plate, using a 48-pronged replicator identical to that described by ROBERTS and ORIAS (1973). The replica clones were then placed in moist chambers within an incubator regulated at 39.5 ± 0.1°, and observed one day later. The use of axenic media allowed substantial growth and made abnormal clones easy to detect, while the exact spatial correspondence of the pattern of the replica clones and the original clones maintained at 23° made it easy to locate the phenotypically normal original clone corresponding to any replica clone that developed abnormally at 39.5°. Putative mutant clones were retested at 39.5°, and if they still manifested the same abnormal phenotype they were then tested for mating type (a nonparental mating type being taken as evidence for recombination) and established as axenic tube cultures by a technique very similar to that described by ORIAS and FLACKS (1973). Tube subcultures placed overnight at 39.5° were silver impregnated (see below) in order to further check the phenotype and establish priorities for genetic analysis. Each such line was maintained at 18°–20° until the time of genetic analysis.

Analysis of expression of mutations

Studies of the expression of these mutations were carried out on cell populations growing exponentially in PPY or TGVS medium within culture tubes or flasks. Methods of assay included cell counting in a model A Coulter Counter (Coulter Electronics), silver impregnation (FRANKEL and HECKMANN 1968), and Feulgen staining. A standard "expression score" (see Table 5) was obtained by scoring silver impregnated cells that were fixed at two generations after the shift from 28° to the test temperature; the actual times of fixation were 5–6 hours post-shift at the supraoptimal temperature (near 39.5°) and 24 hours post-shift at the suboptimal temperature (15°). Other experiments, particularly those used for overall estimates of penetrance, utilized periodic sampling of flask cultures.

RESULTS

1. *Screening of putative mutations.* The procedure we have employed (MATERIALS AND METHODS) allows the detection of temperature sensitive dominant mutations and also recessive mutations that had undergone macronuclear assortment. Screening was specifically for mutations that brought about morphological abnormalities detectable under the dissecting microscope (40X). About 7000 clones were screened. Mutant phenotypes detected at 39.5° fell into three rough classes, which in descending order of frequency were "tiny", "fat", and "monster". The first two classes comprised clones made up of cells that differed from the normal in size and sometimes also proportions, but retained a regular shape. Two "tiny" clones have been genetically analyzed (JENKINS, unpublished), but will not be considered further here as they have no known pertinence to the cell cycle. A "fat" clone was also isolated and analyzed and will be considered in this paper. Our main interest, however, is in the "monster" clones, which are presumed to arise from defects in cell division and/or intracellular patterning. The cells in such clones were generally large and highly irregular in shape, although some clones included a preponderance of fairly regular linear "chains" of connected cells. These "monster" clones were coded arbitrarily when initially isolated and then given "mo" designations (e.g. *mo1*, *mo2*, etc.) after genetic analysis had proceeded to the point at which a simple genetic basis was reasonably established. This paper will concern itself with fourteen of seventeen such mutations at six gene loci that turned out to be defective in specific steps of the cell division process. All mutations were selected in the inbred B strain, except for $mo1^a$, which was originally selected and analyzed in the D strain.

2. *Phenotypes.* The phenotypes of the cell division mutations are summarized in Table 1. All mutant clones of the *mo* series were arrested in specific stages of the division process at the non-permissive temperatures; these stages differed for mutations at different loci but were the same for allelic mutations (see RESULTS, section 4), with the exception of $mo1^a$ and $mo1^b$, where pronounced differences in degree of primary effect were noted. Some of these mutations also manifested other abnormalities. The *fat1* mutation was fundamentally different in that it brought about arrest of cell division at 39.5°, but this arrest was manifested only in some cells, and appeared to be a by-product of a generalized severe reduction in rate of development of all stages. The frequency of cell division of *fat1* homozygotes was also reduced at 28°, although at that temperature arrest of cell division was not seen.

3. *Demonstration that mutations are single-gene recessives.* The protocol for genetic analysis of putative cell division mutants is outlined in the bottom half of Figure 1. It was presumed that the micronucleus of each of the originally selected mutants was heterozygous for a mutation. As the mutant clone consisted of cells of only one mating type, the mutant was crossed to a non-mutagenized parental clone to produce an F_1 generation; half of these progeny were expected to be heterozygous for the mutation in question, the other half to be homozygous for the wild-type allele. The heterozygous progeny were identified by allowing

TABLE 1

Summary of phenotypes of homozygous mutations affecting cell division

Mutation	Fission zone formation*	Division furrowing	Gross appearance after arrest†	Frequency of development‡
mo1[a]	does not take place	does not take place	highly irregular	normal
mo1[b]	partial	partial; arrested	bent chains or heteropolar doublets	normal
mo2§	complete	late arrest	elongated chains	normal
mo3 (all)	complete	late arrest	elongated chains	normal
mo6 (both)	complete	distorted; arrested	collapsed chains or homopolar doublets	normal
mo8 (both)	partial or complete	arrested (sometimes partial)	highly variable	normal
mo12 (both)	variably displaced	arrested	bent chains	normal
fat1	complete	occasionally arrested	enlarged posterior division products	greatly reduced

* The "fission zone" is an equatorial zone of discontinuities in the longitudinal ciliary meridians, that normally appears just prior to the onset of furrowing.

† Appearance after the equivalent of about two division cycles at the restrictive temperature. At subsequent times all of the *mo* homozygotes tend to become highly irregular.

‡ Defined by the frequency of oral development and micronuclear division, which continue in *mo* homozygotes despite arrest of cytokinesis (see FRANKEL, JENKINS and DEBAULT 1976).

§ Assayed in heterozygous assortant.

time for macronuclear assortment and then examining subclones after exposure at 39.5°. F_1 progeny known to carry the mutation were then, whenever possible, crossed with one another to produce an F_2 generation. These F_2 progeny were then scored at 39.5° soon after the cross (7 to 8 generations), before phenotypic assortment of heterozygotes was likely to have occurred. These F_2 progeny all manifested a ratio of abnormal to wild-type progeny consistent with a conventional 3:1 segregation (Table 2). In all except the *mo2* clone, the putative homozygote class was expressed conditionally at the same extreme temperatures as the assorting heterozygotes. The *mo2* clone was exceptional in that the putative homozygote class was abnormal at all temperatures, producing a group of nondividing monsters a few generations after conjugation. The mutated *mo2* gene thus could only be propagated in heterozygous stocks.

Further genetic analysis involved the inbreeding of putative homozygotes to produce an F_3, backcrosses to heterozygous F_1 progeny, and in some cases genomic exclusion crosses of F_1 heterozygotes to A* (III). The F_3 progeny were all tem-

TABLE 2

Inbreeding and genetic analysis of temperature-sensitive cell division mutations

Locus	Allele	F_2 phenotype			$F_1 \times A^*$ phenotype			F_3 phenotype			Backcross phenotype		
		+	ts	Dead	+	ts	Dead	+	ts	Dead	+	ts	Dead
mo1	*mo1*^{a}	33	9	(45)	—	—	—	0	77	(8)	17	20	(23)
	mo1^b	43	13	(4)	—	—	—	0	496	(75)	150	137	(31)
mo2	*mo2*	41	13†	(2)	21	13†	(26)	—	—	—	—	—	—
mo3	*mo3*^{a}	94	23	(6)	22	24	(12)	0	88	(8)	47	42	(1)
	mo3^b	23	7‡	(0)	—	—	—	0	123	(26)	96	90‡	(23)
	mo3^c	20	7	(0)	—	—	—	0	117	(0)	127	117	(2)
	mo3^d	24	5	(0)	—	—	—	0	28	(2)	88	81	(9)
	mo3^e	19	9	(1)	—	—	—	0	68	(14)	72	68	(15)
mo6	*mo6*^{a}	—	—	—	58	19	(100)§	0	85	(3)	130	45‡	(3)
	mo6^b	19	4	(2)	—	—	—	0	113	(4)	172	191	(8)
mo8	*mo8*^{a}	30	6	(20)	—	—	—	0	56	(47)	34	32	(48)
	mo8^b	17	5	(8)	—	—	—	0	133	(63)	81	72	(76)
mo12	*mo12*^{a}	18	6	(6)	—	—	—	0	58	(49)	44	47	(77)
	mo12^b	—	—	—	—	1‖	29	0	34	(198)‖	32	27	(168)‖
fat1	*fat1*	15	5	(9)	—	—	—	—	—	(53)¶	25	24	(60)

All of the data represent pooled results, usually of several crosses of each type. Number of dead clones indicated in parentheses. All pooled data were homogeneous except for the *mo3*^{d} backcross (see text).

† Lines enter division blockage and form monsters at room temperature (see text).

‡ Two classes of ts progeny (see text).

§ 33+, 10ts (14 dead) in $F_1 \times A^*$ cross; 25+, 9ts (86 dead) in subsequent cross of original assortant $\times$ A*.

‖ The single surviving ts line was passed through an additional genomic exclusion cross to obtain other homozygotes that were then crossed with each other ("F_3") and test-crossed ("backcross") to a newly created heterozygote.

¶ All F_3 progeny died within two cell generations after the cross.

perature-sensitive, while the backcross and genomic exclusion progeny generally yielded the expected 1:1 ratios (for exceptions, see below). The highly variable proportions of death following conjugation did not affect these ratios. The backcross data shown in Table 2 represent pooled results of from two to thirteen separate crosses per set; the data from the separate crosses within each set (not shown) are homogeneous with the sole exception of the crosses within the *mo3*^{d} backcross series, in which backcrosses of homozygotes to one F_1 line gave an excess of normal progeny while backcrosses of the same homozygotes to another F_1 line gave the reverse outcome.

In addition to the above example of inconsistent ratios, two lines consistently manifested exceptions to the expected 1:1 backcross and genomic exclusion segregation ratios. The most unambiguous occurred with *mo6*^{a}, which gave clear and consistent 3:1 wild type : temperature-sensitive ratios in two genomic exclusion crosses and in four backcrosses (Table 2), with negligible death (3/180) and nonconjugation (2/180) in the backcrosses. Detailed cytological examination of all of the progeny of one of the four backcrosses revealed that progeny clones classified as "+" were indeed phenotypically indistinguishable from standard B

strain clones. The only anomaly other than the unexpected ratio was the presence of eight lines containing both wild-type and mutant cells in the progeny of two of the four backcrosses. These sectored lines are arbitrarily classified as "ts" in Table 2, but any other classification of these lines does not affect the close fit to a 3:1 ratio.

A second exceptional situation was found in the case of *mo3*^{b}, in which about half of the "ts" progeny clones were highly abnormal, while the other half of the "ts" progeny clones each consisted of some abnormal cells and many others indistinguishable from normal. Thus the true segregation in this case was not 96 +: 90 ts as indicated in Table 2, but rather 96 +: 42 ts/+: 48 ts, clearly a 2:1:1 ratio, suggesting the possible presence of a genetic modifier.

To resolve the uncertainty of the genetic status of the *mo6*^{a} and *mo3*^{b} lines, homozygotes of each were outcrossed to a wild strain, and the resulting F_1 progeny were then crossed among themselves and also backcrossed to the temperature-sensitive homozygote. This time the results, shown in Table 3, are entirely consistent with a single-gene basis for the temperature-sensitive phenotype (3:1 = +: ts segregation in the F_2, 1:1 backcross segregation, data within all sets of crosses homogeneous). Thus the mutant phenotype of the *mo6*^{a} and *mo3*^{b} lines, like those of all of the other mutant lines included in Table 2, can be considered as stemming from recessive micronuclear mutations affecting single gene loci.

As it is possible to explain the ratios originally obtained with the *mo6*^{a} and *mo3*^{b} lines as being due to the combined effects of a recessive gene responsible for the major phenotype plus an unlinked dominant suppressor, an extensive series of crosses was undertaken to attempt to confirm the existence of such a suppressor and isolate it free of the *mo6*^{a} mutant allele. These crosses, as well as some later testcrosses of the original *mo6*^{a} heterozygous assortant to both *mo6*^{a} and *mo6*^{b} homozygotes, provided no evidence for a suppressor. Aberrant ratios in crosses with high viability have also been observed within this study in a completely analyzed F_2 progeny of a double heterozygote derived from a cross of *mo3*^{a} homozygotes with *mo6*^{a} homozygotes, and in crosses carried out by KACZANOWSKI (1975), MCCOY (personal communication) and DOERDER (personal communication). Aberrations of genetic transmission in *T. pyriformis* are unusual and are considered in detail in a separate communication (MCCOY, FRANKEL and JENKINS, submitted).

TABLE 3

Genetic analysis of the progeny of a cross of mo3^b *and* mo6^a *homozygotes with wild-type B strain*

	F_2 phenotype			Backcross phenotype		
Allele	+	ts	Dead	+	ts	Dead
mo3^b	86	26	(50)	70	57	(28)
mo6^a	19	7	(4)	43	41	(9)

The data represent homogeneous pooled results.

4. *Complementation tests.* Homozygotes of the different temperature-sensitive mutations were crossed pairwise with one another, and the progeny were examined before macronuclear assortment allowed substantial expression of recessive alleles in heterozygotes. In each case the progeny clones were either all normal or all temperature-sensitive, indicating complementation and noncomplementation respectively. In all cases in which the F_1 progeny failed to complement, an F_2 was constructed and non-complementation was again observed. Complementation tests involving the *mo2* mutation necessarily employed a *mo2* heterozygote crossed with homozygotes of the temperature-sensitive mutations. A 1:1 progeny ratio would have been expected if *mo2* were allelic with any of the temperature-sensitives, but in fact the progeny of each of these crosses were all wild type.

The simplicity of the complementation data permit their summarization in the form of a complementation grid (Table 4). On the basis of the data, six different *mo* loci have been designated, with one to five alleles in each. Alleles at a single locus are indicated by superscripts.

The criteria for deciding whether allelic mutant isolates are indeed truly independent deserve comment. Independence is obvious when allelic isolates were obtained in different mutagenesis runs. This is true of *mo1*^{a} and *mo1*^{b}, of *mo6*^{a} and *mo6*^{b}, and of two subsets of *mo3*, *mo3*$^{a-d}$ having been obtained in one run and *mo3*^{e} in another. For isolates obtained *within* a mutagenesis run, one cannot have the same certainty, since the clonal isolates that were originally tested had been derived from mutagenized mass cultures. However, the mutant isolates that have been given different superscripts differ reproducibly in the frequency of expres-

TABLE 4

Complementation of mutations affecting cell divisions

Allele	*mo1*^{a}	*mo1*^{b}	*mo2*	*mo3*^{a}	*mo3*^{b}	*mo3*^{c}	*mo3*^{d}	*mo3*^{e}	*mo6*^{a}	*mo6*^{b}	*mo8*^{a}	*mo8*^{b}	*mo12*^{a}	*mo12*^{b}	Wild type (B)
mo1^a	mo	mo	+	+	+	+	+	+	+	+	+	+	+	+	+
mo1^b		mo	+	+	+	+	+	•	+	•	+	+	•	•	+
mo2			mo	+	+	+	+	+	+	•	+	+	+	•	+
mo3^a				mo	mo	mo	mo	mo	+	•	+	+	•	•	+
mo3^b					mo	mo	mo	mo	+	+	+	+	+	+	+
mo3^c						mo	mo	•	+	•	+	+	•	•	+
mo3^d							mo	•	+	•	+	+	•	•	+
mo3^e								mo	+	•	+	•	•	•	+
mo6^a									mo	mo	+	+	+	•	+
mo6^b										mo	+	•	+	•	+
mo8^a											mo	mo	+	+	+
mo8^b												mo	•	•	+
mo12^a													mo	mo	+
mo12^b														mo	+

Complementation tests with *mo2* carried out using heterozygotes, with other mutants using homozygotes. + = wild type (complementing); mo = "monster" (noncomplementing); • = combination not tested.

sion (penetrance) and/or the temperature conditions under which expression is observed (see RESULTS, section 6). Additional evidence for the uniqueness of at least some of the *mo3* alleles will be offered in RESULTS, section 8.

5. *Macronuclear assortment.* The procedure for isolation of mutants was such that the only recessive mutations that could have been detected were those that underwent assortment. However, the kinetics of assortment was examined closely only at the *mo1* locus. At this locus, $mo1^{+}/mo1^{a}$ heterozygotes assorted at the rate of 0.0104 ± 0.0012 pure lines per fission with an output ratio of 59% wild type to 41% temperature-sensitive (p value for 1:1 output ratio: $0.1>p>0.05$). This rate of assortment is not significantly different from the rate of assortment at other loci that have been studied (summarized in SONNEBORN 1974). It is estimated that the time of macronuclear determination was between 2 and 15 fissions after conjugation. This places *mo1* among the group of loci that undergo early determination (BLEYMAN 1971; ALLEN and GIBSON 1973; SONNEBORN 1974).

It should be added here that the phenomenon of early assortment, together with the likelihood of onset of expression when assortment is still incomplete, makes it virtually impossible to decide whether some of the mutations are recessive or incompletely dominant. In *mo3* and *mo6* lines in particular, a small proportion of abnormal cells was observed at 40° in many presumed heterozygous clones even when scoring was performed within 10 fissions of conjugation. This could reflect either a generalized low penetrance of the abnormal phenotype in heterozygotes or expression by the earliest "assortants" that have managed to accumulate a sufficient number of macronuclear subunits determined to express the mutated allele. The fact that many heterozygous clones of $mo6^{b}$ were only slightly penetrant soon after conjugation and subsequently became more so is consistent with the assumption that the early expression was due to the onset of phenotypic assortment. However, it is operationally very difficult to prove which of these two alternatives is correct.

Attempts were made to detect assortment in the direction of normality of phenotype in $mo1^{a}/mo1^{b}$ and $mo6^{a}/mo6^{b}$ heterozygotes. Such assortment might have been expected if one assumed that the two mutations were in different base pairs and that intracistronic recombination could occur in the macronucleus. Our efforts in this direction were unsuccessful, but not sufficiently extensive to disprove any hypotheses.

6. *Expression.* Expression of division blockage of the cell division mutants at various temperatures is summarized in Table 5. The two independently isolated *mo6* alleles were expressed identically at all tested temperatures, while the *mo1*, *mo8*, and *mo12* alleles differed in the straightforward manner that one would expect of alleles with gene products showing varying degrees of "leakiness" of function. This is most striking at the *mo1* locus; the $mo1^{a}$ homozygotes at 36° sometimes manifested a partial inhibition of fission zone formation very similar to that brought about in the weaker $mo1^{b}$ homozygotes at 39.5 to 40°, providing strong evidence for the essential similarity of the action of these two mutated alleles.

TABLE 5

Expression of division blockage in homozygous mutations affecting cell division

Mutagenesis run	Mutation	Expression at (°C)*					Penetrance at 40°†
		40	38	36	22–28	15	
1	*mo1*^{a}	+++++	++++	+++	0	0	100%
2	*mo1*^{b}	++	+/0	0	0	0	~50%
2	*mo3*^{a}	+++	+	+	+	+++	80–100%
2	*mo3*^{b}	++++	+++++	++++	0	0	100%
2	*mo3*^{c}	+	+	+	+	++	<25%
2	*mo3*^{d}	++++	++	+/0	0	0	95–100%
3	*mo3*^{e}	++++	+	+/0	+/0	++++	95–100%
2	*mo6*^{a}	++(+)	+(+)	+	0‡	0	§
3	*mo6*^{b}	++(+)	+(+)	+	0‡	0	§
2	*mo8*^{a}	++	+	+/0	0	0	~50%
2	*mo8*^{b}	+	+/0	0	0	0	~10%
3	*mo12*^{a}	++	+	0	0	0	~50%
3	*mo12*^{b}	+	+/0	0	0	0	<20%

* Percentage of cells in cell division arrest at approximately two generations after the onset of exposure to the restrictive temperature (or in steady-state growth).

+++++ = 75–100% arrested
++++ = 50–75% arrested
+++ = 25–50% arrested
++ = 5–25% arrested
+ = <5% arrested
+/0 = none arrested
0 = substantial increase in expression at later times of assay.

(+) = substantial increase in expression at later times of assay.

† An estimate of the percentage of cells entering cell division that were unable to complete division normally. Includes permanently arrested divisions and long-delayed divisions that generally yielded atypical division products.

‡ Many cells manifest irregularities of ciliary meridians and distorted swimming patterns.

§ Expression of division arrest cumulative and variable.

The situation was slightly more complex for the *mo3* alleles. Although the phenotypes of the different alleles were very similar, the conditions of expression were not. Alleles could be heat-sensitive (*mo3*^{b}, *mo3*^{d}), predominantly cold sensitive (*mo3*^{c}), or sensitive to both heat and cold (*mo3*^{a}, *mo3*^{e}). At room temperature some alleles were weakly expressed and others not at all. The rank order of strength of expression of the alleles at different supranormal temperatures was not highly consistent. Each *mo3* allele appeared to have its own individual range of expression.

With the exception of *mo6* (which will be considered in detail elsewhere), the cell division mutations come to expression within the first cell cycle after the temperature shift. In the incompletely penetrant mutations (again with the exception of *mo6*) it appeared as if there is a rather constant "division failure risk" that becomes established shortly after the temperature shift and remains constant thereafter. A rough estimate of this risk for each mutation is given in the right-hand column of Figure 5. The lower the value, the more gross the approximation, as it then becomes more and more difficult to distinguish long-term blockages from transient delays.

7. *Phenotype on return to permissive conditions.* At least some of the cells homozygous for any of the mutations affecting cell divisions can return to normal cell division and approximately normal shape following prolonged maintenance at 39.5°; such cells reappeared at 28° even if the starting material was a culture that consisted only of sluggish amoeba-shaped monstrous cells (as in *mo1*^{a}) which had spent 12 to 24 hours at 39.5°. Presumably, approximately normal cells could occasionally pinch off from multipolar monsters, as was suggested by SONNEBORN (1932) for a similar situation in *Colpidium campylum*. Such cells produce healthy clones, but at least in *mo1*^{b} and *mo8*^{a} the members of such clones may swim abnormally and propagate inversions of ciliary meridians (NG, personal communication).

8. *Segregation in intra-locus combinations.* Combinations of alleles have already been considered in the section on complementation (RESULTS, section 4). We return here only to those cases in which alleles manifested differences in phenotype or in conditions of expression. The only case of a substantial difference in the phenotype of alleles was encountered with *mo1*^{a} and *mo1*^{b} (Table 1). The phenotype of the *mo1*^{a}/*mo1*^{b} heterozygote was examined at 39.5° and found to be intermediate between that of the two parents; some individual cells of the heterozygote resembled the *mo1*^{a} parent, others the *mo1*^{b} parent, while the bulk were in between. The F_2 progeny of this cross were all abnormal, but the ranges of phenotypic expression of the three possible genotypes overlapped sufficiently to make a search for segregation ratios impractical.

Alleles at each of the other *mo* gene loci are virtually identical in phenotype, but often do differ in the severity of expression and the conditions under which expression occurs. An unambiguous genetic analysis of segregation ratios is possible only in those cases in which there is an all-or-none (or nearly all-or-none) difference in expression under a given set of conditions. This is true for *mo3* alleles under two conditions: at low temperature (15–17°) at which *mo3*^{a}, *mo3*^{c}, and *mo3*^{e} are quite strongly expressed whereas *mo3*^{b} and *mo3*^{d} are not expressed at all, and at 40° at which *mo3*^{c} is only slightly expressed (10–20% of cells arrested) while virtually all cells of *mo3*^{b}, *mo3*^{d}, and *mo3*^{e} and most cells of *mo3*^{a} suffer division arrest. Of particular interest is a comparison among the four alleles (*mo3*$^{a-d}$) which were recovered within the same mutagenesis run. Three such crosses were made (Table 6), involving homozygotes for alleles differing in cold-sensitivity in all three cases, and in heat-sensitivity in one (*mo3*^{c} × *mo3*^{d}). The F_1 progeny were all normal at 17°, indicating that cold-sensitivity is a recessive trait. At 40°, the proportion of arrested cells was near 50% in the *mo3*^{c} × *mo3*^{d} F_1 progeny, indicating an averaging of penetrance at high temperature, perhaps analogous to the averaging of expressivity in the *mo1*^{a} × *mo1*^{b} cross described earlier. The crucial results are in the F_2, in which a 1:2:1 segregation of low:intermediate:high penetrance was observed at 40° in the *mo3*^{c} × *mo3*^{d} cross, while the expected 3:1 segregation of normal : cold-sensitive was observed at 17° in two of the three crosses summarized in Table 6. Surprisingly, the F_2 progeny of the *mo3*^{a} × *mo3*^{d} cross did not segregate at 17°. While this one anomalous case could be interpreted as indicating that the *mo3*^{a} and *mo3*^{d} alleles

TABLE 6

Segregation of conditions of expression in F_2 progeny of crosses among mo3 *alleles*

Cross		Generation	Progeny phenotypes*					
			40°				17°	
			+	ts/+	ts	Dead	+	cs
mo3[a]/mo3[a] × *mo3[b]/mo3[b]*		F_1	0	0	15	(14)	8	0†
(ts, cs)	(ts)	F_2	0	0	43	(17)	29	11‡
mo3[a]/mo3[a] × *mo3[d]/mo3[d]*		F_1	0	0	4	(10)	4	0
(ts, cs)	(ts)	F_2	0	0	19	(67)	19	0
mo3[c]/mo3[c] × *mo3[d]/mo3[d]*		F_1	0	10	0	(14)	10	0
(cs)	(ts)	F_2	15	25	18	(0)§	37	11‖

* 40°: + = less than 20% chains
ts/+ = about 50% chains
ts = all or almost all chains
17°: + = no chains
cs = 50% or more chains

† Only one of two parallel crosses scored at 17°.

‡ Three of the high-temperature sensitive progeny grew very slowly and could not be effectively scored at 17°.

§ An additional two progeny lines entered division blockage at room temperature and therefore could not be scored at high or low temperature.

‖ Ten of these lines became tiny and died at 17°. These were normal at room temperature. At 40°, four were +, two were +/ts, and four were ts.

are identical and resistance to low temperature of *mo3[d]* cells is caused by an inherited and "dominant" cytoplasmic condition, the unusually low viability of all *mo3[a]* × *mo3[d]* crosses renders such an interpretation premature. We prefer to believe, though we cannot prove, that synclones of the *mo3[a]/mo3[a]* genotypic class simply have not survived in the F_2.

DISCUSSION

Phenotypic assortment and the selection of mutations in Tetrahymena

CARLSON (1971), DOERDER (1973), and DOERDER *et al.* (1975) have succeeded in selecting recessive mutations by scoring heterozygotes that had undergone phenotypic assortment. In these studies, a small number of mutations were obtained. We have utilized this same method and obtained 14 temperature-sensitive mutations, in 6 complementation groups, specifically affecting cell division. Four other mutations affecting various aspects of form and pattern were also obtained in the course of this screening program. The number of mutants obtained is a reflection of our decision to analyze only one subclass of morphological abnormalities, namely those manifesting irregular form ("monsters"). Numerous "tiny" and "fat" assortants were discarded without analysis; those few that were analyzed turned out to be simple Mendelian recessives. There is thus every reason to believe that screening procedures designed to select assorting recessive mutations affecting other cellular processes should have a good chance of success. The macronuclear assortment method should thus allow *T. pyriformis* to assume

a position as one of the more amenable organisms for the isolation and analysis of temperature-sensitive mutations.

Our successful experience in isolating mutations affecting cell division has thus far been restricted to use of one chemical mutagen, nitrosoguanidine. Our one attempt at screening of morphologically aberrant mutations following X-ray mutagenesis (5 to 80 kR) was unsuccessful. No such mutations were observed among 16,000 isolates examined; a small number of "tiny" clones were observed but not analyzed further. This failure may be due to a lesser efficiency of ionizing radiation, particularly in inducing temperature-sensitive point mutations (SUZUKI *et al.* 1967).

The properties of the assorting heterozygotes were not in every case identical to those of the corresponding homozygote that was eventually obtained upon inbreeding. The most striking discrepancy was observed with *mo2*, which was initially temperature-sensitive (expressed at 39.5° but not at room temperature) as an assorting heterozygote, yet was 100% penetrant at room temperature as a homozygote. Presumably the *mo2* gene product had a phenotypic effect at high temperature even when macronuclear assortment was incomplete. Possibly a small amount of the wild-type gene product might by itself be able to maintain division at normal but not at elevated temperatures. An interpretation of this general kind is supported by subsequent observations on two sublines of the heterozygous *mo2* assortant. Expression of the *mo2* phenotype at room temperature was observed after the time of original selection in both of the sublines, but the degree of expression fluctuated independently in the two sublines (unpublished observations); this phenomenon is consistent with a model of assortment that assumes a random sorting out of macronuclear subunits determined for expression of one or the other allelic alternatives (ALLEN and NANNEY 1958; NANNEY 1964). Other examples were also observed, most notably in $mo1^a$ and $mo6^b$, in which a weaker degree of expression was detected in the heterozygous assortant than in the homozygote (unpublished observations). Such experiences suggest that many and possibly even all of our mutations were selected when only partially assorted; i.e. the macronucleus still contained some functional copies of the wild-type allele. This in turn raises the possibility that the assortment technique of screening mutations might be of more limited effectiveness in screening for mutations affecting processes for which even a very small amount of the wild-type gene product is sufficient to insure normal gross function.

Number of gene loci participating in the cleavage process

A surprising proportion of the mutations observed were repeats at the same genetic loci. As indicated in Tables 2 and 4, two or more alleles were recovered at five of the six loci (with independent derivations of separate alleles at three); the third and most recent mutagenesis experiment revealed only one new locus affecting cell division (*mo12*), plus two alleles ($mo3^e$ and $mo6^b$) at previously identified loci. This unexpectedly high frequency of repeats suggests that the total number of genes whose products have striking effects on the fission process may not be much larger than the number of genes already identified. However,

such an assertion makes certain assumptions concerning the mutagen, the representativeness of the class of temperature-sensitive mutations, the screening procedure, and the randomness of the distribution of recoverable mutations among loci that all bear some discussion. First, only nitrosoguanidine has thus far been successfully applied, and it is not known to what degree sole reliance on this mutagen biases the outcome. In yeast the extensive use of ethylmethane sulfonate (EMS) did not reveal new *cdc* gene loci not also mutated by nitrosoguanidine (HARTWELL *et al.* 1973).

The representativeness of the class of high-temperature-sensitive mutations is difficult to assess in a eukaryotic organism with very many genes; however, in bacteriophages such as T4 high-temperature-sensitive mutations were found in a large proportion of all known genes (EDGAR and LIELAUSIS 1964), and the distribution of these mutations was such as to support the conclusion ". . . that the temperature-sensitive phenotype is a site specific rather than a gene specific property" (EDGAR, DENHARDT and EPSTEIN 1964). This suggests that, in principle at least, searching specifically for high-temperature-sensitive mutations should not limit one to any subclass of potentially mutable genes.

Our method of selecting mutants carries with it the unavoidable restriction that any recessive mutation that does not undergo macronuclear assortment will never be scored. It therefore becomes pertinent to ask whether such nonassorting mutations might exist. Of sixteen loci in syngen 1 of *T. pyriformis* that had initially been discovered by means *independent* of the assortment phenomenon, fifteen do in fact assort (information from Table 2 of SONNEBORN 1974 and from papers cited therein); thus assortment may well be a general property of the macronucleus of this biological species [the one exceptional case, that of *ts1*, does not speak strongly against this possibility (MCCOY 1973)]. The available evidence thus indicates that screening based on assortment would in itself cause one to miss few, if any, loci. Furthermore, any mutation that brings about a blockage of cytokinesis upon assorting would be expected to produce an easily recognized morphological abnormality, as continued growth in cells unable to complete an already initiated cell division would of necessity bring about the formation of chains or monsters. Of course, other screening methods might be needed for revealing specific blockages of cell cycle stages prior to formation of the fission zone; however, a significant subclass of the many temperature sensitive "fat" lines that we have observed but not analyzed might turn out to be blocked at points before fission zone formation, when continued growth without division would not be expected to lead to morphological distortion.

Perhaps the most severe limitation to the attainment of a reliable estimate of the total number of genetic loci with products essential for cell division is the possible nonrandomness of the distribution of selectable mutant hits among loci. The repeated isolation of mutations at the *mo3* locus, and the peculiar characteristic of some mutations at that locus of being sensitive to *both* heat and cold [a characteristic also observed in some mutated alleles of bacteriophage (COX and STRACK 1971) and of *Drosophila melanogaster* (TASAKA and SUZUKI 1973)] suggests that the successful function of the wild-type allele at this locus involves

some exceptionally finely balanced process that might be upset by virtually any alteration in the structure of the relevant gene products or in the genetic regulation of its quantity (cf. Cox and STRACK 1971). Certain other processes might be far more robust, and scorable mutations at the responsible loci correspondingly rare. Our mutant collection is too small to make statistical testing of this possibility very meaningful; however a bimodal distribution of the frequency of recoverable mutations in complementation groups has been observed in other more extensive collections of mutations, including the *cdc* collection in yeast (HARTWELL *et al.* 1973). It is the possibility of such bimodality of cell division mutations in *T. pyriformis* that renders any estimate of the total number of genes with products required for cell division premature.

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Corresponding editor: S. L. ALLEN