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The functional units of macronuclear assortment in *Tetrahymena thermophila*

By J. WYNNE McCOY

Department of Cell Biology,
Albert Einstein College of Medicine,
Bronx, NY 10461, U.S.A.

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SUMMARY

Macronuclei assorting simultaneously for *H*, *Chx*, *Mpr*, and *co*, and containing only one or two copies of the H^D allele produced several combinations of phenotypes at the other loci, instead of only one or two such combinations. It follows that macronuclear subnuclei, if they exist at all, must frequently exchange parts. Models involving somatic recombination, transient subnuclei, or progressive chromosome fragmentation are discussed as possible explanations.

1. INTRODUCTION

As noted by Raikov (1976), efforts to explain macronuclear assortment in the ciliate *Tetrahymena* (reviewed by Allen & Gibson, 1973) have reached an impasse. Macronuclear assortment produces homozygous or hemizygous segregants during vegetative growth of heterozygotes. It is apparently unique to the genus. Genetic evidence seems to require a macronucleus of diploid subunits (Nanney, 1964; Doerder, 1973), while cytochemical evidence requires haploid subunits (Doerder, Lief & Doerder, 1975; Woodard, Kaneshiro & Gorovsky, 1972). A model of 45 subunits replicating before each fission and then moving randomly to the daughter cells (Schensted, 1958) explains much of the data. Problems arise, however, in explaining results at many loci and with haploid subunits, in accordance with the known DNA content of the macronucleus (Preer (1976) has also noted this difficulty).

A recent theoretical treatment led to the development of new experimental strategies in the study of assortment (McCoy, 1978). In particular, attention is focused on the distinction between the macronuclear subunits physically segregated at each fission (structural subunits) and the subunits identified phenotypically by the alleles they carry (functional subunits). Until recently this distinction was unnecessary. However, once it is admitted that the structural units of the macronucleus must be haploid at the time of cell division (for a variety of reasons), the observation that different loci begin assortment at different times after conjugation (Nanney, 1968; Bleyman, 1971) requires some form of recombination in the construction of haploid units. The functional units of the macronucleus

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could be permanent haploid genomes formed by some recombination process (as recently proposed by Doerder, Lief & DeBault, 1977), whole chromosomes, or pieces thereof. Structural units must include a haploid association phase to prevent macronuclear aneuploidy (see Weindruch & Doerder, 1975, also Nilsson, 1970), but the interphase nucleus could contain, again, haploid genomes, whole chromosomes, or chromosome fragments.

The assortment characteristics of the *H* serotype locus (Nanney & Dubert, 1960) allow a direct determination of recombination during macronuclear assortment. Heterozygotes at this locus often produce macronuclei bearing only one or two subnuclei of one allelic type, as deduced from assortment kinetics (see, for example, Bleyman, Simon & Brosi, 1966).

If the number of phenotypic combinations of other markers recovered with the minority serotype exceeds the number of minority serotype subnuclei initially present, then an intra-macronuclear 'recombination event' of some kind has occurred during assortment, and the functional units of assortment cannot be only permanent haploid genomes.

2. MATERIALS AND METHODS

Assortment parameters are estimated through the use of single cell transfers at known intervals, for a large number of sublines of common origin. Each subline derived from a given progenitor cell may be treated as an independent sample of that progenitor, and the accumulation of stabilized ('pure') sublines as a function of time in fissions gives the initial composition of the progenitor nucleus, by reference to theoretical expectations for various inputs and fission ages. The numerical predictions recalculated by Doerder, Lief & Doerder (1975) from the model of Schensted (1958) are reproduced in Table 1.

Necessary technical details, together with descriptions of strains and media, are given by Allen & Gibson (1973). The *Chx co ts-1 Mpr* stock of *T. thermophila* is the same one developed for recombination studies reported earlier (McCoy, 1977). Marker phenotypes and scoring conditions are described in Table 1 of McCoy (1977). *Chx* and *Mpr* alleles confer resistance to cycloheximide (10 γ/ml) and 6-methylpurine (15 γ/ml) respectively; 0·05 ml culture aliquots to be tested for drug resistance were transferred to 1 ml of sterile 1% peptone containing 1000 units penicillin G and 1 mg streptomycin sulphate. Drug resistance tests were considered positive if any evidence of growing cells was seen after 48 h. The recessive *co* produces a *conical* cell shape and anisotomy (Doerder *et al.* 1975). Homozygotes for *ts-1*, a recessive that does not assort, are killed by exposure to temperatures above 38 °C within 12 h.

Fission ages are counted from the point of common origin of the sublines. Normally, one serial transfer corresponds to 13 cell fissions, the fission-time required for one cell and its progeny to exhaust the nutrients in 1 ml of culture fluid.

In the present experiments, the growth rates of sublines were uniform, and

transfers were performed at 48 h intervals, just as log phase growth was ending. At this stage the cells undergo morphological changes (the 'limited polymorphism' of Corliss (1953) and others, recently studied by Taylor, Gates & Berger (1976) and Nelson & Debault (1978)) that allow the growth rate to be monitored rather precisely. No sublines died during the experiments, and no sublines were consistently slower-growing than the rest or showed any signs of the 'semi-amicronucleate syndrome' (Nanney, 1959).

3. EXPERIMENTAL TEST OF PERMANENT SUBUNIT MODEL

Three separate sets of 1° (primary) subclones were initiated from a cross of *Chx co ts-1 Mpr* (H^D/H^D, strain B background) × C2-2671 (H^E/H^E). Each set was derived from one 18-fission cell derived from a different conjugating pair, and consisted of 30 1° sublines. Sublines were maintained by single cell isolation at 13-fission intervals. When the 1° subclones were 57 fissions from conjugation and 39 fissions (3 transfers) from expansion, they were tested with highly specific anti-Hd and anti-He sera. The latter were prepared from purified antigen (Bruns, 1971). No cross-reaction could be detected between these sera under the normal conditions of their use. Four mixed Hde subclones were identified, two in set 1 and two in set 2. Set 3 had no lines responding to anti-Hd. The Hde lines were expanded 60-fold to give 2° (secondary) sublines that were tested with the same sera after a further 11 fissions. The 2° subclones of 1° subclone 1–21 were found to be 16 Hde and 44 He. By comparison with the expectations for 1:44 and 2:43 inputs at 11 fissions (Table 1), subline 1–21 was very likely derived from a macronucleus with the initial composition 1 Hd:44 He. The probability that 1–21 represents a 2:43 progenitor nucleus is negligible – about 0·002. Similarly, but with slightly less certainty, the initial compositions of the other Hde lines are estimated at 7:38, 5:40, and 2:43. The latter ratio differs significantly from 3:42. The model is very sensitive to input ratio at this fission age, and the goodness of fit of the observations to the discrete expectations indicates the exceptional sensitivity of the serotype assay. Sets 1 and 2 may therefore be compared with the expected values at 39 fissions after expansion to determine their probable initial subunit compositions at the time of selection (18 fissions after conjugation). Each set had 93% pure He, compared with predicted values of 91% and 82% for 1:44 and 2:43 subunit compositions, respectively. The 95% confidence limits (from tables of Snedecor, 1967) on the observed ratio exclude the 3:42 case. The probability that neither set represents a 1:44 or 2:43 progenitor is less than $(0{\cdot}05)^2 = 0{\cdot}0025$, or, conversely, there is a greater than 99·75% chance that at least one set is 1:44 or 2:43 with respect to the *H* locus. If, as suggested by Doerder, Lief & DeBault (1977), there are 66 assorting units, the probabilities are practically unchanged.

Thus, (*Argument 1*) if more than two multiple pure phenotypes could be recovered from both sets in pure Hd lines, macronuclei cannot consist only of permanent pangenomic subunits. As another test (*Argument 2*), if more than one pure type

Table 1 (*a*). *Predictions for macronuclear assortment* (45 assorting units)

No. of fissions after determination	Input Ratio 1:44	2:43	3:42	5:40	10:35	15:30	20:25	22:23	Allele
1	0·247	0·058	0·013	0·001	0·000	0·000	0·000	0·000	A
	0·000	0·000	0·000	0·000	0·000	0·000	0·000	0·000	B
5	0·595	0·351	0·204	0·067	0·003	0·000	0·000	0·000	A
	0·000	0·000	0·000	0·000	0·000	0·000	0·000	0·000	B
10	0·735	0·537	0·391	0·203	0·035	0·005	0·001	0·000	A
	0·000	0·000	0·000	0·000	0·000	0·000	0·000	0·000	B
15	0·801	0·639	0·508	0·316	0·089	0·022	0·004	0·002	A
	0·000	0·000	0·000	0·000	0·000	0·000	0·001	0·002	B
20	0·839	0·702	0·586	0·404	0·149	0·050	0·014	0·008	A
	0·000	0·000	0·000	0·000	0·000	0·001	0·003	0·006	B
25	0·865	0·746	0·642	0·471	0·207	0·083	0·030	0·019	A
	0·000	0·000	0·000	0·000	0·000	0·002	0·009	0·015	B
30	0·883	0·778	0·683	0·525	0·259	0·118	0·049	0·033	A
	0·000	0·000	0·000	0·000	0·001	0·005	0·018	0·027	B

40	0·907	0·821	0·742	0·603	0·346	0·188	0·094	0·070	A
	0·000	0·000	0·000	0·001	0·005	0·017	0·043	0·059	B
50	0·922	0·849	0·781	0·657	0·415	0·250	0·141	0·110	A
	0·000	0·001	0·001	0·003	0·012	0·033	0·073	0·096	B
60	0·933	0·869	0·808	0·697	0·469	0·303	0·185	0·149	A
	0·001	0·002	0·003	0·006	0·022	0·053	0·105	0·133	B
70	0·940	0·833	0·829	0·727	0·513	0·349	0·226	0·186	A
	0·001	0·003	0·006	0·011	0·035	0·075	0·136	0·168	B
80	0·946	0·894	0·845	0·751	0·549	0·388	0·262	0·220	A
	0·002	0·005	0·009	0·017	0·048	0·097	0·166	0·201	B
90	0·951	0·903	0·857	0·770	0·579	0·421	0·294	0·251	A
	0·004	0·008	0·013	0·024	0·062	0·118	0·194	0·231	B
100	0·955	0·910	0·868	0·786	0·604	0·450	0·323	0·279	A
	0·005	0·010	0·016	0·030	0·076	0·138	0·220	0·258	B
120	0·960	0·921	0·883	0·810	0·643	0·497	0·371	0·326	A
	0·008	0·016	0·024	0·044	0·101	0·174	0·264	0·304	B

Table gives proportion of sublines stabilized for each allele at one locus, for different starting compositions of the assorting nucleus (input ratios) and at different times following the start of the assortment process. These figures are abridged from a more extensive table by Doerder, Lief & Doerder (1975)

Table 1 (*b*). *Predictions for macronuclear assortment* (*66 assorting units*)

No. of fissions after mination	Input Ratio 1:65	2:64	3:63	5:61	10:56	15:51	25:41	33:33	
1	0·248	0·060	0·014	0·001	0·000	0·000	0·000	0·000	A
	0·000	0·000	0·000	0·000	0·000	0·000	0·000	0·000	B
5	0·597	0·354	0·208	0·071	0·004	0·000	0·000	0·000	A
	0·000	0·000	0·000	0·000	0·000	0·000	0·000	0·000	B
10	0·737	0·541	0·396	0·210	0·040	0·007	0·000	0·000	A
	0·000	0·000	0·000	0·000	0·000	0·000	0·000	0·000	B
13	0·781	0·609	0·473	0·283	0·074	0·018	0·001	0·000	A
	0·000	0·000	0·000	0·000	0·000	0·000	0·000	0·000	B
26	0·871	0·758	0·658	0·494	0·233	0·105	0·018	0·003	A
	0·000	0·000	0·000	0·000	0·000	0·000	0·001	0·003	B
39	0·907	0·822	0·744	0·608	0·359	0·205	0·059	0·018	A
	0·000	0·000	0·000	0·000	0·000	0·001	0·005	0·018	B
52	0·927	0·859	0·794	0·678	0·450	0·290	0·110	0·044	A
	0·000	0·000	0·000	0·000	0·001	0·003	0·015	0·044	B
65	0·939	0·882	0·827	0·726	0·517	0·360	0·161	0·076	A
	0·000	0·000	0·000	0·001	0·003	0·007	0·031	0·076	B
78	0·948	0·898	0·850	0·760	0·568	0·417	0·209	0·110	A
	0·000	0·001	0·001	0·002	0·006	0·015	0·051	0·110	B
91	0·954	0·910	0·876	0·786	0·609	0·464	0·253	0·144	A
	0·001	0·001	0·002	0·004	0·012	0·025	0·073	0·144	B
104	0·959	0·919	0·880	0·806	0·641	0·502	0·292	0·175	A
	0·001	0·002	0·003	0·007	0·018	0·036	0·096	0·175	B
117	0·962	0·926	0·890	0·822	0·668	0·535	0·326	0·205	A
	0·002	0·003	0·005	0·010	0·025	0·048	0·118	0·205	B

Numbers in this Table were extracted from a more extensive computer printout kindly supplied by Dr F. Paul Doerder.

were recovered from line 1–21 in pure Hd lines, or more than two such pure lines from line 1–18, the same conclusion might be drawn. A positive result from either Argument is logically sufficient to prove the impermanence of macronuclear genomic subunits. The experiment is to seek any exceptional vegetative recombinant progeny indicating that subnuclei are not permanent.

If the lowest number of subnuclei detected in the serotype assay were actually two (or more), then the distribution of nuclei with fewer than two subnuclei expressing Hd, and thus not reacting to the anti-Hd serum, should follow the Poisson distribution, roughly. On this basis, about 37 % of all 1° sublines should be 1 Hd:44 He, and some of these should have been detected by the immobilization of some cells by anti-Hd serum in later transfers. Over a period of 100 fissions and after some 500 serotype assays, no new Hde lines were detected, so that the *clonal* endpoint of the immobilization assay, for these sera and growth conditions, is a single subnucleus. From the fraction of cells immobilized by the anti-Hd serum in clones determined to have 1 or 2 Hd subunits, the *cellular* endpoint is estimated to be around 6 subunits (McCoy, in preparation).

Table 2. *Phenotypes recovered in pure Hd subclones*

Progenitor Subline			
1–21		1–18	
Phenotype	Number recovered	Phenotype	Number recovered
Mpr Chx co	2	Mpr $Chx^{+/r}$ co^+	1
Mpr^+ Chx co	1	Mpr Chx co^+	1
Mpr^+ Chx^+ co^+	2	Mpr^+ Chx co	2
Mpr^+ Chx co^+	6	Mpr^+ Chx co^+	1
Mpr^+ $Chx^{+/r}$ co^+	1	Mpr^+ Chx^+ co^+	1

($Chx^{+/r}$ clones are those having both sensitive and resistant cells, and thus still undergoing assortment for *Chx*. Some of the co^+ clones very likely have some co subnuclei, since the co phenotype is recessive and assortment at this locus began just before the 2° expansions were made.)

From the 2° sublines derived from the four Hde 1° sublines, a number of 3° (tertiary) expansions were made, three from each of five 2° Hde sublines from each 1° Hde line. After 13 fissions, the 60 3° lines were exposed to anti-He serum, and, for each, the cell initiating the next transfer was selected from among the cells least responsive to the antiserum. By this means, strong selection against the He subunits was achieved. Over a period of four successive transfers and selections, 39 pure Hd lines were obtained. Purity was confirmed by tests on a further unselected transfer, using both sera. Among the lines derived from 1–21 and 1–18, pure for Hd, a variety of phenotypes was recovered (Table 2).

Based on *Argument 2*, macronuclei do not consist only of permanent subnuclei by 57 fissions after conjugation, when these lines were expanded. *Argument 1*,

concerning the initial compositions of sets 1 and 2, suddenly became irrelevant when it was discovered that *Chx* and *co* had assorted much later than expected in this cross (McCoy, 1979) As noted earlier, *Argument 2* is by itself sufficient to establish the stated conclusion. As the sample sizes are too small to establish significance between individual classes, unselected outputs were not determined for *Mpr*, *Chx*, and *co*.

4. DISCUSSION

This report gives clear evidence that macronuclear subunits cannot consist only of whole genomes on a permanent basis. The multiplicity of phenotypes recovered in sublines selected for Hd serotype requires *at least* four separate subunit types containing Hd, even if all instances of Chx, Mpr, and co$^+$ phenotypes are undetected mixtures of dominant and recessive alleles (this is unlikely). For subline 1–21 the pure combinations Chx Mpr$^+$ co, Chx$^+$ Mpr co, Chx Mpr$^+$ co$^+$, and Chx$^+$ Mpr$^+$ co$^+$ are absolutely required. Four combinations are also required for line 1–18. This conclusion follows because when any of the recessive phenotypes (co, Mpr$^+$, and Chx$^+$) is found, virtually all subnuclei must be of that type, even though the corresponding dominant phenotypes are ambiguous. Thus, more subunit types appeared than allowed by the number of Hd subnuclei originally present, if subnuclei are taken to be permanent associations. The experimental findings are summarized in Table 3.

Table 3. *Summary of assortment data for test of permanent subunit theory*

Progenitor	Input (estimate)	Probability	Hd types predicted	Observed
1–21	1:44	0·998	1	5
1–18	2:43	0·982	2 pure + 1 mixed	5

The possibility of macronuclear ('somatic') recombinations as a cause for assortment is thus raised, but no confirmed synthetic markers have yet been examined for co-assortment (see McCoy, 1977, 1978). The rate of co-assortment, now developed on a sound theoretical basis (McCoy, 1978), should be important in placing limits on models with somatic recombination. The latter reference also provides the mathematical apparatus for determining possible recombination levels.

There are at least three distinct processes that could result in 'somatic recombination'. First, if chromosomes are maintained in the macronucleus, a classical recombination process could lead to assortment such that linked markers, if close enough together, would show deviations from random assortment. The degree of recombination encountered depends on the number of fissions elapsed since the beginning of assortment. Unfortunately, the rate of assortment would also depend on the distance from the centromere, and numerous additional features would have to be imagined to generate the uniform rate of assortment actually observed.

A second possibility holds that, while there are no chromosomes in the macro-

nucleus (there would be instead some very large number of chromosome fragments), there are genome-sized subunits exchanging parts from time to time. In this case, loci linked on the meiotic map need not show preferential associations during co-assortment, but the rate of co-assortment would differ from that predicted by the existence of permanent pangenomic subunits (McCoy, 1978).

A third possibility is that assortment corresponds to the random distribution of chromosome fragments, but with fragmentation of the genome occurring gradually, over a period of perhaps 50 or more fissions. The application of a suitable array of restriction nucleases might be imagined as a believable mechanism. Of all the schemes so far contemplated, this has the advantage of explaining the available data most economically, including the puzzling temporal aspects of assortment. Proof of this model will be particularly difficult, because it will be necessary to show that discrete, genetically identifiable fragments do actually exist, but that co-assortment for other neighbouring or unlinked markers is at the rate predicted by the absence of subunits. Further, it must be shown for this model, and equally for the first model, that the time of determination is related to map position.

In all these models, haploid genomes must be present in a structural sense before each fission, but only as a necessary vehicle for maintaining genetic balance. Indeed, assortment may occur simply because there are *not* haploid subunits during some part of the cell cycle.

A consideration of the various alternatives seems to indicate that the most important information that can now be obtained on the process of macronuclear assortment will come as a result of experiments designed to monitor co-assortment. Such experiments are necessarily on a large scale. Moreover, such experiments will have to take into account a very large component of variation in input ratio and time of determination, developed in the next report of this series (McCoy, 1979).

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