RECOMBINATION AND ASSORTMENT IN THE MACRONUCLEUS OF *TETRAHYMENA THERMOPHILA*: A THEORETICAL STUDY BY COMPUTER SIMULATION

F. P. DOERDER AND S. L. DIBLASI

Department of Biology, Cleveland State University, Cleveland, Ohio 44115

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ABSTRACT

The compound nature of the macronucleus of *Tetrahymena thermophila* presents multiple opportunities for recombination between genes on the same macronuclear chromosome. Such recombinants should be detectable through their assortment at subsequent amitotic macronuclear divisions. Thus, a macronucleus that is initially *AB/ab* should produce recombinant assortees of the genotypes *Ab/aB*. Computer simulation shows that, when the recombination frequency is two or fewer times per cell cycle, recombinant assortees are produced at experimentally measurable frequencies of less than 40%. At higher recombination frequencies, linked genes appear to assort independently. The simulations also show that recombination during macronuclear development can be distinguished from recombination in subsequent cell cycles only if the first appearance of recombinant assortees is 100 or more fissions after conjugation. The use of macronuclear recombination and assortment as a means of mapping macronuclear genes is severely constrained by the large variances in assortment outcomes; with experimentally small sample sizes, such mapping is impossible.

RECOMBINATION between linked genes is well documented in the diploid micronucleus of the ciliate *Tetrahymena thermophila* (ALLEN 1964; DOERDER 1973; MCCOY 1977). Indeed, recombination is sufficiently frequent that few of the 100 markers assigned to the $N = 5$ chromosomes by the method of nullisomics (BRUNS 1982) show linkage by standard testcross procedures. Recombination has also been suggested for macronuclear genes (ALLEN 1965; DOERDER 1973; ORIAS 1973), but to date no genetic map has been constructed. Mapping of macronuclear genes is important because, in its derivation from a micronucleus at conjugation, the macronucleus undergoes extensive genetic reorganization (reviewed by GOROVSKY 1980). The resulting macronuclear linkages will certainly be determined by molecular means, but a genetic tool is also available. In *T. thermophila* all known genes undergo macronuclear assortment, a stochastic process in which a heterozygous macronucleus gives rise through repeated division to macronuclei which express only one allele. Since macronuclear DNA molecules are sufficiently large so as to contain many genetic loci (MERKULOVA and BORCHSENIUS 1976; WILLIAMS *et al.* 1978; PREER and PREER 1979; YAO and YAO 1981), such linked genes should coassort. That

is, in the absence of recombination, a double heterozygote, *e.g.*, *AB/ab*, should produce only two classes of assortees, one *AB* and another *ab*. Were recombination to occur, two additional classes, *Ab* and *aB*, are expected. The purpose of this paper is to provide a theoretical framework for the interpretation of coassortment experiments in which all four classes appear.

Only two suspected micronuclear linkages have been examined for coassortment in appropriate double heterozygotes (ALLEN 1965; DOERDER 1973). In both cases two recombinant classes of assortees, as well as the two parental classes, appeared in a 1:1:1:1 ratio. If indeed linked in both the micronucleus and macronucleus, either these genes are somatically recombined during macronuclear development or division or, as a result of chromosome fragmentation in development, these genes come to be on different macronuclear linkage groups so that they actually assort independently.

Multiple opportunities for recombination are present each cell cycle; early in the life cycle, the mean G_2 DNA content is 128C, whereas later in the life cycle it is 90C (DOERDER and DEBAULT 1978). Because macronuclear division is regularly unequal and because newly replicated subunits are randomly distributed at division (ORIAS and FLACKS 1975), G_1 macronuclei derived from the same G_2 macronucleus are normally genetically unequal. Thus, a G_2 macronucleus with 50 *AB* and 40 *ab* units may divide into one G_1 nucleus with 22 *AB* and 22 *ab* units and another with 28 *AB* and 18 *ab* units. If in the 50*AB*:40*ab* macronucleus two units recombined, the G_2 distribution would be 49*AB*:39*ab*:1*Ab*:1*aB*. At division the distribution might be 22*AB*:22*ab* and 27*AB*:17*ab*:1*Ab*:1*aB*, where both recombinants go to the same macronucleus. Other distributions are also possible, and by subsequent rounds of replication, recombination and assortment, macronuclei consisting entirely of only one type of these units would be produced.

In this paper we have used computer simulation to answer three major questions. First, is it theoretically possible to detect macronuclear recombination experimentally? That is, given the multiple opportunities for recombination in the compound macronucleus, how much recombination must occur before linked genes appear to assort independently? Second, can recombination occurring during macronuclear development at conjugation be distinguished from recombination occurring in subsequent amitotic cycles? Third, can the frequencies of recombinant assortees provide sufficient information to determine genetic distances and gene order?

This work has appeared in abstract form (DOERDER and DIBLASI 1983).

MATERIALS AND METHODS

The simulation program MACREC has been partially described elsewhere (DOERDER 1979; F. P. DOERDER, unpublished data). The program is designed to simulate the known aspects of macronuclear structure, gene dosage, replication and assortment as described in the introduction. The program permits the user to specify starting ratios of types of assorting units, the recombination frequency (RCF) and the number of clones to be examined. Assorting units are individually counted and are manipulated by reference to tables of random numbers generated anew at each simulation. In the simulations reported here, each simulation involved 500 subclones randomly selected after 18 (sometimes 13) fissions of a single cell; at each 13th fission interval following the

initial subcloning, each subclone was serially recloned until all subclones were stable for one type of assorting unit. In accordance with known life cycle variation in macronuclear DNA content (DOERDER and DEBAULT 1978), the G_1 mean number of assorting units was set at 64 for the first 50 fissions and at 45 after 100 fissions; between 50 and 100 fissions the mean was allowed to gradually (and randomly) decline.

MACREC is written in Fortran and was run on Cleveland State University's IBM 370/158 computer with typical simulations requiring 5–9 min of central processor time.

RESULTS

Detection of macronuclear recombination: The compound nature of the macronucleus presents multiple opportunities for recombination. If, through prior recombination events, recombinant units are already present in a macronucleus, further recombination may produce one of three results, depending upon whether the recombining units are nonrecombinant (P1 or P2, parental) or recombinant (R1 or R2, nonparental). If the choice is P1 × P2, the result is two additional recombinant units, R1 and R2. If the choice is R1 × R2, the result is two parental units, P1 and P2. Because the genetic consequences of these two recombination events have phenotypic consequences, they are in a sense "visible." If, on the other hand, the choice is R1 × P1, P1 × R2, P1 × P1 or any other combination of units, the resulting recombination, although scorable by the simulation program, is experimentally "invisible" because no net genetic, or phenotypic, change results.

In descendants of a single 32*AB*:32*ab* G_1 macronucleus, at some recombination frequency the proportion of recombinant assortees will be equal to the proportion of nonrecombinant (parental) assortees. In other words, at some theoretical RCF the ratio of assortees at the completion of assortment will be 1*AB*:1*ab*:1*Ab*:1*aB*, which is also the ratio expected when the starting macronucleus is 16*AB*:16*ab*:16*Ab*:16*aB*. To determine that theoretical RCF, we simulated, at different RCFs, assortment experiments in which recombinant subunits were produced in a starting macronucleus that contained equal numbers of nonrecombinant units (P1 and P2 only). The RCF was arbitrarily defined in terms of the cell cycle, such that a RCF of 1.0 meant that in each G_2 macronucleus two randomly selected units were recombined. RCFs of less than 1.0 were treated stochastically; for example, RCF of 0.10 meant that recombination occurred in one of ten randomly selected macronuclei or, on the average, once every tenth cell cycle. (Deterministic *vs.* stochastic control of recombination events will be analyzed below.) The results of these simulations are shown in column A (32:32:0:0) of Tables 1 and 2. They are expressed both in terms of the mean appearance (fission) of the first recombinant assortee (Table 1) and of the proportion of recombinant assortees at the completion of assortment (Table 2). By either measure, at RCFs of 2.0 or less, the results are significantly different from those expected for a starting ratio of 16:16:16:16.

The counts of visible (P1 × P2 and R1 × R2) and invisible recombination events were used to derive RCFs based on the number of subunits rather than per cell cycle. For example, in a typical simulation at RCF = 0.10, among the 8,253,894 assorting units counted, the 7029 involved in crossover produced

TABLE 1

Relationship between recombination and appearance of first recombinant assortee for three starting ratios of AB:ab:Ab:aB *subunits*

Recombination events/cell cycle	Appearance of first recombinant assortee (fissions)[a]					
	(A) 32:32:0:0		(B) 31:31:1:1		(C) 30:30:2:2	
	Mean	SD (*N*)	Mean	SD (*N*)	Mean	SD (*N*)
0			66	20.8 (7)[b]	63	12.7 (7)
0.10	83	21.2 (7)	79	17.9 (7)[b]	61	12.4 (7)
0.25	85	17.5 (7)	66	14.3 (7)	55	15.8 (7)
0.50	61	17.9 (7)	61	6.3 (7)	55	11.7 (7)
0.75	63	6.9 (7)	59	8.9 (7)	59	8.9 (7)
1	55	11.7 (7)	53	9.2 (9)	46	4.9 (7)
2	50	6.9 (7)	48	12.7 (9)	42	4.9 (7)
3	47	9.7 (8)				
16:16:16:16	33	4.9 (7)				

[a] Each mean represents *N* simulations each utilizing 500 subclones derived from a single cell.
[b] These means are not significantly different. Actual values are RCF = 0: 70, 57, 83, 70, 31, 96, 57; RCF = 0.10: 96, 109, 70, 70, 51, 83, 70.

TABLE 2

Relationship between recombination and proportion of recombinant assortees at completion of assortment for three starting ratios of AB:ab:Ab:aB *subunits*

Recombination events/cell cycle	Final % recombinant assortees[a]					
	(A) 32:32:0:0		(B) 31:31:1:1		(C) 30:30:2:2	
	Mean	SD	Mean	SD	Mean	SD
0	0.0	—	3.3	1.00	6.1	1.66
0.10	6.3	1.63	9.4	2.01	11.5	2.31
0.25	12.5	1.44	15.5	3.06	15.9	2.40
0.50	19.5	3.27	20.7	2.67	25.7	2.96
0.75	26.9	2.88	25.2	3.33	27.6	2.85
1	30.1	3.48	29.6	3.10	32.1	1.92
2	39.5	2.13	43.3	2.93	44.8	3.85
3	51.8	4.30				
16:16:16:16	49.4	2.16				

[a] Each mean represents the same simulations shown in Table 1.

2090 visible crossover units. The probability that a subunit is of a crossover type is, therefore, 0.00170, and the probability that it is due to a visible crossover is 0.00051. A tabulation of these values is shown in Table 3. The frequency of visible crossover is, as expected, an inverse (nonlinear) function of the RCF; at RCFs of less than 0.10 (not shown) the visible crossovers approach 100% of all crossovers. At higher RCFs, therefore, a given subunit is likely to have participated in multiple recombination events.

The relationship between RCF and the probability of crossover (visible or

TABLE 3

Relationship between RCF and total and visible recombinant events

RCF/cell cycle	Probability of crossover/ subunit[a]	% visible crossover[b]	
		Mean	SD
0.01	0.000176	34.3	2.13
0.10	0.00167	29.0	1.01
0.25	0.00426	23.7	0.92
0.50	0.00846	18.4	1.28
1	0.01702	13.2	0.43
2	0.03404	9.2	0.52

[a] Ratio of total recombinant units to total units in unassorted macronuclei.

[b] Each mean represents the same simulations shown in Tables 1 and 2.

not) is linear, with a slope of 0.0170. This value reflects the relative constancy of the number of subunits that participate in recombination. From the example, the 8,253,894 subunits were distributed among 70,452 unassorted macronuclei for a mean of 117 units/macronucleus. Among all of the simulations, the mean was 118. In a macronucleus with 118 units, if two recombine, the frequency of crossover units is 2/118 = 0.0169, which is the observed slope. Much of the recombination that produces visible crossover units, therefore, occurs early in the life cycle before the G_2 mean DNA content has declined to 90C. This intuitively obvious conclusion is directly observable during the assortment process from the proportions of recombinant subunits. For example, among seven simulations with an input of 32:32:0:0 at RCF = 1.0, at 57 fissions the mean number of G_1 units was 61.2 (SEM = 0.15) and the mean number of clones (out of 500) assorted to either R1 or R2 units was 1.4 (SEM = 0.57). Among the macronuclei of the unassorted clones, the mean proportion of R1 and R2 units was 27.94% (SEM = 0.34). By 100 fissions, 40–60% of clones were fully assorted for any one of the four types.

In the simulations shown in Tables 1 and 2 recombination was defined in terms of the cell cycle. At RCFs of less than 1.0 recombination was controlled stochastically; that is, if RCF = 0.10 and a random number was less than 0.10, recombination occurred. However, at frequencies of 1.0 or more, recombination was not controlled by random numbers; that is, if RCF = 2.0, two recombination events always occurred in each cell cycle. As shown in Tables 1 and 2 both of these modes of control resulted in considerable variance in assortment outcomes. Less deterministic modes of recombination control might be expected to alter these results. To test this idea, simulations were performed in which random numbers were compared to cumulative frequencies of the Poisson distribution to determine the frequencies at which recombination would occur 0, 1, 2, 3 or more times per cell cycle (to calculate these frequencies, the Poisson mean was set equal to the desired RCF). For 32:32:0:0 inputs (other inputs not simulated) the results showed no significant differences from those in Tables 1 and 2. For example, at RCF = 1.0, the mean appear-

ance of the first recombinant assortee was 56 fissions (SD = 10.8, seven simulations); at RCF = 0.10, the mean was 90 (SD = 27.0, six simulations). Although the latter mean and variance are larger than the corresponding entries in Table 1, simulations at other RCF values showed no trend. Similarly, no significant differences or trends were observed in the final percent of recombinant assortees.

Recombination during macronuclear development: In the simulations discussed in the previous section, the macronuclei of the starting cells contained no recombinant units. However, recombination may also occur during the development of macronuclei prior to the first division. If in development two loci come to be on different fragments of micronuclear chromosomes, then, of course, the linkage is broken and the output ratio would be 1:1:1:1 assuming no differential replication of the fragments. Extensive recombination between genes at two loci on the same fragment could also result in this ratio. Assortment experiments can not distinguish between these two possibilities. However, if recombination during development is very rare, then it may be possible to distinguish it from recombination occurring during subsequent amitotic division.

The results of simulations in which the G_1 starting cells contained two or four recombinant units are shown in columns B and C of Table 2. At a RCF of 0.0, meaning that amitotic recombination did not occur, recombinant assortees are produced, as expected. Although rare, the recombinant assortees generally first appeared within 70 to 90 fissions. As the RCF during amitotic division increased, the fission at which the first recombinant assortee appears decreased and the final proportion of recombinant assortees increased. At lower RCFs, however, the variance was considerably larger than at higher RCFs, and at all RCFs the distributions (not shown, but see Figure 1) showed considerable overlap. Recombination during macronuclear development, therefore, is not easily distinguished from recombination in subsequent cell cycles. However, if experimentally no recombinant assortees appear within the first 100 fissions but do so at some time thereafter, one can conclude that recombination during macronuclear development is unlikely and that the observed recombinants are due to recombination in the subsequent amitotic cycles.

Gene mapping: The answer to the question as to whether macronuclear recombination followed by assortment is of sufficient sensitivity to permit gene mapping has been anticipated in the previous sections. It is a qualified yes. Genes located on homologous chromosomes are theoretically mappable relative to each other provided RCFs remain proportional in both development and amitotic division. However, the variance associated with low RCFs is sufficiently high that fine structure mapping is precluded.

Data supporting these conclusions are shown in Figure 1. Unlike the tables, these data are for recombinant assortees scored at 96 fissions after conjugation. This fission age was arbitrarily chosen as a convenient age at which to experimentally assay assorting subclones, both because recombinants formed during development have begun to assort (Table 1) and because considerable experimental effort is required to ascertain completion of assortment among 500

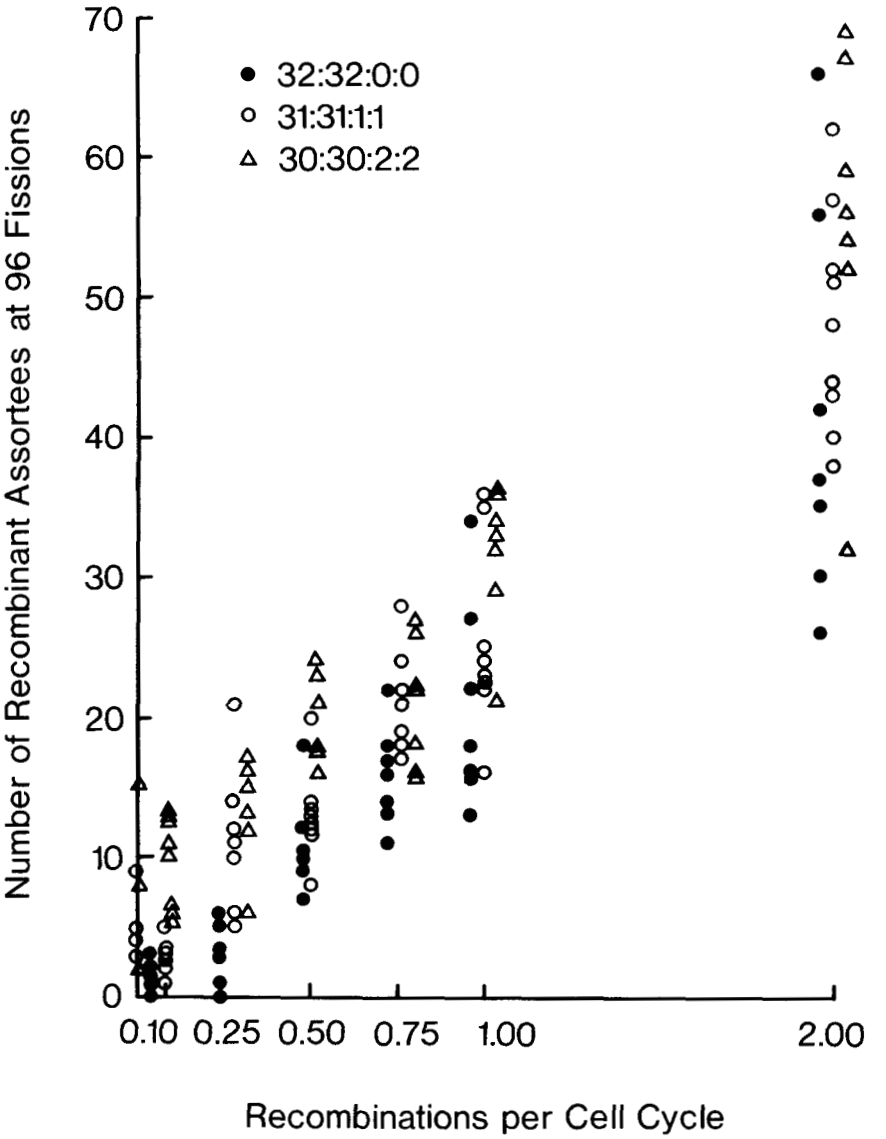

FIGURE 1.—Number of recombinant assortees at 96 fissions for three different starting ratios as a function of amitotic RCF. Each simulation represents the outcome of a single assortment simulation in which 500 subclones are derived from a single macronucleus; for each combination of starting ratio and RCF six to ten simulations were performed. The symbols are offset at each RCF for clarity. Note the considerable overlap both among different RCFs at a given starting ratio and among the three starting ratios at a given RCF. These data are from the same simulations reported in Tables 1 and 2.

clones. These data show, in support of the conclusions of the previous sections, that experimentally measured assortee frequencies are not easily transformable into meaningful RCFs. For example, if, at 96 fissions, 15 recombinant assortees are observed (out of 500 subclones), one cannot interpolate with certainty

TABLE 4

Recombination and assortment among 100 subclones with a starting ratio of 32AB:32ab:0Ab:0aB

	Simulation no.	No. of recombinants at: 96 fissions	End of Assortment	First recombinant (fission)
RCF = 0.10	303	0	9	130
	304	0	8	104
	305	0	7	104
	306	0	6	117
	307	0	6	117
	308	0	4	221
	Mean		7.0	130
	SD		1.8	46.8
RCF = 1	297	1	25	78
	298	4	36	65
	299	0	29	104
	300	0	28	104
	301	0	24	104
	302	4	29	65
	Mean	1.5	28.5	87
	SD	2.0	4.2	19.6

either the RCF or the input ratio. Nevertheless, depending upon the precise outcomes of repeated experiments, a crude map may be possible. For example, for hypothetical loci *A*, *B* and *C*, experimentally observed values of 25 *AB* recombinants and ten *BC* recombinants are sufficiently different with respect to possible RCFs to permit ordering of the loci if *AC* recombinants total 40 or 50. On the other hand, if as a reflection of the variance, the number of observed *AC* recombinants is 30, no map is possible. Given the variation of possible outcomes for any given RCF, any map that is drawn is subject to considerable uncertainty.

Sample size in recombination experiments: In the simulations reported here, 500 subclones were scored. A review of the literature shows that assortment experiments always use considerably fewer. For some purposes a lower number may suffice (F. P. DOERDER, unpublished results), but in other experiments it will not. To illustrate, suppose it is desired to score for recombination in assorting subclones at 96 fissions, as in Figure 1. But suppose that, due to the difficulty of determining the phenotypes, only 100 subclones can be scored. What can be learned? The answer is suggested in Table 4. For RCF = 0.1, no stable recombinants were seen at 96 fissions, and for RCF = 1.0 recombinants were seen in only three of the six simulations. Yet, when 500 clones were scored, as in Figure 1, zero values were rare (*e.g.*, one in seven for RCF = 0.10). In addition, when 100 clones were scored, the fission at which the first recombinant assortee appeared was considerably delayed (*cf.* Table 1); the mean proportions of recombinant assortees at the end of assortment were,

however, unchanged. Thus, for a smaller sample size, rare recombination is likely to be missed. This conclusion is not unexpected given the randomness associated with the assortment process and indicates that sample size must be carefully considered for each hypothesis to be tested. Unfortunately, given the diversity of possible hypotheses, we are unable to present a meaningful table of optimum sample sizes.

DISCUSSION

We have shown by computer simulation that linkage between macronuclear genes should be experimentally demonstrable by assortment when the frequency of recombination is less than three times per cell cycle. We have also shown that assortment experiments are not likely to readily distinguish recombination during development from subsequent amitotic recombination unless the former is sufficiently rare so that recombinant assortees do not first appear until well after 100 fissions past conjugation. Finally, we have shown that the frequencies of recombinant assortees should permit in some circumstances the construction of a crude genetic map.

Recombination between macronuclear genes is possible at any stage of the life cycle. If recombination is rare, then it should produce parental and recombinant assortees in frequencies that suggest linkage. Both the timing and frequency of recombination can influence the frequency of recombinant assortees. For instance, recombination early in macronuclear development at the 4C (haploid equivalents) stage could result in a 1:1:1:1 ratio of assorting units, whereas later recombination at the 64C stage could result in a 31:31:1:1 ratio. Thus, recombination in early development would tend to obscure any subsequent recombination. RCFs may be different at different stages of the life cycle. Recombination during macronuclear development may be higher, relative to total gene dosage, than during subsequent amitotic cycles. In addition, recombination might not occur in macronuclei that skip an S phase. Simple assortment experiments would not distinguish among these possibilities.

Relatively few experiments to map macronuclear genes have been performed. In large part this is because micronuclear linkages have been difficult to demonstrate. Indeed, the assignment of nearly 100 loci to the $N = 5$ micronuclear chromosomes has been through the use of nullisomic strains rather than through conventional testcrosses of double heterozygotes (BRUNS 1982; P. J. BRUNS, personal communication). Testcrosses that have been reported have, unfortunately, yielded ambiguous results, demonstrating linkage in some crosses but not in others (DOERDER 1973; MCCOY 1977); the offending loci have yet to have their linkages confirmed by the method of nullisomics.

In two cases in which suspected micronuclear linkages have been examined by macronuclear assortment (ALLEN 1965; DOERDER 1973), no evidence of macronuclear linkage was found. If the micronuclear linkages are subsequently verified, the lack of coassortment means either that the loci are on different macronuclear chromosomes or that recombination is very frequent. The computer simulations presented here demonstrate that low levels of recombination

produce recombinant assortees at frequencies sufficient to establish linkage. Therefore, if closely linked micronuclear genes (as determined by testcrosses or molecular methods) continue to show independent macronuclear assortment but are shown by molecular techniques to be on the same macronuclear chromosome, frequent macronuclear recombination would be indicated.

ORIAS (1973) proposed that macronuclear recombination is responsible for peculiarities of assortment for the isozymes of the *P-1* (acid phosphatase) locus (ALLEN 1971). Heterozygotes frequently assort to express not two, but three, stable types of subclones. Orias suggested that the stable third isozyme (P_3) is the result of intraallelic recombination between the genes for P_1 and P_5 which are the stable isozymes of the majority of assortees. According to Orias' scheme, the recombinant would have a charge intermediate between the other two stable isozymes. The simulations reported here are consistent with Orias' interpretation if the hypothetical recombination event occurs after macronuclear development. Thus, ALLEN (1971) reported no stable P_3 clones in an interval of 117–160 fissions after conjugation, although at 117 fissions the isozyme was present in a minority of unassorted clones. Unfortunately, the published data are insufficient either to extrapolate the time of the initial recombination event or to estimate the frequency of its occurrence.

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Corresponding editor: S. L. ALLEN