T SEROTYPE DIFFERENTIATION IN TETRAHYMENA

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ONE of the most interesting aspects of the T serotype system is the differentiation of the serotype heterozygotes during vegetative multiplication to express only one of the parental serotypes. Similar phenotypic assortment attributed to allelic repression has been found in all other genetic systems examined in *Tetrahymena* (reviewed by NANNEY 1964)

This paper will be concerned with the timing of T serotype differentiation, the kinetics of stabilization of pure types and the distribution of parental types among the stabilized lines for two of the three T heterozygotes. The third combination was not studied because only one differential antiserum was available for this heterozygote. The comparison of the H serotype and T serotype differentiations reveals similarities and differences between the two systems which bear on the basic nature of this phenotypic assortment. Since all crosses and vegetative pedigree lines were maintained at 25°C, and samples from these were tested at 40°C, the T serotypic differentiations occur in the absence of their expression. A similar separation of "determination" and "expression" is common in the development of higher forms and suggests an analogy between T serotype differentiation in *Tetrahymena* and differentiation in embryonic cells of higher organisms.

MATERIALS AND METHODS

The strains used in this study were representatives of the inbred families A, homozygous for T^A and H^A ; A3, homozygous for T^B and H^E ; B2, homozygous for T^C and H^C ; and D1, homozygous for T^C and H^C .

The culture and breeding methods and serological procedures were described in the preceding paper. Cultures were transferred from 25°C bacterized peptone to 40°C axenic peptone by adding about 0.05 ml from a starved bacterized peptone culture tube to an axenic peptone tube containing antibiotic (1×10^6 units penicillin and 1 g streptomycin per liter).

To determine the distribution of parental serotypes among stabilized sublines of heterozygotes an index, the "output ratio" was used (NANNEY *et al.* 1963; BLEYMAN, SIMON and BROSI 1966). For serotype Hx, the output ratio is $100(2H_x + H_{xy})/2N$, where N is the number of lines typed, H_x is the number of sublines scored as pure for Hx, and H_{xy} is the number of sublines still manifesting both serotypes. Thus the output ratio for serotype Hx is the percent stable Hx lines plus one half the percent unstable lines. It follows that the output ratio for serotype Hy would be 100 minus the output ratio for Hx. Since the ratio of the two pure types obtained from unstable lines approaches one after a short lag period the output ratio should approximate the initial ratio of the two types.

RESULTS

Differentiation of the Tbc heterozygote: Tac and Tbc cultures tested a few fissions after conjugation are completely immobilized by both parental antisera.

If these cultures are transferred serially and the "leftover" culture after each isolation is tested for serotype, a wide range of serotype responses is found in the derived lines after about 50 fissions. For example, in the case of a Tbc heterozygote, a continuous series of types is generated from those which are completely immobilized by anti-Tb and do not react with anti-Tc to those which are completely immobilized by anti-Tc and do not react with anti-Tb. Among the intermediate types, some cells in a given test depression will be immobilized and other cells will be unaffected by the test antiserum, thus giving a "mixed" reaction. The intermediates can be classified into those in which more cells are immobilized by anti-Tb than by anti-Tc, those in which the responses to the two antisera are equivalent, and those in which more cells are immobilized by anti-Tc than by anti-Tb. These intermediate cultures give rise to all types of derivative cultures, while the cultures scored as "pure" for one of the parental types usually give rise to derivative cultures of the same pure type.

Quantitative information on the Tbc heterozygote was first obtained from a vegetative pedigree experiment in which sublines from a Tbc ($T^{B/C}$, $H^{C/E}$) heterozygote were tested for H and T serotypes 65 fissions after conjugation (BLEYMAN, SIMON and BROSI 1966). At this time about 20% of the sublines were pure for either Tb or Tc, but 95% of the sublines were pure for H serotype. The distribution of parental serotypes among the stabilized lines was determined by computing an output ratio as described in METHODS. The output ratios for different sublines varied between 36 and 53 for the Tc serotype with an output ratio of 46 obtained on the total sample. This was in contrast to the eccentric ratios of 0 to 15 found for the Hc serotype in the same experiments.

To obtain an accurate estimate of the rate of stabilization of a Tbc heterozygote the following procedure was employed. One hundred and twenty lines were selected from the tubed fifth transfer cultures of an A3 $\times$ D1 cross (Tbc) which still gave strong reactions with both parental antisera. These were transferred four more times and tested for T serotype at each transfer. This was done by tubing samples from each transfer and testing them at 40°C. Since in the H serotype system some lines once scored as pure give rise to derivative sublines which were scored as intermediate, each pure line was transferred and tested two additional times after being scored pure before being classified as stabilized pure. In the H system these "reversions" were shown to be artifacts of the scoring procedure, since the more times in a series a culture is scored pure, the smaller the number of derived cultures which are scored intermediate in serotype (NANNEY and DUBERT 1960). In particular, in the H system, 21% of cultures scored once as pure give intermediate reactions in a derived culture, 9% of the cultures scored twice serially as pure give rise to intermediate cultures, and only 3% of cultures scored three times in a series as pure ever give intermediate cultures.

It was on the basis of this knowledge that the procedure of scoring each pure line two additional times was adopted. This proved less satisfactory for the T system, because tabulation of the final results revealed about 26% reversions after one previous pure culture, and 14% reversions after two previous pure

TABLE 1

Phenotypes of the next isolation cultures in lineages which had tested "pure" for different numbers of transfers

No. of previous pure cultures	Pure	Phenotypes of derived cultures	
		Intermediate	Percent intermediate
1	116	30	26
2	84	12	14

cultures (Table 1). Extrapolating from this, one would expect perhaps 7% reversion after three serially scored pure cultures, instead of the 3% observed in the H system. An error of this type could cause a constant rate of stabilization to appear to increase with time, since the estimated number of newly stabilized pure cultures/transfer would be inflated, particularly for later transfers, by "false stables". In fact, the results show a slightly increasing rate of stabilization with time. Table 2 shows the fraction of pure types obtained at each transfer. The fraction is estimated to be 0.0118 ± 0.0004 pure types/fission, and is close to the 0.0113 obtained for the H serotypes (NANNEY and DUBERT 1960) and "unstable" mating types (ALLEN and NANNEY 1958). The figures obtained were 0.0113 ± 0.0004 for mating types, and 0.0112 ± 0.0018 for H serotypes, based on daily transfers, assuming eight fissions per day. When cultures were transferred every other day, assuming 13 fissions per transfer, and tested for H serotypes, a rate of 0.013 ± 0.0015 was obtained. The rate for the T serotypes of 0.0118 ± 0.0004 thus overlaps with the rate of 0.013 ± 0.0015 found for the H serotypes on an alternate day transfer schedule, although it is lower. The difference is not significant.

TABLE 2

Rate of stabilization of pure types from T serotype heterozygotes

Parental genotypes	Transfer	New pure types			Fraction stabilized	Average fraction stabilized per:	
		Tb	Tc	Total		Transfer*	Fission
<i>T^B/T^B × T^C/T^C</i>							
(C × B2)	first	3	14	17	.142		
	second	8	8	16	.155		
	third	6	8	14	.161		
						.153	.0118 ± .0004†
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		Ta	Tc	Total			
<i>T^A/T^A × T^C/T^C</i>							
(A × B2)	first	3	11	14	.130		
	second	6	8	14	.149		
	third	9	4	13	.163		
	fourth	7	3	10	.149		
						.148	.0114 ± .0001†

* One transfer is assumed to be equal to 13 fissions.

† Standard error.

Two types of error in scoring for the T serotypes would decrease the number of pure types. The switchover point for the H-T serotypes is 37°C, so the occasional cultures appearing "mixed" for one T serotype and negative for the other serotype, may actually be mixed for H and T serotypes, but pure for the T serotype, and thus misclassified as intermediate. The other source of error consists of cultures which are sluggish after being transferred from bacterized peptone tubes at room temperature to 40° peptone tubes and which might be misclassified as showing a positive response.

To summarize for the Tbc heterozygote, output ratios were close to 45 for the Tc serotype and the rate of stabilization of pure types in older cultures was 0.0118 ± 0.0004 per fission, close to that found for the H serotypes. Twenty percent of sublines derived from a Tbc heterozygote were pure for one parental serotype at 65 fissions after conjugation.

Differentiation of the Tac heterozygote: Since differentiation was not observed until 50 fissions after conjugation in preliminary experiments, no early pedigree analysis was made. Instead nine Tac heterozygotes were selected and tested at each transfer after conjugation until a few mixed reactions appeared at the fourth transfer. At this time each of these lines was expanded to 12 and transferred and tested at least four more times. The data in Table 3 show that the output ratios for these heterozygotes were close to 50. The percentage of pure types at 78 fissions was approximately 13%, about the same as that for the Tbc heterozygotes at 65 fissions. In fact, the stabilization of pure lines appeared to lag by about one transfer in this experiment, as compared with the Tbc experiment. However, it is difficult to determine how much of the variation was due to slight differences in the number of fissions per transfer and how much to actual differences in the time of phenotypic differentiation. Cell counts were not made, but cultures were assumed to have undergone 13 fissions after two days. Tabulation of the fraction of new pure types per fission for the Tac heterozygote gave a rate of stabilization of 0.0114 ± 0.0001 , not significantly different from the one determined by the other experiment (Table 2).

Time of differentiation of T heterozygotes: If the rate of stabilization of pure types and the relative frequency of the two parental types among the stabilized lines (output ratio) are known, the expected fraction of pure types at any number of fissions after phenotypic differentiation has begun can be calculated using the

TABLE 3

Tc output ratios and fraction pure types for T heterozygotes at various times after conjugation

Parental genotypes	Fissions after conjugation	Output ratio	Fraction pure	Total sublines
$T^B/T^B \times T^C/T^C$ (A3 $\times$ D1)	65	46	.19	471
$T^A/T^A \times T^C/T^C$ (A $\times$ B2)	78	54	.13	120
	118	51	.47	120

TABLE 4

*Total fraction stabilized lines at different fissions after fixation for different input ratios**

No. fissions after fixation	Input ratio					
	1/44	5/40	10/35	15/30	20/25	22/23
1	.25	.00	.00	.00	.00	.00
10	.74	.20	.04	.01	.00	.00
20	.84	.40	.15	.05	.02	.01
30	.88	.52	.26	.12	.07	.06
40	.91	.60	.35	.20	.13	.12
50	.92	.66	.43	.28	.21	.20
60	.93	.70	.49	.35	.28	.27
70	.94	.74	.54	.41	.34	.34
80	.95	.77	.59	.47	.41	.40
90	.95	.79	.63	.52	.46	.45
100	.96	.82	.68	.58	.54	.53
120	.97	.85	.73	.64	.59	.59
140	.97	.87	.77	.69	.65	.65
160	.98	.89	.81	.74	.70	.70
180	.98	.91	.83	.77	.74	.73
200	.98	.92	.85	.80	.77	.76

* The values were derived from the print out from a digital computer (SCHENSTED 1958).

equations given by SCHENSTED (1958). On the basis of a constant rate of 0.0113 for the H serotype system, the expected fraction of pure sublines at any fission after differentiation was calculated for different input ratios by SCHENSTED (1958; see Table 4). By computing the percent pure lines at different fissions after conjugation and comparing the values with those in the table, the time at which phenotypic differentiation begins can be determined.

It can be seen from the table that for a 22/23 input ratio 12% pure types would be expected at 40 fissions if phenotypic differentiation takes place at the first cell division after conjugation. For the Tbc heterozygote, 15% pure types were obtained 65 fissions after conjugation and for the Tac heterozygote 13% pure types were obtained 78 fissions after conjugation. Thus, there is a lag of at least 25 fissions before serotype differentiation occurs in the Tbc system and 35 fissions for the Tac system. (In the experiment described above, 20% of the Tbc cultures tested as pure at 65 fissions; however, they were only tested once. When some of these lines were used in the rate experiment a 26% reversion of cultures testing once as pure was obtained. Therefore, the correct number of stabilized pure cultures at this time would be 20% minus 5.2% or 14.8%. This is close to the 12% expected at 40 fissions). Since the stabilization data for the Tbc systems were obtained from cultures transferred on alternate days, i.e., under conditions which accelerate fixation, the estimate of a 25 fission delay is minimal.

In order to determine more exactly the time of differentiation of the Tac heterozygote, the following experiment was done. Four F_1 pairs were expanded to caryonides and 15 cells were taken randomly from each caryonide to obtain a total of 60 subcultures for each of the four pairs. These were carried through 19 serial daily transfers and tested for serotype every third transfer starting with

TABLE 5

Total fraction stabilized lines at successive fission intervals

Fissions	Expected fraction 1:1 input	Observed fraction*	Total lines	Estimated time of differentiation in fissions
20	.01	..	240	30
30	.06	..		
40	.12	..		
50	.20	.03		
60	.27	..		
70	.34	.13		
80	.40	..		
90	.45	.26		
100	.53	..		
110	.56	.40		

* Actual observations were made at 49, 70, 91 and 112 fissions.

the seventh transfer. Cell counts showed an average of seven fissions per transfer. The results of this experiment are given in Table 5. There was no significant difference between different pairs or caryonides, so the total fraction of stabilized lines was computed for successive fission intervals. The results of 3%, 13%, 26% and 40% for 50, 70, 90 and 110 fissions after conjugation, respectively, are close to the expected fraction for a 1:1 input ratio of 1%, 12%, 27% and 40% for 20, 40, 60 and 80 fissions, respectively (see Table 4). Thus, the beginning of phenotypic differentiation of the Tac heterozygote appears to be 30 fissions after conjugation.

In summary, the output ratios, kinetics of stabilization and timing of phenotypic differentiation appear similar for the Tac and Tbc heterozygotes. The evidence for this is provided by the experiments described in this paper and by numerous serotype tests done on progeny from F_1 , F_2 and testcrosses when they became mature for mating type at 60 to 90 fissions after conjugation. Pure types were frequently found at this time, but a majority of lines had to be transferred a few more times before they could be classified as a pure type.

DISCUSSION

A model proposed (ALLEN and NANNEY 1958; NANNEY 1964) to explain the differentiation of unstable mating types and H serotype heterozygotes can also be applied to the T serotype differentiations. In brief, the model proposes that the ciliate macronucleus is compounded by replication of diploid subnuclei. When the compounding is almost or entirely complete, the subnuclei differentiate to express only one allele at any given locus. The time of this allelic repression varies for different loci and possibly for different alleles. In subsequent divisions these differentiated subnuclei are mechanically sorted out and sublines pure for the parental types are obtained.

The evidence for this model was obtained from vegetative pedigree experiments involving the H serotype systems and unstable mating types. Pedigree analysis

of H heterozygotes showed that the rate of production of stabilized pure lines becomes constant within about 20 divisions after mixed types first appear and is equal to .0113 (NANNEY and DUBERT 1960). The total fraction of new pure types is constant, although the relative values of the two subtypes, Hx and Hy may vary initially. Very similar behavior has been found in clones unstable for mating type (ALLEN and NANNEY 1958). Most caryonides give rise to cells of only one mating type. However, some caryonides yield cells of two different mating types, usually with one type predominating. These "unstable" caryonides were the subject of a pedigree analysis which showed that they could be considered as macronuclear mosaics containing assorted subnuclear units (ALLEN and NANNEY 1958; SCHENSTED 1958). The unstable caryonides after an initial period of equilibration yield sublines pure for mating type at a constant rate of 0.0113/fission. From this value the number of assorting macronuclear subunits can be estimated at 90 immediately before cell division (SCHENSTED 1958). The combined rate is identical for all unstable lines and the rates for the two mating types approach equality as stabilization proceeds. These results are compatible with the assortment of stable subnuclear units, but would not be expected if such units replicated differentially.

Phenotypic differentiation has been found in all genetic systems examined in Tetrahymena. These include the *mt*, *H* and *T* serotype loci and three loci controlling isoenzymes. A comparison of the properties of the different systems is relevant to the proposed model. Differentiation of alleles at different loci has been followed in the same heterozygotes in several cases (ALLEN 1965; BLEYMAN, SIMON and BROSI 1966). These experiments have shown that phenotypes controlled by different loci, even linked loci, are distributed randomly among differentiated sublines. The output ratios of stable pure types are different for some loci. This ratio is highly eccentric for the *mt* and *H* loci, but close to equality for the *E-1*, *E-2*, *P-1* and *T* loci. There are different ratios for the different *H* allelic combinations. The *P-1* locus has a third stable type found in low frequency which requires the presence of both parental alleles (ALLEN, MISCH and MORRISON 1963). The time of the phenotypic differentiations has been established for the H serotype system, as occurring in different cases before the first, second or third macronuclear division after conjugation (NANNEY, NAGEL and TOUCHBERRY 1964; BLEYMAN, SIMON and BROSI 1966). Estimates for the other systems give 25 or more fissions after conjugation for the *T* locus, and at least 40 to 50 fissions for the *E-1*, *E-2* and *P-1* loci (ALLEN 1965 and unpublished). Thus all systems in which phenotypic differentiation occurs substantially after the time of development of the new macronucleus have output ratios close to equality. As described, the *mt*, *H* and *T* loci all give approximately the same rate of stabilization of pure lines once sorting out has begun. The rate of stabilization is more difficult to study with the enzyme loci, but sublines initiated after 80 fissions give stable types at similar rates for the *mt*, *H*, *E-1* and *E-2* loci (ALLEN 1965).

Diploid subnuclei in the ciliate macronucleus have not yet been demonstrated cytologically. We know that the macronucleus is compound from its large size and DNA content, but electron microscopy does not reveal subunits. Therefore, other alternatives must be considered. Instead of the epigenetic allelic repression

proposed in the above model, actual genetic assortment of chromosomes could also lead to subnuclei of pure types (NANNEY 1964). The chromosomes in the macronucleus might be independently assorted or they might be organized into haploid or diploid sets. If no chromosomal association occurs, homozygous subnuclei might be produced by random segregation; macronuclear division is by constriction and occasionally a particular chromosome might be omitted by chance from a given macronucleus. This model would lead to vegetative assortment in which the assorting units would be chromosomes. If one assumes haploid replicative units, no chromosome imbalance would occur, but sublines could receive different proportions of the homologues and would eventually become pure for one of the parental types. In the absence of extensive somatic crossing over, either of these models of genetic assortment would result in simultaneous fixation of all alleles on a particular homologue. The *E-1* and *mt* loci are linked by 25% recombination and can therefore be tested for association in the differentiated sublines. No relationship between *mt* and *E-1* assortment in pure sublines could be demonstrated, however (ALLEN 1964). Thus, extensive somatic crossing over must be assumed to randomize the expected association if these models are retained. The third genetic alternative retains the idea of diploid subunits, but postulates extensive somatic crossing over, which eventually renders all subnuclei homozygous.

The models of genetic assortment must make the additional assumption of differential replication of chromosomes or haploid sets at a particular period in the cellular proliferation to explain the eccentric output ratios. Since the initial allelic input is 1:1 for all loci, and since output ratios of 95:5 are not uncommon in the case of H serotypes, differential replication must be assumed. However, there is no evidence for differential replication during the assortment period, so this must occur at a definite period early in the cell cycle. Different output ratios and times of segregation of pure types are found for the different H heterozygotes, requiring the differential replication to be allele specific in degree and timing (NANNEY *et al.* 1963). In addition, cells heterozygous at three different loci with output ratios close to equality (*Hcd*, *Tbc*, *E-1bc*) begin segregating pure lines at three distinct times in the life cycle (BLEYMAN, SIMON and BROSI 1966; ALLEN 1965). Such precise sequential timing is not compatible with random loss of chromosomes or haploid sets or with random somatic crossing over. The appearance of a stable *P-1* type (ALLEN, MISCH and MORRISON 1963) requiring both parental alleles to be present is also evidence against a model of genetic assortment. Finally, in the case of the mating type locus, even homozygotes assort; therefore genetic assortment is definitely ruled out as a sufficient explanation for the behavior of this locus.

To summarize, since it is difficult to reconcile the hypothesis of genetic assortment with the available facts, the model of subnuclear differentiation and epigenetic assortment is preferred. That all loci examined in *Tetrahymena* should have evolved this control mechanism seems surprising until the special nature of these loci is considered (NANNEY 1964). They all involve codominant alleles which were isolated from nature. Null mutants and new mutations might behave

quite differently. Earlier papers (NANNEY 1964; ALLEN 1965; BLEYMAN, SIMON and BROSI 1966) have drawn attention to possible parallels between allelic repression in *Tetrahymena* and similar phenomena in higher organisms. One example is the finding of only allotype a or allotype b gamma globulin in individual cells of the mouse heterozygote *a/b* (WEILER 1965).

In at least two of the systems in *Tetrahymena* differentiation appears to take place in the absence of expression of the trait. In the mating type system the macronuclei are differentiated at the caryonide level, but mating types are not expressed until 60 to 80 fissions after conjugation. In the T system, heterozygotes which were obtained from crosses and transferred at room temperature are often sorted out at the T locus when samples are tested at 40°C, 100 fissions after conjugation. Thus the state of the gene is clearly separated from its expression. Sequential nuclear differentiation and separation of determination and expression are common in the development of higher forms. Thus, allelic repression in *Tetrahymena* may be analogous to differentiation in embryonic cells of higher organisms.

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SUMMARY

T heterozygotes at first express the serotypes of both parents, but differentiate during cellular multiplication to express only one of the two types. The two parental types are represented approximately equally among the differentiated T heterozygotes and the kinetics of stabilization are very similar to those found for the H serotypes and unstable mating types. The time of T serotype differentiation was estimated to be about 30 fissions after conjugation. T serotype differentiation is compared with similar phenotypic assortment found in other genetic systems of *Tetrahymena*. The data support a model of epigenetic allelic repression in preference to a model of genetic assortment of chromosomes or subnuclei. Since all crosses and vegetative pedigree lines were maintained at 25°C and samples from these were tested at 40°C, the T serotype differentiations are established and assorted in the absence of their expression.

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