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Variability in the timing and outcome of macronuclear assortment in *Tetrahymena thermophila*

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SUMMARY

New experimental designs have detected unexpectedly large variations in the time at which macronuclear assortment begins, and in the ratios of the stabilized products. Variation is detected between strains, and, within strains, between conjugating pairs. The *Chx* locus gave results ranging from late assortment (40–60 fissions) to early assortment with skewed input, indicating the existence of some relation between the parameters of input ratio and time of determination.

1. INTRODUCTION

Ciliate protozoans of the genus *Tetrahymena* show a remarkable temporal regulation of gene expression that has led investigators to speculate on stochastic or genetically programmed mechanisms of biological time-keeping (Nanney, 1964, 1968, 1974; Bleyman, 1971). A crucial piece of evidence was the observation that the timing of macronuclear assortment is locus specific (data reviewed and summarized by Allen & Gibson, 1973). This paper reports data showing that this assumption is not entirely valid – large variations in both input ratio and time of determination have been encountered. While the temporal problem remains very real, there is evidently more involved than simply commencing assortment for a given locus after a set number of fissions have elapsed. The use of the multiple marker stocks described in earlier papers (McCoy, 1977, 1979) also revealed other new features of assortment. Probably the most interesting of these is the existence of a continuum of input ratio and determination time.

2. MATERIALS AND METHODS

Procedures follow exactly those cited previously (Allen & Gibson, 1973; McCoy, 1979). The few modifications used here will be mentioned as individual experiments are discussed. Reference will be made to theoretical predictions (Doerder *et al.* 1975, 1977, and unpublished) included in tabular form in the preceding paper. Marker phenotypes for *Chx*, *Mpr*, *co*, and *ts-1* are described in the previous paper (McCoy, 1979). The *H* locus has a number of alleles identified by the immo-

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bilization and agglutination of cells when exposed to a characteristic monospecific antiserum (Nanney & Dubert, 1960).

Strain *Chx co ts-1 Mpr* 18–21 has been described earlier (McCoy, 1977, 1979). Dr F. P. Doerder provided a strain bearing the *r4* allele (Doerder, unpublished; Sonneborn, 1975; McCoy, 1977); a stock used in the present experiments was derived by three successive backcrosses to strain B derivatives. The additional *co* stock was also the product of successive backcrosses of the original isolate (Doerder *et al.*, 1975) to strain B. Wild type strains are the same ones used in previous experiments (McCoy, 1977, 1979).

3. RESULTS

In the preceding paper (McCoy, 1979) heterozygotes were constructed between a multiple marker stock, *Chx co ts-1 Mpr* (McCoy, 1977) and strain C2. Three sets of subclones were derived from cells isolated at 18 fissions past conjugation. Each set represented one cell from a different conjugating pair, and contained 30 sublines. These sublines were also used in obtaining the data reported here. Three more sets of sublines were obtained from a cross of the marker stock to a derivative of strain D (strain I1, Allen & Lee, 1971). All sets were transferred by single cell isolations at 13-fission intervals. Sublines were tested at each transfer for the Mpr^+, Chx^+, and co phenotypes (*ts-1* was not tested because it does not assort, McCoy, 1973). Sublines showing these phenotypes were considered 'pure' (stabilized) if all succeeding transfers showed no evidence of the dominant allele.

From the fraction of pure types at each transfer, the input ratio (for loci already assorting at the time the initial cell for each set was isolated: 18 fissions for the C2 crosses and 0 fissions for the D crosses) or the time of determination (the time at which assortment commences) could be estimated for each set by reference to the theoretical predictions cited above.

The results are summarized in Table 1, with data from earlier experiments. Complete data are contained in Table 2. Data for *co* are here pooled because not enough fixations to the co phenotype had occurred to give significant estimates for the individual sets. No figures for *co* are available for the B × D sets; instead, data from an earlier experiment are substituted. When the B × D experiment was terminated, there were only 4 co out of 120 total sublines. Previous experiments (unpublished and Doerder *et al.*, 1975) showed that co sublines are generally slower growing than wild type, and that their rate of appearance in the earlier strain B situation was consequently only about 30% of the expected rate. The slower growth rate actually predicts a rate of appearance very close to that observed in the B background. In the B × C2 cross, however, the rate of appearance was normal, and the growth rate of the co subline was *at least* 85% of normal, as measured both by cell number and by time of exhaustion of the constant food supply. If the growth rate depends strongly upon genetic background, the time of determination for *co* cannot be estimated in the B × D cross. If the more normal performance in the B × C2 crosses is simply the result

Table 1. *Summary of assortment data*

Locus	Est. input	Est. time of determination	Genetic background
co	22:23	54	B × C2 (pooled data)
	22:23	20	B
Chx	22:23	18	B × C2
	22:23	49	B × C2
	22:23	62	B × C2
	22:23	14	B
	22:23	0	B × D
	22:23	0	B × D
	12:33	0	B × D
Mpr	24:21	0–18	B × C2
	25:20	0–18	B × C2
	30:15	0–18	B × C2
	36:9	0	B × D (three replicates)

of eliminating various deleterious alleles during backcrosses to B, then the B × D cross gives an estimated time of determination of about 60 fissions after conjugation.

No variation is recorded for *Mpr* (but see below), except that the input ratios are significantly less extreme in the B × C2 cross. Because the B × C2 sets were started from 18-fission cells, the evidence concerning immediate assortment of *Mpr* is not conclusive. Tremendous variation is conclusively demonstrated for the *Chx* locus, however. In the B × C2 cross, one set began assorting as soon as it was isolated, while the other two sets began assorting at 49 and 62 fissions. In the B × D cross, all sets began assortment *immediately*, although with differing inputs.

These results provide striking evidence for variation between strains and for variation between pairs within strains. Variation between pairs is further documented in an experiment monitoring the assortment of serotype *r4* (Doerder, unpublished; Sonneborn, 1975; McCoy, 1977) in 15 sublines from each of 20 pairs from a single cross. Sublines tested at 26 fissions past conjugation with anti-Hd (wild type, does not immobilize r4 cells) and anti-He sera (the latter prepared against purified He antigen, Bruns, 1971; immobilizes r4 cells as well as the homologous He cells, although anti-r4 serum does not immobilize He cells) gave the distribution of apparent pure r4 sublines shown in Table 3. The average inferred input ratio is 8:37. Comparison of the observed frequency distribution with either binomial or Poisson expectations shows an extremely significant heterogeneity. That is, the number of sublines pure for r4 is not randomly distributed among pairs. The variation recorded here is real, and suggests three 'modes' of input.

Table 2. *Macronuclear assortment*

Strains ...	B, C2 Mpr+			B, C2 Chx+			B, C2 co			B, D Chx+			B, D Mpr+	B co
A ...	Pair 1	Pair 2	Pair 3	Pair 1	Pair 2	Pair 3	Pair 1	Pair 2	Pair 3	Pair 4	Pair 5	Pair 6	(pooled)	(300 sub-lines)
Fissions	Fraction of sublines pure for phenotype A													
	Initial cells selected at 18 fissions past conjugation													
18														
26	—	—	—	—	—	—	—	—	—	0·00i	0·00i	0·20(35)	0·42(39)i	—
39	—	—	—	—	—	—	—	—	—	0·03(25)	0·07(22)	0·30(34)	0·43(37)	0·000
44	0·00i	0·03(25)i	0·13(32)i	0·00t	0·00t	0·00i	0·00t	0·00t	0·00t	—	—	—	—	—
52	—	—	—	—	—	—	—	—	—	0·10(23)	0·10(23)	0·33(32)	0·44(36)	0·014
65	—	—	—	—	—	—	—	—	—	0·17(23)	0·20(23)	0·40(33)	0·46(34)	0·036
70	0·10(22)	0·10(22)	0·27(30)	0·00	0·00	0·07(19)	0·00	0·00	0·00	—	—	—	—	—
78	—	—	—	—	—	—	—	—	—	0·20(22)	0·20(22)	0·40(31)	0·46(33)	0·075
83	0·17(22)	0·23(27)	0·27(28)	0·03(52)	0·03(52)	0·13(21)	0·00	0·03(52)	0·07(42)	—	—	—	—	—
91	—	—	—	—	—	—	—	—	—	—	—	—	—	0·097
96	0·17(21)	0·27(26)	0·30(28)	0·10(46)	0·03(65)	0·20(22)	0·03(65)	0·13(38)	0·07(55)	—	—	—	—	—
104	—	—	—	—	—	—	—	—	—	—	—	—	—	0·123
109	0·30(25)	0·33(27)	0·43(30)	0·17(43)	0·07(68)	0·30(25)	0·07(68)	0·17(42)	0·10(59)	—	—	—	—	—
122	0·40(28)	0·33(25)	0·47(31)	0·17(56)	0·13(63)	0·33(25)	0·10(72)	0·23(35)	0·17(55)	—	—	—	—	—
Estimated no. A-type subunits	24	25	30	23	23	22	23	23	23	23	23	33	36	23
Estimated time of determination	0–18	0–18	0–18	49	62	0–18	68	42	53	0	0	0	0	20*

(i) Number in parentheses is estimated input of A subunits.
(t) Number in parentheses is estimated time of determination.
* Estimated by fitting a least-squares line and comparing x-intercept with those of other, more normal curves.

Table 3. *Distribution of pure r4 sublines from a heterozygote, within individual pairs (15 sublines per pair)*

No. pure sublines	No. of pairs with this distribution
0	5
1	2
2	2
3	4
4	0
5	0
6	0
7	2
8	2
9	2
10	0
11	0
12	0
13	0
14	1
15	0

Six of the 20 pairs had moderately skewed input, one had a great excess of r4, and 13 had equal proportions of r4 and $r4^+$.

4. DISCUSSION

(i) *Statistical analysis of assortment data*

There is not yet any clear method for determining whether an observed assortment experiment fits a theoretical curve, using typical sample sizes. In fact, because there is no estimating equation, there is not even a method for finding the best-fitting theoretical curve, except empirically. It is possible, however, to estimate the probability that an observation differs from a given expectation.

(I) Test of the identity of two experimental assortment curves: For each fission point sampled, and for which at least one pure line was recovered in one of the experimental sets, a 2×2 contingency table is constructed. Contingency χ^2 values (each with 1 D.F.) for each of the k fission points are summed and the resulting χ^2 variate is interpreted with k degrees of freedom, since there is no necessary connection between the individual points. For sets 1 and 2 at the *Chx* locus, a value of 2·66 with 4 degrees of freedom is obtained, giving a 0·6 probability that both curves result from identical underlying distributions. A similar analysis shows that set 3 differs significantly from that distribution ($P = 0{\cdot}001$).

Where the expected fraction pure is very low, exact methods should be used, and the exact P values may be used to generate a χ^2 component appropriate for the test described above. Where all the individual p's can be determined, the relation $\chi^2 = -\Sigma 2 \ln p$ may be used, where χ^2 has $2k$ D.F. for k classes. However, it is most often of little interest if two distributions are simply inhomogeneous. What is desired is the probability that the results are consistently different – for this

purpose it is necessary to evaluate both tails of the expected distributions, separately.

(II) Goodness of fit of experimental to theoretical curves: Observed numbers of sublines pure and impure at each fission point are compared to expectations based on the same sample size at each point. The resulting χ^2 variate is again considered to have k D.F., as the method of generating the expectations has no direct connexion with the data. By this procedure a lower limit is set on the time of assortment for *Chx* in sets 1 and 2, lumped; times less than 35 fissions are ruled out at the 0·05 level for the 45-subunit model. For the 66-subunit model, times less than about 25 fissions are ruled out.

It is to be noted that data for all individual sets are in good statistical agreement with expectations by these same criteria. If sources of variation other than those already discussed were important, significant variation within sets should also be present. The finding of large variation among but not within sets, then, verifies the adequacy of the present methodology. Explanations for anomalous assortment curves reported previously will be discussed below.

The procedures given here are conservative in that the D.F. might be further discounted. Non-parametric tests are also useful, and may in some cases allow further distinctions. For example, intercomparison of the three *Mpr* curves for sets 1, 2, and 3 by the χ^2 procedure gives $P = 0{\cdot}5$. But if ranks are assigned at all fission points, set 3 has the highest rank in all six samples. A probability of $2 \times (1/3)^6 = 0{\cdot}0027$ is obtained for the hypothesis that set 3 is homogeneous with the other two sets.

By combining these tests, informative conclusions can be drawn from manageably small samples. In Table 2 the non-significant differences in estimated parameters are indicated by horizontal lines.

A set of 38 computer-generated pedigrees was used to check the correctness of the methodology developed above. The input for the initial model nucleus was A and a subunits in equal numbers. At each 'fission', subunits were partitioned randomly according to the Schensted model. The sample size for each pedigree was 32 sublines; the fission ages sampled were 52, 65, 78 and 91. Each pedigree closely resembles a typical assortment experiment. The 38 χ^2 values for fit to expected frequencies gave $\overline{\chi^2} = 3{\cdot}002$ and $\hat{\sigma}^2 = 7{\cdot}024$. The χ^2 distribution has mean = D.F. and variance = 2 D.F., so this type of comparison appears to follow χ^2 with k-1 D.F., although the variance is a bit higher than expected. The 741 pairwise comparisons (including self-comparisons) of these 38 pedigrees were tested by the same kind of χ^2 analysis, as a means of estimating the probability distribution for 'χ^2' calculated as described above for the two pedigree comparison case. Here the mean χ^2 was 3·201 and $\hat{\sigma}^2 = 9{\cdot}954$. The variance is too high to allow the use of the true χ^2 distribution. However, it was found that the use of k instead of $k-1$ D.F. properly estimated the 0·01 and 0·05 levels, while with $k-1$ D.F far too many cases were judged significant at both levels

This analysis suggests that, were it nor for the lack of independence of observations within a pedigree, $k-1$ D.F. would apply. But a discrepancy is introduced

by the failure of this assumption, and it may be factored out, to a close approximation, by using k D.F. In the case of goodness of fit to Schensted predictions, it appears that χ^2 with $k-1$ D.F. may actually be a better estimator of probability than χ^2 with k D.F. Indeed, for the case where longer fission intervals are taken between observations, the successive observations depend far less strongly on previous values, and k–1 D.F. will again apply.

As the simulations above did not use Yates's correction, it is apparent that the methodology must apply fairly well to the small numbers situations that occur in almost all assortment pedigrees. The inclusion of an extra degree of freedom in all values reported here also helps prevent errors from this source.

(ii) *Variability of assortment parameters*

Variation of both time of determination and input ratio is documented in Table 2 for *Chx* and *Mpr*, even in the small samples used here. Variation of *co* is documented for B × B *vs.* B × C2 crosses, since the former case gives an estimated time of determination around 20 fissions, after suitable correction for the observed slow growth rate, while the latter heterozygote gives an estimated time of 54 fissions in the absence of abnormal growth rate.

A further example of variation is found in Table 4 of Bleyman & Bruns (1977), containing assortment data for *Mpr* and *Chx* for 8 testcross progeny heterozygous at both loci, at about 26 fissions after conjugation (Bleyman, personal communication). The fraction pure for Mpr^+ varies from 3 % to 44 %, and the inferred input ratio from 5:40 to 20:25. Homogeneity χ^2 is highly significant, $p = 6{\cdot}2 \times 10^{-11}$ The *Chx* data are even more heterogeneous. Two clones began assorting early for *Chx* (with skewed input), while the remaining 6 were either later assorting or early assorting but with equal input (these possibilities are not distinguishable in the data). Because the clones contain unknown and varying amounts of the strain D genome, these data do not bear on the question of strain differences.

The assortment data for *r4* (Table 3) are distributed in three classes. The gaps between these classes are suggestive of some feature of the system that leads either to equality, or to strong inequality within each exconjugant, for this early assorting locus. Thus, the one pair most strongly expressing r4 would correspond to the case of both exconjugants choosing strong inequality. The intermediate class would have one exconjugant voting for and one against equality. The low-r4 class would correspond to the case of both exconjugants voting for equality. Under this model, a probability of 0·224 for an exconjugant choosing strong bias for r4 gives an expected distribution of 12·04:6·96:1·00, compared with the observed 13:6:1, an excellent agreement. A similar study using individual exconjugants would therefore be extremely interesting as a test of this model.

In spite of ample documentation for variation of assortment parameters, it cannot be argued that the regularity of assortment is entirely a mirage. The 45-fold kinetics developed by Schensted (1958) and reinvestigated by Orias & Flacks (1975) and Orias & Newby (1975), closely approximate all results obtained to date. This is true whether the subline expansions were made at 18 or at a much

greater number of fissions after conjugation. Doerder, Lief & DeBault (1977) have noted a few apparent exceptions, especially in the occurrence of 'slow' curves when assortment commences late. But the reader will easily be able to verify from the tables in the previous paper that the result of starting with a mixture of determination times (determination occurs, say, over a period of 30 fissions) will be an aberration in the early part of the assortment curve only, such that the time of determination estimated from early transfers is less than the time estimated from later transfers. This anomaly is shown in an extreme form in the co-assortment data of Doerder (1973) for *r – 1* and *r3* accompanied, however, by considerable death. The data for other late-assorting loci are difficult to compare. Phillips (1967) gives data for *T* which seem very uniform. That is, they agree closely with 45-fold kinetics. Data of Allen (1965, 1971) for the esterases and *P – 1* do show an anomaly of the sort described above. These experiments differed in design. Phillips used a large number of sublines from a few caryonides, while Allen used one subline from each of many pairs. Allen thus probably sampled some caryonides determined very early for these loci. These observations tend to indicate that most variation is between caryonides, rather than between sublines within caryonides, even for traits beginning assortment long after the sublines have been established.

Doerder & DeBault (1975) and Doerder *et al.* (1977) have found that the DNA content of young macronuclei is high enough to account for about 66 haploid genomes, and that the decay to the matured value of 45 occurs slowly after about 50 fissions (for A × B and B × B crosses – strains C2 and D have not been examined in this way). What is not known is the actual size of the functional macronuclear genome at this time. There is evidence for limited changes in macronuclear DNA content. Most of the change is compensated by chromatin elimination (Cleffman, 1967). While the extra 45% is probably not due to the amplified DNA coding for ribosomal components (Yao, Kimmel & Gorovsky, 1974), it is not known that only such DNA is amplified, or that young macronuclei have the same degree of amplification. If enough amplification occurs, the theory of assortment remains unchanged. However, if the number of genome copies increases to 66, the appearance of pure types for early assorting loci during the first 50 fissions would be greatly curtailed. For loci beginning assortment immediately, input ratios estimated at successive transfers would be less and less extreme, by comparison with expectations based on 45 subunits. The *Mpr* and *Chx* data should bear on this question. The B × D set 5 data for *Chx* show such an effect in which the estimated input of *Chx* decreases from 35 to 31 over a period of 65 fissions. The pooled *Mpr* data (B × D) show a similar effect – the estimated input decreases from 39:6 to 33:12 over a period of 50 fissions. The effect does not appear to be a general one, however, and might result from a distribution of *input ratios* within pair 6 and (for *Mpr*) within all three pairs. (See also set 3, *Chx*.) An equal mixture of 40:5 and 22:23 inputs, for instance, produces an initial apparent input of about 39:7 which moderates to 33:12 after about 50 fissions. An equal mixture of 35:10 and 22:23 nuclei gives an expected initial estimate of 33:12, moderating gradually to 29:16 after about 100 fissions. In view of the variation within pairs already

known from the assortment of *H* serotypes (Bleyman, Simon & Brosi, 1966), this seems a reasonable explanation. It is also clear that small trends, even if very general in occurrence, cannot be taken as proof of the 66 subunit model.

Doerder *et al.* (1977) show (their Fig. 3) that the times of first appearance for pure phenotypes at a variety of loci agree generally with a model in which assortment of 66 haploid subunits begins immediately after conjugation. But they do not demonstrate that whole assortment curves can also be made to fit their model. Data for later fissions have been ignored, as have the sources of variation demonstrated in this paper.

Also, the new 66-subunit model seems to invoke a strange coincidence: all the loci heretofore designated as 'late' and for which only one allele can be scored as pure would now have to be produced from 36:30 inputs, roughly – i.e. the pure phenotypes we are able to score are fortuitously all biased in the same direction if assortment is assumed to begin immediately from a 66-ploid nucleus. Even without this difficulty, Table 2 contains assortment curves that simply do not fit – if they are produced by 66-ploid nuclei, assortment cannot have begun until at least 25 fissions after conjugation.

The data in Table 2 contain other suggestive regularities. All loci beginning assortment beyond about 10 fissions show the same input ratio, about 22:23, or equal inputs of the two alleles. This feature has been noted many times before (Allen & Gibson, 1973). But here the *Chx* locus breaks a barrier of sorts: the input ratio is 22:23 when *Chx* assorts late, but is as low as 12:33 when it assorts immediately after conjugation. Evidently there is some kind of built-in gradient, such that early and highly skewed assortment is only the end of a continuum ranging all the way to late and equal assortment.

For *Mpr* and *Chx* (*co* is exceptional for technical reasons – see above) the assortment parameters in the strain D crosses are either (*a*) earlier, where possible, or (*b*) more skewed, compared with the C2 crosses. The probability of this circumstance is estimated to be 0·005 by chance alone, based on ranks. Thus, there seems to be a strain difference in assortment behaviour. The results parallel the differences for meiotic recombination in the same strains (McCoy, 1977), although this is probably coincidental. The determination times previously reported for other loci (Allen & Gibson, 1973; Sonneborn, 1975) may have to be reinterpreted in light of these findings, since they are based on crosses into a variety of genetic backgrounds. Indeed, assortment data based on only a small sample of caryonides, or on a series of caryonides represented only by one subline each, may not give a very informative view of the range of determination times available to any particular locus.

Even more interesting, and worth further investigation, is the possibility that determination times and input ratios tend to be correlated among different loci undergoing assortment in the same nucleus. That is, if a set of sublines is earlier than the norm for one locus, it will also tend to be earlier for other loci. In the C2 crosses, set 3 assorted very early for *Chx*. It also had the most skewed output for *Mpr* and produced conical sublines earlier than the other B × C2 sets. If it can be shown that this circumstance happens more often than expected by chance alone,

it will indicate an intrinsic stepwise control of assortment throughout the genome.

The data in the report of McCoy (1979) and the data on DNA content cited earlier argue very strongly for the impermanence of subunits. Further experiments may allow limits to be placed on the rate at which (theoretical) subunits must exchange parts to give the observed numbers of different phenotypic combinations. The assortment of closely linked markers has not been followed, so there are no data bearing on somatic recombination mechanisms for assortment. It is still crucial to determine the relationship between map position and determination time. Unfortunately the answer to this question will have to await substantial new progress in recovering, characterizing, and mapping mutants with readily scored phenotypes.

The studies reported above lead to the following generalizations concerning macronuclear assortment:

(1) The functional units of assortment are not entire genomes, whether haploid or diploid. However, segregation of haploid structural units at each fission is logically necessary.

(2) Tremendous variation is found between strains and between pairs, both in input ratio and time of determination.

(3) There is no conclusive evidence for such large variation within pairs, and especially within caryonides. Variation within pairs is suspected, however, in several instances involving loci determined at conjugation.

(4) Variation of the sort documented here is capable of explaining most, if not all cases of apparent anomalous assortment behaviour. Hence, new experimental strategies are required to distinguish between hypotheses.

(5) The hypothesis that sorting out correlates with map position cannot be tested until better maps are available and until more assortment data are gathered.

(6) Input ratio and determination time are related, so that some loci cannot be easily classified as early or late assorting.

(7) There is some evidence to suggest that assortment sometimes is coordinated within a nucleus, over many loci.

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