MACRONUCLEAR GENETICS OF TETRAHYMENA
I. RANDOM DISTRIBUTION OF MACRONUCLEAR GENE COPIES IN *T. PYRIFORMIS*, SYNGEN 1

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ABSTRACT

The objective of this study was to test the idea that the macronuclear (somatic) genetic information is randomly distributed at each cell division in *Tetrahymena pyriformis*, syngen 1. We took advantage of a quick and reliable test for the detection of stable vegetative segregants in clones heterozygous for *ts2*, a heat-sensitive mutation. Clones that originated from cells inferred to contain very few (1–3) copies of the *ts2*$^+$ allele in their macronuclei were selected for pedigree analysis. Experimental results were compared with the results of a computer simulation of the experiment. Our results are fully consistent with the predictions of the ALLEN-NANNEY-SCHENSTED model concerning the replication and distribution of functional gene copies. This model proposes that the macronucleus contains many functional copies of a given gene that are duplicated once during the cell cycle and are randomly distributed to the two-daughter nuclei. Our work has provided a more sensitive test of the assumption of randomness than was previously available. Our evidence for complete randomness suggests that 45, the effective number of macronuclear gene copies previously inferred from the segregation rate, indeed represents the *actual* number of copies. This conclusion, coupled with previously available evidence that the macronucleus is approximately 45-ploid, suggests that the segregating genetic units in the macronucleus are in effect haploid. This appears to remove the need to postulate inter-allelic repression to account for the phenomenon of phenotypic assortment. Our results, as well as those of others, also are inconsistent with any simple form of the master-slave hypothesis of ALLEN and GIBSON.

TETRAHYMENA is a ciliate with a diploid germ line and the characteristic separation of germ line (micronuclear) and somatic (macronuclear) genetic information. The micronuclear information, maintained in storage for the sexual progeny produced after conjugation, is copied and distributed faithfully at each binary fission during asexual propagation. Much evidence indicates, however, that this is not true of the macronuclear genetic information. CLEFFMAN (1968) and DOERDER *et al.* (1974) have provided cytological evidence for frequent inequalities in the amounts of macronuclear DNA distributed to daughter cells. ALLEN and NANNEY (1958) and SCHENSTED (1958), in companion papers, inferred the random distribution of macronuclear genetic information involved in

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mating-type determination. This remarkable study dealt with the distribution of "differentiated" states of the same macronuclear allele, but analogous observations were soon made in the simpler case of heterozygous cells containing two different alleles of a simple mendelian gene in their macronuclei (NANNEY and DUBERT 1960). These observations have been extended to heterozygotes in all but one of the genetically determined traits studied in syngen 1 (see SONNEBORN 1974). This apparent randomness in the distribution of genetic information leads to a situation where vegetative descendants of a heterozygous cell cease to express one of the two different alleles of a gene. This phenomenon has been termed "phenotypic assortment" (SONNEBORN 1974). It has also been variously called interallelic repression, allelic exclusion, macronuclear drift and allelic assortment.

The model of macronuclear replication and distribution, proposed by ALLEN, NANNEY and SCHENSTED to explain their observations, stands without serious challenge after many more experimental observations. This model assumes that the macronucleus contains a large number of functional copies of a given gene that in the case of a homozygote are all alike, but that are different in the case of a heterozygote, some expressing one allele and the others the alternative allele. During each cell cycle, each copy duplicates itself, producing two daughter copies which are functionally identical to it. At the time of macronuclear division, all the daughter copies are randomly distributed to the daughter macronuclei, so that the two daughters of a given gene copy have equal probability of going to the same or to opposite daughter macronuclei. Each macronucleus is assumed to get exactly one half of the copies present at the time of cell division. It follows that in a cell lineage originating from an *Aa* heterozygote, the ratio of the number of functional macronuclear *A* alleles to that of *a* alleles can drift aimlessly in successive daughters, until either one of two stable, endpoint ratios are reached: 0 or 1. No further change in macronuclear composition or phenotype can occur in the descendants of a cell that has reached one of these two states. Thus in a clone derived from a heterozygote with mixed macronucleus, the distribution of functional *A* (or *a*) alleles becomes increasingly bimodal, and concentrated at 0 and *N* (the total number of functional macronuclear gene copies) as the clone increases its age measured in fissions. The original model made additional assumptions about the organization of the segregating units ("subnuclei") and their degree of ploidy. Since these are not directly related to the kinetics of distribution, we use the assumption-free term "functional gene copies" instead of "subnuclei", and will mainly consider that subset of the ALLEN-NANNEY-SCHENSTED assumptions that deals strictly with the replication and distribution of functional gene copies. We refer to this subset as the "random distribution of equivalent copies" (RDEC) model. The other assumptions in the ALLEN-NANNEY-SCHENSTED model are treated in the DISCUSSION.

The predictions of the above model convincingly fit the data on the kinetics of segregation originally obtained by ALLEN and NANNEY (1958). From the rate of appearance of stable segregants and the assumptions stated above, it was inferred that the number of functional macronuclear gene copies is 45. The type of data

gathered by ALLEN and NANNEY, however, did not efficiently test the idea that distribution was completely random. As they pointed out, the real number of copies could be higher than 45; but there could be a small bias in the distribution, resulting in a higher probability that the two daughters of a copy would go to the same daughter macronucleus. This would probably not detectably change the segregation kinetics, the rate, or the estimated number of copies. An equal and opposite argument can be made if the number of copies is less than 45 and there is a tendency for two daughter copies to go to opposite daughter macronuclei. Thus, from the data of ALLEN and NANNEY one can only infer an "effective" number of copies, without knowing how well it approximates the "real" number of copies.

The isolation of a heat-sensitive mutant (ORIAS and FLACKS 1973), with the attendant possibility of using heat as a very strong selective agent, allowed a quick and reliable method of detecting heterozygous cells which are stable heat-sensitive segregants. This, in turn, made it possible to specifically test whether or not distribution of macronuclear genetic information in syngen 1 is completely random, by studying cells that have very few functional copies of the wild-type allele left in the macronucleus. This paper describes experimental evidence and results of computer simulation consistent with the idea that indeed the distribution of macronuclear genetic material is completely random, and that the assorting genetic units of the syngen 1 macronucleus are haploid. Our results also provide further evidence against the idea that the replication and distribution of most of the macronuclear DNA proceeds according to any simple form of the master-slave model of ALLEN and GIBSON (1973).

MATERIALS AND METHODS

Strains: The heterozygotes used in this study were the F_1 progeny of strains H9–6 and CA10, belonging to inbred strain D, syngen 1, *T. pyriformis*. Strain H9–6 (mating type II) is homozygous for a recessive heat-sensitive mutation, designated *ts2* (ORIAS and FLACKS 1973; ROBERTS and ORIAS 1973a). Strain CA10 (mating type V) is homozygous for the *ts2*$^+$ allele and also carries a cytoplasmically inherited mutation to chloramphenicol resistance (ORIAS and FLACKS 1973; see also ROBERTS and ORIAS 1973a); the cytoplasmic mutation is not relevant to the study, since only chloramphenicol-sensitive F_1 progeny were selected for the study.

Culture media and methods: All the work was performed with cells in pure (axenic) culture in 2% proteose peptone medium supplemented with 0.1% yeast extract and inorganic salts (PPY medium). The composition of the medium as well as the routine methods used for performing crosses and handling cells have been previously described in full detail (ROBERTS and ORIAS 1973a; ORIAS and FLACKS 1973).

Microclone temperature shift test: This represents a special kind of growth test which was performed repeatedly to test for "purity" of the macronuclear composition of a cell. The test was performed as follows. Heterozygous cells were singly isolated in individual drops of PPY medium on a petri plate and were incubated at 30° for 20 ± 4 hours, producing microclones of 100–200 cells. The plate containing those drops was then transferred to 40° and incubated for 3 ± 1 additional days. The results were scored as "+" if *any* healthy cells (elongated, transparent, colorless) were seen swimming around, and as "—" if *all* the cells were immobile, rounded and darkly pigmented. Homozygous wild-type clones invariably gave a positive growth test while homozygous heat-sensitive clones or heterozygous clones which had been repeatedly scored as pure, invariably gave a negative test. Since the spontaneous rate of "reversion" of heat-sensitive segregants must be less than 10^{-6} (unpublished observations), such an event is not

expected to confuse the results of this type of growth test. The adequacy and rationale of this test are considered in the RESULTS section.

Computer simulation methods: Expected results for the experiments performed were obtained by a computer simulation that involved many repetitions of the experiments. An IBM 360/75 computer was used, and all the programs were written in Fortran IV H language. Different subprograms were written to accomplish individual steps in the sequence of computations to be described below, and were extensively checked individually to be certain of the reliability of their performance. A brief description of the most important features of the programs used is given below.

a. Generation of random numbers: Random numbers were obtained by multiplying a 31-bit odd integer by the constant 65539, and discarding the high order bits (RANDU subroutine, IBM, see reference). The number thus obtained served two purposes: (1) when divided by $2^{31}-1$, it yielded a number between 0 and 1, the desired random number; and (2) when multiplied by the constant it gave the next integer to be used. For a given simulation, the starting integer was the time of day, obtained from the computer clock in digital form (expressed in hundredths of a second) and incremented by 1 if it was even.

b. Expansion of the clones: Where the experimental design called for expansion to about 100–200 cells, the intial cell was expanded to 128 cells by 7 rounds of doubling. The macronuclear composition of the original cell was specified at the outset; the composition of daughter macronuclei at subsequent divisions was determined using the procedure described in the next section. If a sample of 24 out of the 128 cells had to be selected, this was done by random number generation. Where the experimental design called for selecting 24 cells from a population which had been expanded for 13 generations, it would obviously have been wasteful to generate the composition of each of the 8192 cells prior to randomly selecting 24 of those cells. In such cases, a 13 random-bit string was generated for each of the 24 end cells, the bit at each location in the string indicating whether the end cell had been derived from the anterior or posterior daughter at that cell division. It was only necessary then to generate the composition of the cells along the selected lineages.

c. Replication and distribution of macronuclear units at cell division: Replication and distribution were assumed to occur according to the assumptions described by SCHENSTED (1958). Replication was accomplished by multiplying by 2 the number of copies of each allele (totaling 45 prior to replication). The distribution was accomplished by generating a random number, between 0 and 1, and using it to determine the number of copies going to each daughter macronucleus by reference to a table constructed according to the probability of every possible partition. As a check on the whole procedure, a simulation of the original experiments of ALLEN and NANNEY (1958) was performed. This simulation yielded an average equilibrium rate of stabilization per fission (*Rf*) of 0.0115, a value which is not significantly different from that determined experimentally by ALLEN and NANNEY (0.0113).

RESULTS

Test for the stabilization of a heterozygous cell

The analysis described here was made possible by the availability of a rapid and reliable test for the stabilization of the clone. This test, which has been described in the MATERIALS AND METHODS section as the "microclone temperature shift test" (MCTS test), relies on the great selective advantage of cells expressing the *ts2*$^+$ allele at 40°. The test consists of allowing the cell to be tested to give rise to a microclone (100–200 cells) at 30° and then shifting the temperature to 40° and looking for the presence or absence of survivors after 3 days. If survivors were absent, the cell was characterized as having stabilized for the expression of the *ts2* allele, (i.e., having lost all functional copies of the *ts2*$^+$ allele, and containing only functional copies of the *ts2* allele). Otherwise the cell was considered

to still have at least one functional copy of the *ts2*$^+$ allele. Because the interpretation of subsequent experiments so heavily depends upon the reliability of this test, we provide here experimental evidence and theoretical arguments which support the idea that indeed most, if not all, pure *ts2* segregants were correctly identified by the MCTS test.

The possibility that some cells might still be unstable but yet score as pure (stable) by the MCTS test was explored by the following experiment. Forty-six cells, from a microclone known by a previous test to have originated from a nearly stable cell, were isolated in separate drops of PPY medium. After about 20 hours at 30° the contents of each drop (containing 100–200 cells) were equally divided. One portion was immediately shifted to 40° and was scored after 3 days. The other portion was added to a tube containing 0.5 ml of PPY medium and incubated at 30° for 3 more days. Each tube culture increased to an estimated 2×10^5 cells (about 18 fissions). The entire content of each tube was then added to a separate petri plate containing 10 ml of PPY medium and incubated at 40° for 3 days, at which time the plates were scored using the same criterion as for the drops. The following results were obtained. 37 microclones gave negative results by both methods; 8 microclones gave positive results by both methods, and 1 gave a negative result in the drop but a positive result in the plate. If a cell had been nearly pure after only 7–8 fissions, but scored as pure because no descendant had reached the threshold number of *ts2*$^+$ copies required to survive the shift to 40°, further cell divisions prior to the temperature shift should have increased the likelihood of such descendants and should have resulted in clones which scored as negative after 7–8 fissions but positive after 13. The rarity of such cases permits the conclusion that the microclone temperature shift test is a simple and reliable test for the detection of most, if not all, heterozygous cells that have become pure for the expression of the recessive heat-sensitive allele. Indeed, the only exceptional microclone found could well be the result of a somatic "reversion" of a pure segregant to the wild-type phenotype, rather than a rare segregant for a progenitor that was not pure. Such a possibility seems reasonable if it is considered that there were about $37 \times 2 \times 10^5$ or about 7.5×10^6 cells. A spontaneous somatic reversion frequency of one per 7.5×10^6 would not be inconsistent with the failure to see spontaneous revertants in other experiments (unpublished).

The adequacy of the MCTS test can also be supported on independent theoretical grounds based on the RDEC model. According to this model, a microclone started from a cell with even a *single* functional copy of the *ts2*$^+$ allele will often contain at least one cell with as many as 10 *ts2*$^+$ copies at the time of the temperature shift (Table 1). This number would seem sufficient to allow at least one cell from nearly every microclone (if not every one) to survive the transfer to 40°.

Thus the MCTS appears to be a reliable and convenient tool to probe the kinetics of segregation of allele copies in the macronucleus, both on empirical and theoretical grounds.

TABLE 1

Distribution of number of ts2$^+$ *alleles among the eighth-fission descendants as a function of the number (n) of* ts2$^+$ *alleles present in the progenitor cell, obtained by computer simulation*

	Percentage of cells containing a given number of *ts2*$^+$ alleles								
n	0	1	2	3	4	5–6	7–8	9–10	>10
0	100	—	—	—	—	—	—	—	—
1	70	6.9	7.2	5.3	3.8	4.6	2.0	0.4	—
2	48	9.7	10.4	8.7	6.9	9.7	4.9	1.4	0.1
3	34	9.7	11.5	10.4	9.5	14.5	8.2	2.1	0.2
4	24	9.4	11.5	11.2	11.0	18.5	11.1	3.3	0.3

Is there selective pressure against heat-sensitive segregants?

In order to test the mechanism of distribution, it is necessary that there be no differential rates of reproduction at 30° among cells possessing the various macronuclear genotypes. Two lines of evidence suggest that this condition was adequately approximated. We have shown that *ts2* homozygotes, assumed to have the same macronuclear genotype as heat-sensitive segregants of a *ts2*$^+$/*ts2* heterozygote, have the same growth rate at 30° as a wild-type isogenic homozygote (*ts2*$^+$/*ts*$^+$) (ORIAS and FLACKS 1973). The following experiment provides a second line of evidence that differential growth rates did not significantly affect the results of this study.

One of the heterozygous clones with a high frequency of stable heat-sensitive segregants described in the next section was propagated by making daily 250-fold dilutions and incubating at 30°. The frequency of heat-sensitive segregants was determined regularly by doing 192 single cell isolations and performing the MCTS test described above. The results are shown in Figure 1. In the absence of differential growth rates, the frequency of heat-sensitive segregants would have been expected (see Table 6, ALLEN 1971) to increase slowly and reach an asymptote of 98% (assuming that the progenitor cell of this clone had only 1 *ts2*$^+$ copy and 44 *ts2* copies). The results up to about 30 fissions do not differ significantly from the expected slow climb, but the frequency of heat-sensitive segregants then begins to decrease, having dropped to about 10% by 100 fissions.

From the above experiment we draw two conclusions. (1) There appears to be a slight selective advantage associated with the *ts2*$^+$ allele that appears to become important and evident only after 40–60 fissions. (2) During the first 15–20 fissions the selective disadvantage of the cells with mainly *ts2* alleles is negligible. This is the period during which the observations were made for all the subclones described below. Thus we believe our observations are not distorted by differential growth rates.

Composition of clones derived from nearly stable cells

The object of this series of experiments was to study the frequency of stable heat-sensitive segregants among clones which could be inferred to be nearly stable, that is to have originated from cells containing very few functional copies

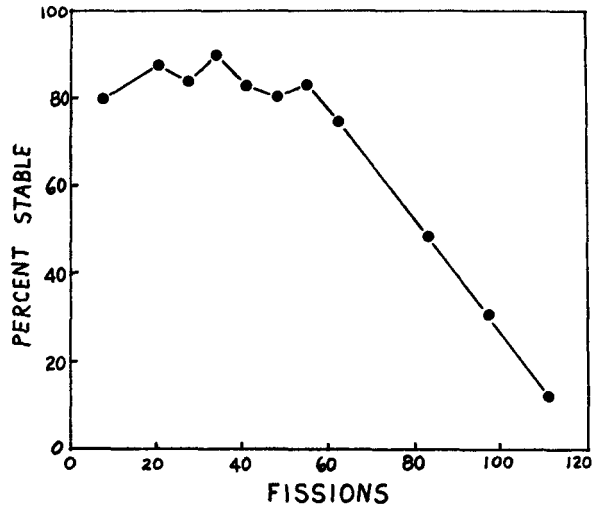

FIGURE 1.—Test for differential growth rate of stable heat-sensitive segregants of a *ts2*$^+$/*ts2* heterozygote. From a stationary cell culture of a heterozygote, previously characterized as containing a high frequency of stable heat-sensitive segregants, a number of single-cell isolations were made and incubated at 30°. The next day, 24 single cells isolations were made from each subclone and these were subjected to the MCTS test for stable segregants. One culture (0.03 ml), in which 19 out of the 24 cells tested were stable heat-sensitive segregants, was added to 7.5 ml of PPY medium and incubated at 30°. A new transfer (0.03 ml into 7.5 ml of fresh medium) was made every day; 192 single-cell isolations were also made every day from the grown culture and were subjected to the MCTS test. From the dilution factor (250) used at each transfer, we estimate that the culture grew at the rate of 8 fissions per day. The number of stable heat-sensitive subclones (expressed as the percentage of 192) are plotted in the ordinate.

of the *ts2*$^+$ allele. To this end, the composition of a large number of clones, 7–8 fissions old, was determined. The design of the experiment, diagramed in Figure 2, was as follows. Many cells (24–48) likely to be nearly stable were singly isolated into separate drops of PPY medium in a petri plate. The drops were incubated at 30° for 20–24 hours, producing clones of 100–200 cells. We arbitrarily call each of these a *B* clone, to save words in further description. One of these is represented in Figure 2 and labeled with the letter *B*. In order to determine the fraction of stable *ts2* cells in each *B* clone, 24 of its cells were isolated at that point (step 4, Figure 2) and tested for stability by the MCTS test. Each *B* clone could then be characterized by the number of stable heat-sensitive cells among the 24 tested. This number will be called *S* and can vary from 0 to 24.

It was asserted above that the cells used to generate the *B* clones were likely to be nearly stable. This was accomplished by obtaining these cells from a clone (*A* clone, Figure 2) that had been previously determined to have itself originated from a nearly stable cell. Such *A* clones were selected by the criterion that at age 7–8 fissions at 30° (step 2, Figure 2) at least 30% of its members were pure for the *ts2* allele; by the time the *B* clone progenitors were isolated the *A* clones had meanwhile undergone another 5–6 fissions at room temperature. A

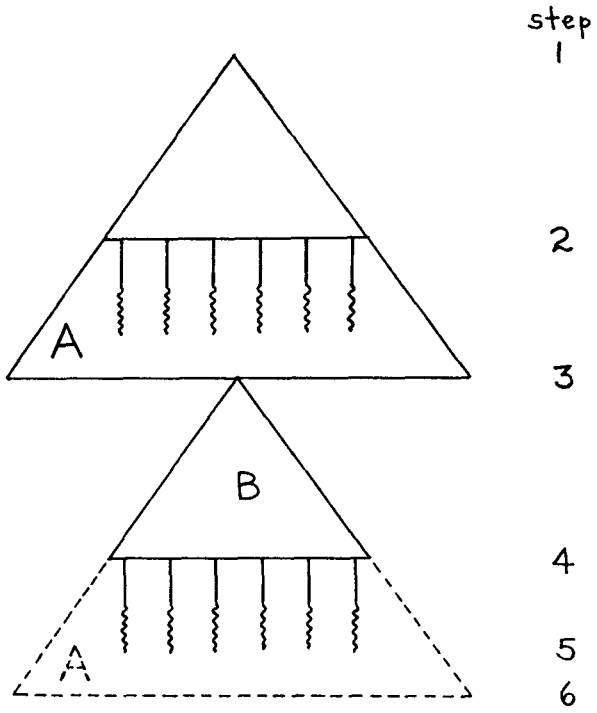

FIGURE 2.—Diagramatic representation of the protocol used to study the composition of clones obtained from nearly stable cells. (A) represents the clone used as a source of cells likely to be nearly stable and used to initiate the clones (B) whose composition was to be determined. The triangles represent the expansion of a clone. The vertical lines represent MCTS tests of single cells, 6 (out of the 24) being shown in each case; the straight part of the line represents the incubation at 30° for 1 day, the wavy part of the line represents the incubation at 41° for 3 additional days.

The schedule of operations was as follows. (1) A single heterozygous cell was isolated into a drop of PPY medium and incubated at 30° for 20 ± 4 hours. (2) The drop was transferred to and kept at room temperature for 3–6 additional days; 24 single cells were isolated to PPY medium and tested by the microclone temperature shift test. (3) After determining that clone *A* contained at least 30% stable cells, 24–28 single cells were isolated in drops of PPY medium and incubated at 30° for 20 ± 4 hours. (4) From each *B* clone, 24 cells were isolated and tested by the microclone temperature shift test (vertical lines); the *B* clones were transferred to and kept at room temperature. (5) End of the experiment. The results were scored and each *B* clone was assigned a number from 0 to 24 according to the number of isolates scored as "—". (6) Selected *B* clones containing at least 30% stable cells were then used as the equivalent of *A* clones (i.e., they serve as source of cells to start new *B* clones).

cyclical procedure was initiated whereby *B* clones shown to have at least 30% stable cells become *A* clones, the source of new *B* clones. The 30% boundary was arbitrarily selected so as to include clones likely to have originated from a cell with three or fewer functional *ts2*$^+$ alleles in its macronucleus (see Table 1).

The distribution obtained by pooling the results of scoring a total of 495 B clones is shown in Figure 3, where the number of *B* clones has been plotted against *S*. The figure shows a peak at $S = 17$, and no evidence of other peaks for

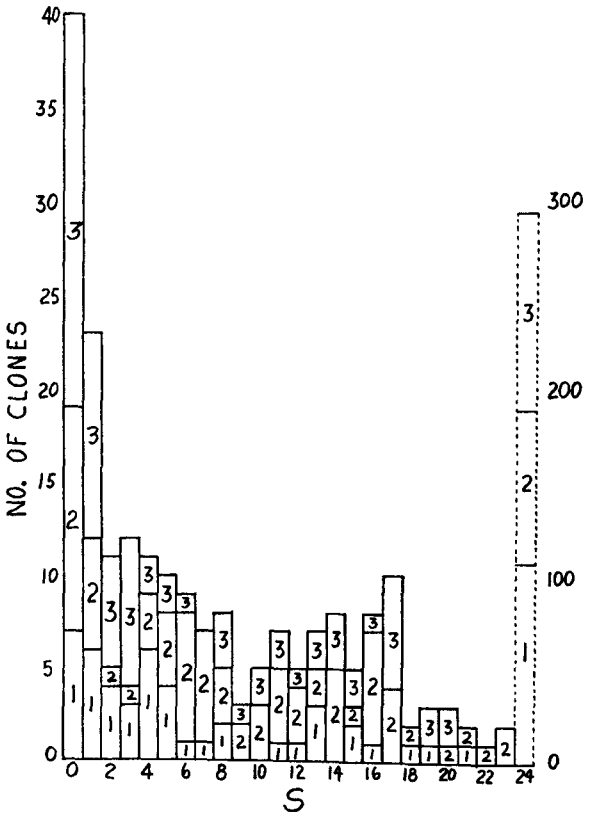

FIGURE 3.—Cumulative distribution of all the clones studied. The abscissa represents the class to which a given *B* clone belongs; the experimental basis for the classification is described in the text and under Figure 2. The ordinate represents the number of clones found in each class. The right ordinate applies only to the 24–class; the left ordinate to all other classes. The segments within each bar show the number of clones derived from progenitor cells with various inferred values of *n*, indicated by the numerals in the segment. The value of *n* was inferred from the number of stable heat-sensitive segregants present at stage 2, Figure 2. Boundaries used to determine *n* were arbitrarily placed at the halfway point between the % of sensitive segregants expected, from the computer simulation, for consecutive values of *n*.

values of *S* between 17 and 24. This discontinuity fits very well with the predictions of the RDEC hypothesis: unstable cells with only one functional copy (the least non-0 number of *ts2*$^+$ copies) are expected to give rise to 70% stable cells (Table 1), a value which corresponds to $S = 17$. Thus for *S* between 17 and 24, only the steadily decreasing tail of the peak at $S = 17$ is expected.

In order to determine how far off the assumption of random distribution could be and still get a peak not significantly different from 70%, we performed computer simulations in which bias in either direction from randomness was deliberately introduced. This was done by allowing in separate simulations a random fraction of the gene copies to be distributed at every fission either (a) always to opposite daughter macronuclei or (b) always to the same, randomly selected,

TABLE 2

Distribution of clones obtained by a computer simulation of the experiment described in Figure 2, using the RDEC model

n	0		2		4		6		8		10		12		14		16		18		20		22		24
1	39	24	20	13	19	11	11	10	12	12	9	10	8	8	8	9	6	6	6	6	2	1	2	0	948
2	100	47	32	21	16	16	28	20	24	18	21	16	13	13	13	14	17	13	1	12	6	2	0	0	737
3	166	60	41	57	28	20	23	25	22	20	21	22	21	20	17	17	8	12	15	13	6	3	1	1	561

n represents the number of *ts2*$^+$ segregating units in the cell used to initiate the *A* clone (see Figure 2)..
A total of 1200 B clones were generated for each initial value of *n*.

daughter macronucleus; the remainder of the copies was distributed strictly randomly. The analysis indicates that if there is a deviation from randomness, in either direction, equivalent to as little as 10% perfectly ordered gene copy distribution (and 90% truly random distribution), our 70% peak would have differed significantly (at the 5% level) from the expectations. Since the distribution of the last few copies is examined, this analysis is, within the experimental error, completely independent of the total number of assorting units, at least for $N > 10$.

In view of evidence considered in the DISCUSSION, the most attractive alternative to 45 units assorting completely at random is 23 diploid units distributed with such a bias that an equivalent average rate of stabilization per fission (Rf = .0113) is obtained. In order to determine how much bias would be required, we have performed computer simulations assuming $N = 23$ and using different degrees of bias. The results indicate that the required bias is given by a combination of approximately 45% disjunctional and 55% truly random distribution. This amount of bias would lower the first non-100% peak by a highly significant amount, according to the analysis of the previous paragraph. Thus our experimental results cannot be reconciled with the idea of 23 diploid assorting genetic units. These computer simulations will be considered in more detail in a separate publication (ORIAS, manuscript in preparation).

The following attempt was made to determine whether or not all of the results of Figure 3 are in quantitative agreement with the expectations of the RDEC model. A computer simulation of the experiment described in Figure 2 was generated, using $N = 45$. Three sets of 1200 B microclones were separately generated with cells having 1, 2 and 3 $ts2^+$ copies, respectively, in the macro-

TABLE 3

Comparison of the predicted and observed total distribution of B *clones*

n	CLONES CLASSES									TOTAL
	0	1–2	3–4	5–7	8–10	11–14	15–18	19–23	24	
O	8	10	9	6	2	5	4	2	107	153
1										
E	5.0	5.6	4.1	4.1	4.2	4.3	3.5	1.4	121	
O	12	7	4	16	8	14	12	5	82	160
2										
E	13.3	10.5	4.9	8.5	8.4	7.3	6.0	2.7	98	
O	21	17	10	3	6	8	8	4	105	182
3										
E	25.2	15.3	12.9	10.3	9.6	12.1	7.9	3.6	85	
O	41	34	23	25	16	27	24	11	294	495
P										
E	43.5	31.5	21.9	22.9	22.2	23.8	17.4	7.7	304	

E: expected; O: observed; P is a pool of all the clones.

Classes have been pooled so as to give about 20 expected clones in each pool.

The difference between the expected and observed results in the total pool (row P) is not statistically significant (P(X^2) = .5–.6).

nucleus. The results of this simulation are shown in Table 2. To compare the experimental results with the predictions, it was necessary to classify each of the *A* clones used in the biological experiment according to the number of functional *ts2*$^+$ copies of its initial cell; this was done according to the number of stable cells found at age 7 fissions (step 2, Figure 2), using the values in Table 1 (see legend to Figure 3). Given the predicted frequencies for each type of clone and the actual number of clones of each type used, the number of *B* clones expected from the computer simulation for each value of *S* could be determined. The comparisons between observed and expected results are shown in Table 3. The agreement is good in the individual comparison, and is remarkably good when the total results are compared. The latter comparison is probably more reliable, since it tends to average out unavoidable mistakes made in the classification of the clones with regard to the number of functional *ts2*$^+$ copies in their initial cells.

Clones derived from two daughters of a nearly stable cell

This series of experiments was designed to study the distribution of *ts2*$^+$ allele copies to the two daughters of the same nearly stable cell. Cells from an *A* clone (at step 3, Figure 2) were fed by making a dilution into PPY medium at 30°. Two hours later, dividing cells were isolated into drops of the same medium. In 15–30 minutes, one daughter was removed to a separate drop. Pairs of sister clones so obtained were then treated as *B* clones of the previous work.

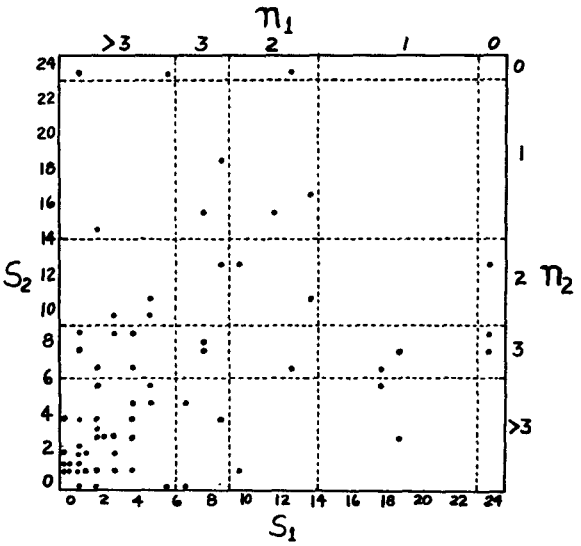

FIGURE 4.—Relationship between the composition of sister *B* microclones. The composition (*S*) of the clone derived from one daughter of a dividing cell is plotted along the bottom abscissa (S_1); that of the other daughter, along the left ordinate (S_2). Each point represents a different pair of sister *B* clones. The dotted lines arbitrarily divide the graph on the basis of likely initial value of *n*, inferred as in Figure 3, and shown on the top abscissa for one daughter (n_1) and on the right ordinate for the other daughter (n_2). Fourteen 0–0 pairs and 76 24–24 pairs were also observed but are not shown in the graph.

The results obtained for 156 sets of sister *B* clones are shown in Figure 4. While the numbers are too small to test meaningfully the quantitative agreement with an expected distribution, two observations are worth noting. (1) Many points are widely scattered from the main diagonal, providing a striking confirmation of unequal distribution, and (2) the results are qualitatively in agreement with the model of random distribution.

DISCUSSION

Random distribution of allele copies in the macronucleus

The results obtained with daughter subclones (Figure 4) and, more rigorously, the cumulative distribution shown in Figure 3 support the idea that allele copies are randomly distributed in the macronucleus of Tetrahymena (ALLEN and NANNEY 1958; SCHENSTED 1958). In particular, the experiments described here provide a more sensitive test of the assumption of randomness than had been possible previously, since they examine the distribution in cells having very few functional copies of the *ts2*$^+$ allele. Also because of this, frequencies of stabilization we have dealt with are very insensitive to variation in the total number of *ts* gene copies (*ts2*$^+$ plus *ts2*), and these frequencies neither are affected by, nor *directly* comment on, that number (previously estimated to be 45, as discussed below).

The original experiments of ALLEN and NANNEY (1958) permitted the conclusion that if the distribution was random, then the observed rate of stabilization demanded that the total number of allele copies be 45 in the G1 macronucleus. The number could be larger or smaller depending on whether the two daughter copies of an allele tend to segregate preferentially to the same or opposite daughter macronuclei, respectively. The results reported here support the assumption of truly random segregation and, *taken together* with those of ALLEN and NANNEY, are interpreted to mean that 45 is the *real* number of segregating units.

The lack of a peak of clones between 70% and 100% stable *ts*$^-$ cells (Figure 3) is noteworthy. The peak with the highest non-100 percentage must be attributed to progenitor cells with the least non-0 number of *ts2*$^+$ alleles, namely 1. The agreement between the observation and the prediction of the model substantiates the idea that each copy nearly always replicates at each cell division. If a lone functional *ts2*$^+$ copy frequently failed to replicate, then the probability of giving rise to a stable daughter at the first division would be ½ (rather than ¼) with a consequent displacement of the 70% peak toward a higher percentage after 7–8 fissions. This finding is in agreement with the report of ANDERSON, BRUNK and ZEUTHEN (1970) that there is no detectable amount of macronuclear DNA which fails to replicate at every cell division in strain GL.

Are the segregating units haploid or diploid?

The assorting genetic units were originally assumed to be diploid (ALLEN and NANNEY 1958; NANNEY 1964). However, the following evidence taken as a whole leads us to the hypothesis that in syngen 1 the assorting genetic units are haploid. WOODWARD, KANESHIRO and GOROVSKY (1972) showed by cytospectro-

photometric methods that the G1 macronucleus in syngen 1 has about 45 times the amount of DNA of the haploid complement. ALLEN and GIBSON (1972) confirmed these findings and furthermore provided evidence that at least 80% of the DNA sequences in the macronucleus are unique, having a kinetic complexity approximately equal to that expected for the haploid micronuclear complement. YAO and GOROVSKY (personal communication) have recently directly established that 80–90% of the DNA sequences of the micronucleus are present in the macronucleus. All these experiments taken together strongly imply that the Tetrahymena macronucleus, as a first approximation, can be considered to be simply 45-ploid in terms of its DNA composition. Our conclusion that 45 is the real number of segregating genetic units, taken together with the 45-ploidy of the macronucleus, implies to us that *the assorting genetic units in syngen 1 are effectively haploid* and that *most (if not all) macronuclear gene copies are functional copies*, i.e. they are expressable. This, to us, represents the *simplest* interpretation of all the cytochemical, molecular and genetic evidence taken as whole. Suggestions that the assorting units are haploid have previously been made by NILSSON (1970) on the basis of cytological observations in strain GL and by ALLEN and GIBSON (1973) as a feature of their master-slave model (see below).

The haploidy of the segregating units and the notion that most (if not all) macronuclear gene copies are expressed remove the need to postulate inter-allelic repression in heterozygotes (NANNEY 1964) as the basis for the expression of a single allele by each segregating unit, originally assumed to be diploid. This should not be interpreted as a suggestion that interallelic repressions do not

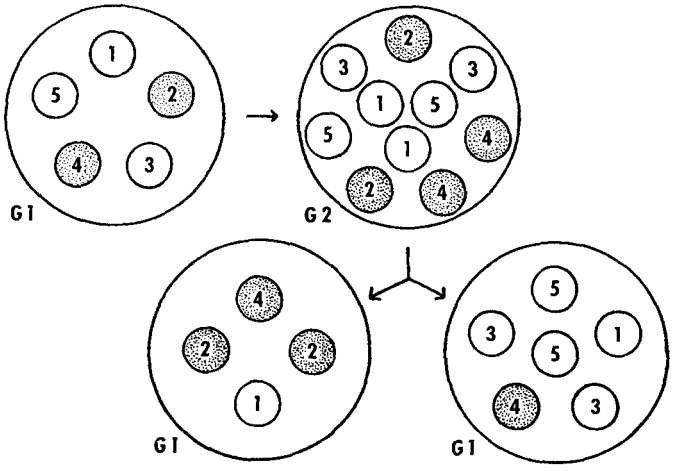

FIGURE 5.—Illustration of the model of replication and random distribution of macronuclear gene copies during binary fission in syngen 1 of *T. pyriformis*. The largest circles represent the macronuclear membrane. Small circles represent haploid copies of the same gene in an *Aa* heterozygote. The open circles represent *A* alleles and stipled circles *a* alleles. The allele copies are numbered to show their fate. Only 5 copies are shown, instead of 45, for simplicity's sake. G1 and G2 refer to stages in the cell cycle.

occur at all in Tetrahymena; indeed, the macronuclear differentiation involved in mating type determination (NANNEY 1956) could well have such a basis. What is being suggested here is that the phenotypic assortment seen in *most heterozygotes* studied is not based on interallelic repression, but on the complete loss of one of the two alleles from the macronucleus. A direct test of this hypothesis has not yet been performed.

Thus, our analysis favors the following simple modification of the original model of the syngen 1 macronucleus (NANNEY 1964). Most genes are represented by approximately 45 haploid copies, on the average. These copies are all identical in homozygotes, but initially of two allelic types in heterozygotes. They are duplicated once during each cell cycle and are distributed completely at random to the two daughter macronuclei at the time of cell division. The two daughter macronuclei get approximately the same total number of copies. This model is illustrated in Figure 5.

Basis for randomness in distribution

Neither these results nor others previously reported give clues concerning the basis for the randomness in distribution. Discrete chromosomes have not been seen in the macronucleus of Tetrahymena or other holotrichous ciliates (although they are readily seen in the early stages of macronuclear differentiation in hypotrichous ciliates (PRESCOTT, MURTI and BOSTOCK 1973). It is possible that the daughter DNA molecules, whatever their organization, are not permanently attached to any macronuclear structure regularly segregated at the time of division (if any such structure exists); in such case, a rather thorough mixing of the macronuclear contents must be assumed to occur during the G2 period. Alternatively, there may be an attachment site on each DNA molecule tending to accomplish a regular segregation of the two daughter molecules to different macronuclear daughters; the retention of the nuclear membrane during macronuclear division could provide such attachment sites. If such were the case, it is necessary to further assume that extensive recombination (crossing over) must occur in the marconucleus between homologous segments of nonsister DNA molecules to randomize the distribution of alleles. Since the most rigorously studied heterozygotes all give the same stabilization rate, such that the number of copies must be 45, it must be further assumed that all the genes rigorously studied must be located far enough from their hypothetical kinetochore that they can be completely randomized by crossing over. The above speculations should be qualified by stating that the existence of macronuclear crossing over has not been established. Certain observations made with acid phosphatase-1 heterozygotes have been attributed to this process (ORIAS 1973) but alternative interpretations have also been proposed (ALLEN 1971). The random vegetative segregation of markers which are linked in their micronuclear (meiotic) transmission (ALLEN 1965; DOERDER 1973) could also be explained by frequent macronuclear crossing over, but an alternative explanation based on extensive chromosomal fragmentation in the macronucleus is also possible

(ALLEN and GIBSON 1973). Precedent for such fragmentation exists in the distantly related ciliate Stylonichia (PRESCOTT, MURTI and BOSTOCK 1973).

The existence of a locus in syngen 1 which has not yielded stable segregants for the recessive allele [*ts1*, McCOY (1973)] is puzzling in view of the many examples of random distribution. The observation may turn out to be an important clue toward understanding the mechanism of distribution. It seems unlikely that adverse selective pressure accounts for the failure to isolate the segregants (McCOY, 1973 and personal communication). Unfortunately, there is as yet no mathematical treatment which would indicate how strong the selective disadvantage must be in order to prevent the isolation of recessive segregants. In the case of the *ts2* locus there appears to be some selective pressure against the recessive allele (Figure 1), but this pressure is not strong enough to prevent the appearance of the stable recessive segregants.

The evidence against the master-slave model

ALLEN and GIBSON (1973) have proposed an entirely different model for the replication and distribution of macronuclear genetic information. Their model postulates two types of haploid gene copies, masters and slaves. A slave cannot replicate; a master replicates at each cell cycle in both of two alternative modes, to make two identical master copies and many slave copies. Thus in the macronucleus of an *Aa* heterozygote, there would be an *A* master, an *a* master, many *A* slaves and many *a* slaves. Concerning distribution, masters are distributed equally; i.e., the two daughters of a master normally go to different daughter macronuclei. The slaves are assumed to be randomly distributed. The appearance of stable segregants is attributed to rare failures in the equal distribution of masters, an event which the authors call "misreplication". We will refer to this model as the "master-slave" (M-S) model.

The M-S model, in its simplest form outlined above, is inadequate to explain the original experimental observations of ALLEN and NANNEY (1958), because it provides no basis for the range of initial rates of stabilization among the clones they analyzed, a phenomenon amply confirmed in this work. This phenomenon is readily explained by the RDEC model on the basis of different initial ratios of the two types of functional macronuclear gene copies. Under the assumptions of the simple M-S model, however, stability is all-or-nothing, strictly dependent on whether there are two or one kind of masters in the cell; the number and kinds of slaves are irrelevant to the kinetics of stabilization. Clones with high initial rates of stabilization (nearly stable clones, in our terminology) should be those rare clones (with a frequency on the order of 1%) in which misreplication of one master occurs during the first two fissions. The consequent kinetics should show an *Rf* that begins high but drops to equilibrium value within six fissions; this was not observed by ALLEN and NANNEY. Furthermore, the first non-100% peak of the cumulative distribution (Figure 3) should occur at 50%, containing the clones with the earliest possible "misreplication", namely during the first cell cycle after isolation, and, furthermore, this peak should contain only about 1% of the clones; clearly the results are in serious conflict with the predictions.

In attempt to circumvent some of these problems, ALLEN and GIBSON (1973) proposed that the rates of misreplication are variable. These authors have proposed neither rules governing variation of the misreplication rate, nor its pattern of clonal transmission. Clearly to account for the original observations, the misreplication rate has to first go up and then down in some clones, only up in others, always stopping their fluctuation at 0.0113. Indeed the hypothetical misreplication rate behaves exactly as if determined by a small number of particles randomly distributed. Thus nothing of explanatory valued is gained, and much is lost, by the whole concept of master and slave copies.

DOERDER (1973) has argued against the M-S model on the basis of independent genetic evidence obtained in a study of the early segregation kinetics for two epistatic loci; but there is no quantitative mathematical treatment of this problem to serve as a basis of rigorous comparison.

ANDERSEN (1972) has obtained the most rigorous biochemical evidence against the hypothesis of a master-slave type of replication and distribution of most or all of the macronuclear DNA. His experiments using DNA density-labelling in strain GL demonstrate that, within the limits of detection, all the macronuclear DNA replicates semiconservatively onced uring each cell cycle. The M-S model predicts a type of replication which would *appear* as conservative, because about half of the macronuclear DNA (the old slaves) would remain intact. and about half (the new slaves) would be synthesized completely from new precursors. The ribosomal DNA, present at a ploidy level of close to 10,000 per macronucleus (ENGBERG and PEARLMAN 1972; YAO, KIMMEL and GOROVSKY 1974), also appears to replicate semiconservatively (ENGBERG, MOWAT and PEARLAN 1972). None of the work, however, excludes the possibility that a minor fraction of the DNA, too small to be detected by the methods used, replicates according to the master-slave mechanism.

Consequences of the random distribution of macronuclear genetic information

The occurrence of random macronuclear gene distribution has a number of consequences, some of which have already been pointed out and successfully exploited. Firstly, this mechanism makes possible the isolation of recessive mutants in the heterozygous state (CARLSON 1971; DOERDER 1973). This provides a very useful solution to the general and difficult problem of isolating recessive mutants in organisms with a diploid germ line. Secondly, it makes possible the isolation of somatic mutants in spite of the large ploidy of the macronucleus; in principle it is necessary to mutate only one copy of a macronuclear gene, with random distribution increasing the number of copies to the point where a "phenotypic threshold" is reached. Variants likely due to spontaneous or induced macronuclear mutation have been found by NANNEY (1959), MCCOY (1973), BYRNE (personal communication), ORIAS (unpublished results) and SILBERSTEIN, ORIAS and POLLOCK (in preparation). Strong evidence for the macronuclear basis of somatic mutations to cycloheximide resistance, based on the kinetics of phenotypic assortment, has recently been provided (ORIAS and NEWBY, in preparation). If amicronucleate Tetrahymena strains, frequently

isolated from natural habitats (ELLIOTT and HAYES 1955), also distribute macronuclear genes randomly, mutants could arise in such strains. This would be a source of evolutionary plasticity that could account for their continued survival. Indeed, BYRNE (personal communication) reports the isolation of 6-methylpurine-resistant mutants in amicronucleate strain GL that he attributes to the induction of macronuclear mutations. Thirdly, the process leads to the formation of so-called "functional heterokaryons" (BRUNS and BRUSSARD 1974), or strains in which the macronucleus expresses a phenotype which is not a simple consequence of the dominance or recessiveness of alleles in the germ line. Such strains have been exploited to eliminate parental and non-conjugant cells in mass mating (ROBERTS and ORIAS 1973b; BRUNS and BRUSSARD 1974). Fourthly, random distribution should make possible the detection of rare crossing over events in the macronucleus (ORIAS 1973), again by increasing the number of copies of the recombinant allele to the point where the recombinant phenotype is detectable. A suitable pair of mutations to test this possibility is not yet available. Lastly, heterozygotes which still have a mixed macronucleus should be avoided when trying to characterize the physiological or biochemical properties of new mutants, because phenotypic variability is expected from culture to culture of the same clone; only homozygotes or stable heterozygous segregants should be used for these purposes.

The problem of young heterozygotes

The most detailed studies that support the RDEC model of distribution of macronuclear genetic information have been made in heterozygous clones older than 50–70 binary fissions post-conjugation. Studies of younger heterozygotes have shown that the model is not necessarily applicable to them. The most significant problem is that heterozygotes at certain loci (but not at others) behave as though there is a delayed onset of random distribution and segregation (see review by SONNEBORN 1974). The first interpretation given to this observation was based on the idea that the segregating units are diploid, and that allelic exclusion involves a functional differentiation so that only one allele is expressed by each segregating unit (NANNEY 1964). Random distribution could only begin to give stable segregants after this differentiation has occurred; thus the time of onset of segregation is equated to the time of functional differentiation or determination of the diploid segregating units. Our conclusion that the segregating units are haploid is in conflict with NANNEY's (1964) model of interallelic repression. While the haploid model is in excellent agreement with biochemical and cytological observations, neither a simple haploid nor diploid models can account for the delayed onset of random distribution for certain loci without additional *ad hoc* assumptions. Indeed, the M-S model, discussed in the previous section, arose as an attempt to find a solution to this problem. Further insights into the mechanism which accomplishes the distribution of macronuclear DNA, and its developmental aspects, may hold clues to this important unsolved question.

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