SEQUENTIAL NUCLEAR DIFFERENTIATION IN TETRAHYMENA[1]

LEA K. BLEYMAN, ELLEN M. SIMON AND RUTH BROSI[2]

Department of Zoology, University of Illinois, Urbana

Received March 7, 1966

NUCLEAR differentiation is often responsible for phenotypic differences among genically identical lines in ciliates and is demonstrated by patterns of assortment in vegetative pedigrees. The first example of such a nuclear differentiation (SONNEBORN 1937, 1939) was the "caryonidal inheritance" of mating types in syngen 1 of *Paramecium aurelia*. In this case, macronuclei usually differentiate in an "all-or-none" fashion early in their development, and mating types are distributed at the cell divisions at which whole new macronuclei are assorted. Macronuclear differentiation is also the basis for mating type determination in the Group B syngens of *P. aurelia*, which appeared initially to manifest "cytoplasmic inheritance" (SONNEBORN 1954; NANNEY 1957). In syngen 1 of *Tetrahymena pyriformis*, macronuclear differentiation is again responsible for intraclonal mating type differences, but the diploid subunits of a single compound macronucleus often differentiate to express different mating types and then continue to assort for many cell divisions (NANNEY 1956; ALLEN and NANNEY 1958). Submacronuclear differentiation and vegetative assortment have also been demonstrated to underlie intraclonal variations in the H-immobilization antigens (NANNEY and DUBERT 1960) and the esterase and acid phosphatase isozymes (ALLEN 1960, 1961; ALLEN, MISCH and MORRISON 1963).

The specificity of the H-immobilization antigens is controlled by alleles at the *H* locus. Within each of the 11 inbred strains of syngen 1, *T. pyriformis*, one of four alleles (H^A, H^C, H^D, *and* H^E) is found in homozygous condition. Heterozygotes at the *H* locus initially express both antigenic types, but in the course of cellular proliferation produce sublines expressing only one or the other serotype. This intraclonal differentiation is interpreted as due to the repression or inactivation of one of the two alleles in each subnucleus. The probabilities of repression of the two alleles determine the ratio of the two kinds of pure subclones, which is characteristic of the allelic combination. An analysis of the distribution of this "output ratio" in vegetative pedigrees shows that the H antigenic determination occurs either before or soon after the first macronuclear division, as an uncoordinated event among the subnuclei (NANNEY, NAGEL and TOUCHBERRY 1964). The timing of allelic repression has been studied in three of the possible six heterozygotes at the *H* locus. The objective of the present study was to extend and complete the analysis of the remaining allelic combinations. The distributions within

[1] This investigation was supported by Public Health Service grant GM-07779 to D. L. NANNEY.

[2] Predoctoral fellow of the Public Health Service (grant GM-22,479); the work on the T antigens constitutes a part of the work undertaken for the Ph.D. degree.

the vegetative pedigrees of mating types and of another antigenic marker were also examined to ascertain the temporal relationships of various kinds of nuclear differentiation within the same developing macronuclei.

MATERIALS, METHODS AND COMPUTATIONS

Four inbred strains of *Tetrahymena pyriformis*, syngen 1, were used as parents of heterozygotes: D1 (H^C/H^C, mt^D/mt^D), A (H^A/H^A, mt^A/mt^A), A3 (H^E/H^E, mt^A/mt^A) and F (H^D/H^D, mt^F/mt^F). The genes mt^A, mt^D and mt^F, though independently isolated are very similar and possibly identical. Each permits the development of mating types I, II, III, V and VI in a characteristic pattern and in characteristic frequencies.

Figure 1 summarizes the procedure for establishing vegetative pedigrees and the terminology employed. Each experiment begins with the isolation of individual conjugating pairs from a mating mixture (either a mixture of two cultures—referred to as a mass mating, or a mixture of the progeny of two cells isolated into one depression of a slide—referred to as an isolation mating). The pairs are isolated into depression slides containing a few drops of culture medium. When the now genetically identical exconjugants separate at the end of conjugation, they are placed in separate depressions and examined frequently. At the first fission after conjugation, the two newly developing macronuclei established in each exconjugant are distributed to the daughter cells, which serve as the progenitors of the "caryonides," *i.e.*, lineages within which all the

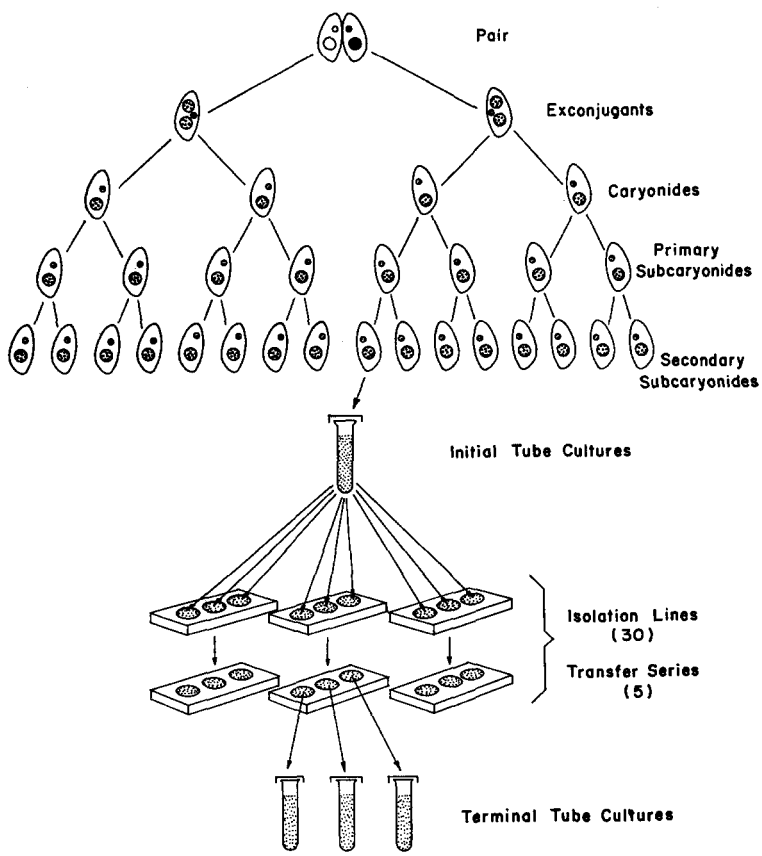

FIGURE 1.—Establishment of vegetative pedigrees.

cells contain a descendant of a common primordial macronucleus. The two "sister caryonides," derived from each exconjugant, are isolated to yield a total of four derivatives from each original pair. At the second postzygotic cell division the new macronucleus divides for the first time, and the daughter cells are designated as the progenitors of primary (1°) subcaryonides—four from each exconjugant or eight from each pair. The final bifurcation in these experiments comes at the third fission, when the secondary (2°) subcaryonides are established. The 16 pedigreed lines from each pair are then allowed to proliferate in their individual depression cultures through approximately 12 more cell divisions.

To provide a quantitative evaluation of the properties of the various lineages, 30 single-cell isolations are made from each of the 16 lines representing each pair. The cultures are not sexually mature at this time, so the mating types cannot be detremined. Moreover, the assortment of the serotypes has not progressed far enough to provide a reliable estimate of the relative frequencies of the alternative pure types. Hence, each of the 30 sublines is carried through five serial isolation steps, in which one cell is transferred from a grown depression culture into fresh medium. The terminal culture is then tubed and tested in standard fashion for mating types and serotypes.

The next problem consists of the identification of quantitative discontinuities within the pedigrees. The method has been described in detail earlier (NANNEY *et al.* 1964). The first step is to determine the "output ratio" for each of the sets of 30 sublines. This is defined by a simple percentage formula, $100\,[(2\,Hx + Hxy)/2N]$, where N is the number of subcultures tested, Hx is the number of cultures pure for one of the two antigenic types, and Hxy is the number still manifesting both serotypes. The raw output ratios are then converted by an arc sine transformation for use as "X" in the analysis of variance. Table 1 lists the formulae used to determine the sources of variance among the pedigreed sublines and the method for extracting the components of variance.

The number of subunits present in the macronucleus at the time of differentiation is estimated by assuming independence of subnuclear differentiation and fitting the arrays of output ratios to expansions of various powers of the binomial $(p + q)^N$. The power to which the expansion is raised represents the number of subnuclei (2, 4, 8, 16, etc.) present at the time of differentiation, and the terms of the expansion represent the classes of output ratios, from 0 to 100. The power array which most closely approximates the experimental distribution indicates the smallest number of independent subnuclei which could be present at the time of differentiation (NANNEY *et al.* 1964).

RESULTS

1. *Analysis of the cross, A × D1* (H^A/H^C; mt^A/mt^D): The alleles H^A and H^C have been analyzed previously in combinations with H^D, in crosses of A × B and D1 × B, respectively. In the H^A/H^D heterozygotes, H antigenic determination occurred before the first macronuclear division; in the H^C/H^D heterozygotes it occurred after the first macronuclear division but before the second. For the new cross six exconjugant clones derived from a mass mating (N–64–119) of strains A and D1 were expanded through 2° (secondary) subcaryonides. Two of these were co-conjugant clones (derived from the two members of a single pair). The output ratios and mating types were determined for each of the 30 sublines of each 2° subcaryonide (Table 2). The analysis of variance (Table 3) shows that the largest variation occurs between 2° subcaryonides within 1° subcaryonides. The H^A/H^C combination was characterized by a highly eccentric output ratio. Only about 4% of the sublines were Hc in phenotype. This output is close to the limits of resolution of a procedure which involves only 30 sublines and may contribute to a high error component incorporated in the variance for 2° sub-

TABLE 1

Analysis of variance formulae

Formula	Variance calculated	Mean square = Components of variance*
1. $SX^2_{\text{total}} - \frac{(SX)^2}{N}$	Total	
2. $\sum\left[\frac{(SX_p)^2}{16}\right] - \frac{(SX)^2}{N}$	Between pairs	$\frac{\text{equation 2}}{\text{df}_\text{p}} = 16V_p + 8V_e + 4V_c + 2V_s + V_{ss}$
3. $\sum\left[\frac{(SX_e)^2}{8}\right]$ — (1st term in equation 2)	Between exconjugants, within pairs	$\frac{\text{equation 3}}{\text{df}_\text{e}} = 8V_e + 4V_c + 2V_s + V_{ss}$
4. $\sum\left[\frac{(SX_c)^2}{4}\right]$ — (1st term in equation 3)	Between caryonides, within exconjugants	$\frac{\text{equation 4}}{\text{df}_\text{c}} = 4V_c + 2V_s + V_{ss}$
5. $\sum\left[\frac{(SX_s)^2}{2}\right]$ — (1st term in equation 4)	Between 1° subcaryonides, within caryonides	$\frac{\text{equation 5}}{\text{df}_\text{s}} = 2V_s + V_{ss}$
6. Equation 1 — (equation 2 + 3 + 4 + 5)	Between 2° subcaryonides, within 1° subcaryonides	$\frac{\text{equation 6}}{\text{df}_\text{ss}} = V_{ss}$

* p=pairs; e=exconjugants; c=caryonides; s=1° subcaryonides; ss=2° subcaryonides; df=degrees of freedom.

TABLE 2

Serotype output ratios and mating types in cell lineages from A × D1 heterozygotes

Subclone	Exconjugant clone	Output ratio (Hc)	Mating type*	Exconjugant clone	Output ratio (Hc)	Mating type*
1	A†	5	VI	D	12	I
2		10	VI		0	I
3		5	VI		3	I
4		5	VI		0	I
5		8	I		0	I
6		12	I		5	I
7		2	I		3	I
8		2	I		0	I
1	B†	18	I	E	0	VI
2		7	I		7	VI
3		0	I		5	VI
4		3	I (III)		0	VI
5		5	V		0	VI (I)
6		10	V		2	VI
7		8	V		3	VI (I)
8		2	V		3	VI
1	C	7	I	F	6	VI
2		0	I (VI)		0	VI
3		3	I		3	VI
4		2	I (VI)		5	VI
5		0	I		2	VI
6		5	I		0	VI
7		13	I		3	VI
8		3	I		5	VI

* Minority mating types (in parentheses) = three or less per 30 sublines.
† Clones from the same conjugating pair (co-conjugants).

caryonides. The small variances (actually producing negative components) between caryonides and between 1° subcaryonides indicate that the H^A/H^C output ratio is not a caryonidal characteristic, as it was for H^A/H^D, nor is it a 1° subcaryonidal characteristic, as with H^C/H^D. Differentiation must occur after the second macronuclear division.

Because differentiation occurs at least as late as 2° subcaryonides, the output ratios of these subclones were used in the binomial analysis. The output arrays

TABLE 3

Analysis of variance of output ratios computed in Table 2 (A × D1)

Sources of variation	Degrees of freedom	Mean squares	Components of variance*
Between exconjugants	5	72.7	$V_e = 8.4$
Within exconjugants, between caryonides	6	5.8	$V_c = -8.6$
Within caryonides, between 1° subcaryonides	12	40.4	$V_s = -7.3$
Within 1° subcaryonides, between 2° subcaryonides	24	54.9	$V_{ss} = 54.9$

* See footnote of Table 1 for explanation of symbols.

TABLE 4

Comparison of experimental and theoretical distributions for H^A/H^C *2° subcaryonidal output ratios. Theoretical distributions represent binomial expansions* $(0.04 + 0.96)^N$

N	Classes	Expected	Observed	P from chi-square
4	1–3	0.4	0	
	4	6.8	36	> .001
	5	40.8	12	
8	1–7	1.9	0	
	8	11.5	36	> .001
	9	34.6	12	
16	1–15	6.4	6	
	16	16.6	30	> .001
	17	25.0	12	
32	1–30	6.6	8	
	31	11.1	13	
	32	17.3	15	.80
	33	13.0	12	
64	1–60	5.2	8	
	61	6.8	4	
	62	10.6	9	> .001
	63	12.4	9	
	64	9.4	6	
	65	3.5	12	

were compared to the distributions calculated for independent differentiation when 4, 8, 16, 32 and 64 subunits were present (Table 4). All the distributions can be rejected at the 1% level except that for $N = 32$.

With respect to mating type, the sets of 30 related cultures were usually uniform, but occasionally a second type appeared in low frequency. The four sets comprising a caryonide were also usually homogeneous, not only with respect to the majority type, but also in manifesting the same minority type (subclones 2 and 4 of clone C and 5 and 7 of clone E). The similarity between sister caryonides (subclones 1 to 4 and 5 to 8) in clones C to F is probably coincidental; previous large scale studies (NANNEY 1956) have detected no tendency for sister caryonides to be alike in mating type.

2. *Analysis of the cross, A3 × D1* (H^C/H^E, mt^A/mt^D, T^B/T^C): The H^E allele had previously been examined in combination with H^A, and differentiation was found to occur at least as late as primary subcaryonides. In this cross, by an isolation mating (N–65–80), it is combined with H^C. In addition to H-serotypes and mating types, one other system of nuclear differentiation was examined. Cells of syngen 1, when grown for brief periods at 40°C in a 1% peptone medium containing 0.4% liver extract, lose their ability to be immobilized by specific H antisera and acquire new specificities (BROSI, in preparation). These specificities are controlled by a series of alleles unlinked to either *H* or *mt*. Heterozygotes at the *T* locus, like those at the *H* locus, manifest vegetative assortment of phenotype. The sublines of only one pair were tested for T serotypes.

The results of the pedigree analysis are presented in Table 5. With respect to

TABLE 5

Serotype output ratios and mating types in cell lineages from A3 × D1 heterozygotes

Subclone	Pair	Output ratio (Hc)	Mating type*	Pair	Output ratio (Hc)	Output ratio (Tc)	Mating type*
1	A	0	II	C	5	43	V–VI
2		5	II		8	42	V–VI
3		3	I (VI)		7	50	V–VI
4		2	I–VI		15	42	V–VI
5		0	II		0	42	I
6		0	II		5	43	I
7		0	I		0	43	I
8		2	I–VI		2	47	I
9		0	III		0	52	I–VI
10		0	III		0	47	VI–I
11		2	I		3	38	I–VI
12		0	I		2	47	VI–I
13		5	III		0	50	II
14		2	III		0	53	II
15		8	I		0	55	II
16		8	I		0	45	II
1	B†	0	II				
2		3	II				
3		0	II				
4		0	II				
5		0	II				
6		0	II				
7		0	II				
8		0	II				

* First type is majority type; parentheses indicate three or less of minority type.
† Pair B is represented by one exconjugant clone only; the other clone died.

mating types, pair C showed the expected caryonidal pattern; half-pair B manifested a (probably) coincidental similarity of sister caryonides; but pair A showed a 1° subcaryonidal distribution of mating types. The significance of this observation will be discussed later.

The analysis of variance for the H antigens (Table 6A) shows that H-antigenic determination is caryonidal, i.e., differentiation occurs before the first macronuclear division. The T serotypes were determined only for pair C. At the time of testing, when most of the sublines were pure for H serotype, nearly all were

TABLE 6

Components of variance for the output ratios in Table 5 (A3 × D1)

Set	V_e	V_c	V_s	V_{ss}*
A. Hc, all clones	— 0.9	28.2	1.3	18.4
B. Hc, pair C	18.3	33.1	3.5	19.2
C. Tc, pair C	— 0.03	— 1.1	3.4	6.2

* See footnote of Table 1 for explanation of symbols.

TABLE 7

Serotype output ratios and mating types in cell lineages from A3 × F heterozygotes

2° sub-caryonides	Pair	Output ratio (Hd)	Mating type*	Pair	Output ratio (Hd)	Mating type*	Pair	Output ratio (Hd)	Mating type*
1	A	10	II	D	17	VI	G	10	VI (III)
2		12	II		20	VI		27	VI
3		20	II		10	VI		33	VI
4		25	II		12	VI		48	VI
5		18	I (VI)		3	VI		20	III
6		13	I–VI		10	VI		28	III
7		5	VI–I		8	VI		30	III
8		7	VI–I (III)		3	VI		13	III
9		5	II		7	I		10	I (III)
10		17	II		2	I		15	I
11		17	II		2	I		22	I–III
12		22	II		0	I		5	I (III)
13		13	III		18	VI		10	VI
14		5	III		25	VI		20	VI
15		5	III		12	VI		20	VI
16		5	III		17	VI		15	VI
1	B	3	VI	E	3	II	H	13	I
2		2	VI		0	II		5	I
3		5	VI		3	II		7	I
4		3	VI		2	II		5	I
5		15	I		0	III		7	I
6		7	I		5	III		0	I
7		12	I		10	III		3	I
8		8	I		5	III		5	I
9		3	II		8	I		3	VI
10		0	II		8	I		2	VI
11		2	II		2	I		0	VI (I)
12		3	II		2	I		0	VI (I)
13		2	VI		8	I		7	VI–I
14		3	VI		5	I (VI)		3	VI (I)
15		3	VI		13	I (VI)		0	I–VI
16		2	VI		10	I (VI)		0	VI–I
1	C	5	I	F	5	VI (I)	I	18	I–VI
2		8	I		10	VI		15	VI–I
3		3	I		22	VI		10	I–VI
4		13	I		7	VI		20	VI–I
5		12	VI		8	VI		23	VI
6		12	VI		13	VI		23	VI (III)
7		18	VI		5	VI		12	VI (III)
8		5	VI		18	VI		18	VI–III
9		7	III		12	III (V)		10	III
10		3	III (I)		23	III		23	III–I
11		8	III		17	III		22	I–III
12		0	III		18	III (V)		27	I–III
13		5	III (V)		7	VI (I)		4	II
14		5	III		10	VI		8	II
15		18	III (V)		7	VI		10	II
16		13	III		5	VI (I)		12	II

* First type is majority type; parentheses indicate three or fewer of minority type.

still showing mixed responses to anti-T sera. Moreover, the analysis of variance for T types (Table 6C) shows no major discontinuities within the vegetative pedigree. The conclusion to be drawn is that the T types began sorting much later than the H types. This conclusion will be documented and supported further in a later publication (BROSI, in preparation).

A binomial analysis of H types in caryonides excludes $N = 8$ and $N = 16$ ($P = >.001$ and $<.02$) but $N = 32$ ($P = .70$) and $N = 64$ ($P = .5$ to $.7$) cannot be rejected. Significantly, in pair A the H serotypes are fixed prior to the first macronuclear division; mating types are fixed between the first and second divisions; and T serotypes are fixed (by analogy with pair C) much later.

3. *Analysis of the cross A3 × F* (H^E/H^D, mt^A/mt^F): This cross, completing the series of H heterozygotes, was initiated as an isolating mating (N65–108); nine pairs were expanded through 2° subcaryonides and the 30 sublines of each were tested for H serotypes and mating types (Table 7). The mating type distribution followed the caryonidal pattern. A majority (61%) of the caryonides were pure for one mating type. Where secondary mating types were present, they were the same within sister subcaryonides. Unlike any of the previous crosses, however, the pairs manifested significant heterogeneity with respect to the time of H differentiation (Table 8). Pairs B, C, D, F, and I have a caryonidal pattern; pairs A, E and H have the major discontinuity at the level of 1° subcaryonides; pair G differs from all the others and appears to differentiate even later. The output ratios are not the cause of this heterogeneity, since the same ratio (compare pairs A and F; B and E) may be associated with either a caryonidal or a 1° subcaryonidal differentiation.

Under these circumstances, a combined analysis of variance of all the pairs would blur any discontinuities. When this cross is treated as two major subgroups, with pair G not included in either one, the time of differentiation for each group is clearly seen (Table 8). Therefore, the binomial analysis was based on caryonidal outputs in one case and on 1° subcaryonidal outputs in the other. In both

TABLE 8

Components of variance for the output ratios of the pairs in Table 7

Pair	Component of variance					Output ratio
	V_p	V_e	V_c	V_s	V_{ss}	
A	. . .	— 6.4	4.0	*22.6*	15.3	12:88
B	. . .	11.8	*17.0*	4.1	13.7	5:95
C	. . .	— 4.5	*10.6*	— 2.6	37.8	8:92
D	. . .	16.4	*44.1*	7.5	16.4	10:90
E	. . .	10.6	— 2.3	*15.7*	21.2	5:95
F	. . .	—10.0	*18.9*	— 9.6	28.8	12:88
G	. . .	30.7	— 8.2	5.9	45.5	20:80
H	. . .	20.9	— 7.3	*33.8*	0.1	16:84
I	. . .	8.5	*19.2*	4.6	15.3	4:96
A, E, H	24.1	9.6	— 5.4	*24.1*	12.2	. . . .
B, C, D, F, I	11.7	1.0	*22.0*	— 0.8	22.4	. . . .

TABLE 9

Binomial analysis of H^E/H^D *output ratios*

N	P values for 20 caryonides Pairs B, C, D, F, I	P values for 24 subcaryonides Pairs A, E, H
8	> .001	> .001
16	.2	.02
32	.7–.8	.05–.10
64	.2–.3	> .001

groups, an adequate fit is obtained for a value of $N = 32$ (Table 9). Although the fit for 1° subcaryonides is close to rejection at the 5% level all the other values of N except $N = 32$ can be rejected. The fact that the binomial analysis indicates a similar macronuclear compoundness at the time of differentiation in these two groups (and in the preceding crosses) suggests that H differentiation occurs at a particular time in the cell cycle, but can occur in different cell cycles (prior to the 1st, 2nd or 3rd macronuclear division) in individual pairs.

DISCUSSION

The theoretical basis for employing vegetative pedigrees in the analysis of nuclear differentiation in ciliates has been set forth earlier (NANNEY 1964; NANNEY *et al.* 1964), but a recapituation may be helpful. ALLEN and NANNEY (1958) first showed that mating type assortment in clones of unstable mating type in Tetrahymena could be interpreted according to a mosaic model for the macronucleus. The number of assorting subnuclei was estimated at 45 immediately after a nuclear division, or 90 just before. Subsequently, NANNEY and DUBERT (1960) demonstrated that the vegetative assortment of H serotypes in heterozygotes conformed to a similar pattern and yielded a corresponding value for the number of assorting units. These studies were concerned primarily with the behavior of "mixed" macronuclei after they had been established.

Other studies have been designed to characterize the initial events. Mating type differentiation in three genotypes (mt^A/mt^A, mt^A/mt^B and mt^B/mt^B) has been shown (NANNEY and ALLEN 1959) to occur prior to the first macronuclear division; a majority of caryonides manifested a single mating type, and most of the remainder yielded a single predominant type. The two caryonides from a single exconjugant show no correlation in their majority mating type (NANNEY 1956). When H differentiation was found to occur at different times in different heterozygotes, the question was raised whether the time of mating type determination might not also be variable, so that all the kinds of macronuclear differentiation in a particular cross occur at the same time. To answer this question the different kinds of nuclear differentiation had to be assessed simultaneously. As has been noted, with the exception of one exconjugant, all the pairs examined in the present series displayed caryonidal patterns for mating type distribution, even when other patterns were found for other traits.

With respect to mating types, the minority mating types produced in mixed

macronuclei provide some additional information about the timing of differentiation. Presumably the subnuclei assume their hereditary properties during the maturation of the macronuclear anlagen, as their number increases from one to perhaps 90. (We may note, however, that mating type *manifestation* is ordinarily delayed until 50 or more fissions later.) If the differentiations should occur early in macronuclear development, when relatively few subnuclei are present, the ratio between the most common type and the least common type of subnucleus will be relatively low. Thus, if differentiation occurs when only four subnuclei are present, any minority type will comprise at least a fourth of the total, and after assortment has been completed a quarter of the pure sublines should be of that type. The usual rarity of the minority type (confirmed in this study) implies a late fixation of subnuclei, at a time when at least 32 subnuclei are present. The fact that one mating type is usually found in great excess in a caryonide also indicates that the differentiation with respect to mating types is not random for the many subnuclei present within a macronucleus at the time of determination. The differentiations within a single macronucleus are "coordinated" in some way.

The studies on H antigenic differentiation are similar in rationale, but require somewhat different techniques. The qualitative dissimilarities so obvious in vegetative pedigrees for mating types are not apparent for serotypes; most of the caryonides produce some of each of the alternative pure subclones and only quantitative differences can be examined. For this reason the lineages have been assessed by an analysis of variance (NANNEY *et al.* 1964) to identify discontinuities in the pedigrees. If, for example, differentiation occurs prior to the first macronuclear division, the sublines of a caryonide will tend to be alike, but sister caryonides—representing separate differentiative events in the same cell—will be less correlated. If differentiation occurs between the first and second macronuclear divisions a discontinuity will be observed at the next cell division, etc.

In the previous study three H genotypes were examined (Table 10). The H^A/H^D heterozygotes showed caryonidal homogeneity; the H^C/H^D heterozygotes showed a discontinuity at the next division, indicating differentiation between the first and second macronuclear divisions; the H^E/H^A heterozygotes, expanded only as far as 1° subcaryonides, differentiated at least this late and possibly later.

TABLE 10

Summary of the times of differentiation for H antigens

Cross	Genotype	Output ratio	Time*	N†	e‡	Reference
A × B	H^A/H^D	87:13	c	32	8½	NANNEY *et al.*, 1964
A3 × F	H^E/H^D	90:10	c, sc (ssc)	32	18	Tables 7, 8, 9
A3 × D1	H^E/H^C	98: 2	c	32	5	Tables 5, 6
C × A	H^E/H^A	81:19	sc-ssc	32	8	NANNEY *et al.*, 1964
D1 × B	H^C/H^D	43:57	sc	8	20	NANNEY *et al.*, 1964
A × D1	H^A/H^C	96: 4	ssc	32	6	Tables 2, 3, 4

* Time of fixation: c = caryonidal discontinuity; sc = 1° subcaryonidal discontinuity; ssc = 2° subcaryonidal discontinuity.
† N = the minimum number of subnuclei at the time of H antigen fixation.
‡ e = the number of exconjugant clones analyzed.

The current study completes the set of possible heterozygotes with the four available alleles. The H^E/H^C heterozygotes again show a caryonidal pattern, and the H^A/H^C heterozygotes show a discontinuity only at the level of 2° subcaryonides, indicating a fixation at least two fissions later. The H^E/H^D manifest a heterogeneity not thus far encountered in other genotypes, perhaps because the others have not been represented by such large samples. Some of the pairs have a caryonidal pattern and others show a discontinuity at the level of 1° subcaryonides. One pair may have an even later differentiation.

These observations indicate that each *H* heterozygote has a characteristic time (or distribution of times) at which the subnuclei become fixed, and that H fixation is independent of mating-type fixation. Some differentiations occur, as do those for mating type, prior to the first macronuclear division, some occur before the second, and some before the third. The patterns of timing are not clearly related to particular alleles; all four alleles are represented in each of the three fixation intervals. The output ratios, which do appear to depend on allelic combinations (NANNEY *et al.* 1963), are not obviously related to timing. The two most eccentric outputs (H^C/H^E and H^A/H^C) are associated in one case with caryonidal discontinuities and in the other with 2° subcaryonidal determination. The output ratio nearest equality (H^C/H^D) is intermediate in its timing.

While the variance analysis identifies the fission interval during which differentiation occurs, it does not indicate the precise time within that interval. The binomial analysis (NANNEY *et al.* 1964) was designed to provide evidence on this point. It attempts to interpret the distribution of output ratios on the basis of the numbers of independent foci of differentiation present at the time of differentiation. Thus, if only four subnuclei are present and these are independently fixed. different whole nuclei may have very different output ratios, corresponding to 0:4, 1:3, 2:2, etc. If, on the other hand, 32 independent foci are present, the distribution of outputs will be much less dispersed. The observation that caryonidal outputs (where caryonidal determination has been established) fit well the formula $(p + q)^{32}$, indicates that differentiation occurs at least as late as the 32 unit stage. It does not set an upper limit, because any irrelevant source of variation (experimental or sampling error for example) will disperse the distribution and make fixation appear to have occurred when fewer units were present. Thus, fixation may occur as late as the 90-unit stage, immediately before macronuclear division, and the discrepancy between 90 and 32 may represent the dispersion resulting from uncontrolled variation. The fact that all the sets (caryonidal, 1° subcaryonidal, etc.) yield a best fit with 32 units may suggest that the fixation occurs at the same time within the cell cycle, regardless of which cycle is involved. The available information does not however, establish this interpretation. The binomial analysis is probably not sensitive enough to fix precisely the time of differentiation within later cycles. It does demonstrate, however, that the number of independent foci present at the time of differentiation is sufficiently high to rule out any large amount of "coordination" such as has been postulated for mating type differentiations.

The third system of nuclear differentiation employed in these studies was that

of the T antigens. These are alternatives to the *H* antigens, expressed at "*t*orrid" (40°C) temperatures. Their determinants are unlinked to *H* or to *mt* (BROSI, in preparation), which are themselves unlinked (NANNEY 1960). The *T* alleles manifest vegetative assortment of phenotypes, much like the *H* alleles, but the output ratios for T and H may be very different. Moreover, no point of discontinuity within the early pedigrees has been demonstrated for the T system, indicating that the differentiations for T occur at some time after the last bifurcations. Hence, within the same pedigrees, three different systems of nuclear differentiation can be demonstrated to be occurring in their own characteristic patterns and with their own specific timing. ALLEN (1965) has provided similar evidence for the independence of vegetative assortment for mating types and esterases; this case is particularly significant because it involves genetically linked loci (ALLEN 1964). Thus, all the available data indicate that the various systems of nuclear differentiation are independent of each other and may be locus-specific.

Although these studies make some progress toward characterizing the mode and timing of the nuclear differentiations, they provide little understanding of the mechanism. "Allelic repression" appears to be almost universal for genes in Tetrahymena, but parallels may be found in other diploid organisms. Aside from sex-linked repressions, recent pertinent studies are those on gamma-globulin forming cells in the mouse (WEILER 1965) and the rabbit (PERNIS *et al.*, 1965) which express only one of the two alleles at an autosomal locus. *H* allelic repression in persistent heterozygotes of Salmonella (IINO 1964) may provide another analogy.

From the point of view of ciliate cytogenetics, these observations go far toward a resolution of the persistent and vexatious problem of macronuclear organization (see NANNEY 1964). SONNEBORN's (1947) genetic studies on Paramecium have long been interpreted as indicating that the macronucleus is composed of diploid subnuclei, but alternative interpretations are difficult to exclude. For example, a macronucleus with large numbers of randomly assorting chromosomes (or haploid subnuclei) would be expected to show considerable phenotypic stability even though constant assortment occurred at each fission (KIMBALL 1943). The observations on vegetative assortment in Tetrahymena raised again the possibility that haploid units (or individual chromosomes) of the macronucleus are segregating; the differences between Paramecium and Tetrahymena might then reflect nothing more than the degree of macronuclear compoundness. The fact that all known genetic loci in Tetrahymena manifest phenotypic assortment was particularly disquieting, and raised serious questions concerning the hypothesis of allelic repression. A more careful study of the various systems, however, reveals properties which can be reconciled with a genetic assortment only through many and improbable assumptions. The locus (and allelic) specificity with respect to the time at which assortment begins and to the output ratios of the alternative types is particularly difficult to explain on a genetic basis. To be sure, "allelic repression" is not a "mechanism" which can explain the patterns of assortment, but a search in the area of genic regulation now appears much more profitable than a further exploration in the realm of transmission irregularities.

One other problem raised by these studies deserves brief comment. Quite aside from the mechanism of allelic repression is the mechanism of ordering the various events in time. Sequential differentiation is of course a feature of all developing organisms and examples of sequential gene activation can be found (e.g., see INGRAM 1963 on hemoglobin changes). But the changes occurring in ciliates may occur over long periods of time (SIEGEL and COHEN 1963) in an essentially constant environment. The esterases in Tetrahymena may not differentiate until as late as 40 cell divisions after conjugation (ALLEN 1965) and other differentiations may occur even later. The ciliate changes, unlike those in multicellular organisms, cannot be readily explained on the basis of effects of a progressively changing environment, but suggest an intrinsic long-range timing mechanism to which various differentiative events are geared in different ways.

SUMMARY

Three interstrain heterozygotes of syngen 1, *T. pyriformis* were analyzed for the time of differentiation of the H antigens, the mating types and, in part for the T antigens. The time of H antigenic differentiation varied, occurring either before the first macronuclear division, or one to two divisions later. In all cases, the minimum number of subunits present in the macronucleus was the same. The allelic combination at the *H* locus determined the degree of repression of one allele over the other and the time of fixation, but the pattern of timing could not be simply related to the individual alleles. The time of mating type differentiation was, except in one pair, before the first macronuclear division, and thus showed no correlation to the timing of H antigenic differentiation. Another antigenic marker, T, was independent of both mating type and H antigenic type in the lateness of its differentiation, and radically different from the H antigen in output ratio. These various traits manifest discrete localized sequential differentiations within genically identical macronuclear subunits.

LITERATURE CITED

ALLEN, S. L., 1960 Inherited variations in the esterases of Tetrahymena. Genetics **45**: 1051–1070. —— 1961 Genetic control of the esterases in the protozoan *Tetrahymena pyriformis*. Ann. N. Y. Acad. Sci. **94**: 753–773. —— 1964 Linkage studies in variety 1 of *Tetrahymena pyriformis:* a first case of linkage in the ciliated protozoa. Genetics **49**: 617–627. —— 1965 Genetic control of enzymes in Tetrahymena. Brookhaven Symp. Biol. **18**: 27–54.

ALLEN, S. L., M. S. MISCH, and B. M. MORRISON, 1963 Genetic control of the acid phosphatases in Tetrahymena: formation of a hybrid enzyme. Genetics **48**: 1635–1658.

ALLEN, S. L., and D. L. NANNEY, 1958 An analysis of nuclear differentiation in the selfers of Tetrahymena. Am. Naturalist **92**: 139–160.

INGRAM, V. M., 1963 *The Hemoglobins in Genetics and Evolution.* Columbia University Press, New York (pp. 97–131: The switch from fetal to adult proteins.)

IINO, T., 1964 Genetics of flagellar antigen in Salmonella. Proc. 11th Intern. Congr. Genet. **3**: 731–740.

KIMBALL, R. F., 1943 Mating types in the ciliate Protozoa. Quart. Rev. Biol. **18**: 30–45.

NANNEY, D. L., 1956 Caryonidal inheritance and nuclear differentiation. Am. Naturalist **92:** 139–160. —— 1957 Mating type inheritance at conjugation in variety 4 of *Paramecium aurelia*. J. Protozool. **4:** 89–95. —— 1960 The relationship between the mating type and the H serotype systems in Tetrahymena. Genetics **45:** 1351–1358. —— 1964 Macronuclear differentiation and subnuclear assortment in ciliates. 22nd Symp. Soc. Study Develop. Growth. pp. 253–273.

NANNEY, D. L., and S. L. ALLEN, 1959 Intranuclear coordination in Tetrahymena. Physiol. Zool. **32:** 221–229.

NANNEY, D. L., and J. M. DUBERT, 1960 The genetics of the H serotype system in variety 1 of *Tetrahymena pyriformis*. Genetics **45:** 1335–1358.

NANNEY, D. L., S. J. REEVE, J. NAGEL, and S. DE PINTO, 1963 H serotype differentiation in Tetrahymena. Genetics **48:** 803–813.

NANNEY, D. L., M. J. NAGEL, and R. W. TOUCHBERRY, 1964 The timing of H antigenic differentiation in Tetrahymena. J. Exptl. Zool. **155:** 25–42.

PERNIS, B., G. CHIAPPINO, A. S. KELUS, and P. G. H. GELL, 1965 Cellular localization of immunoglobulins with different allotypic specificities in rabbit lymphoid tissue. J. Exptl. Med. **122:** 853–876.

SIEGEL, R. W., and L. W. COHEN, 1963 A temporal sequence for genic expression: cell differentiation in Paramecium. Am. Zoologist **3:** 127–134.

SONNEBORN, T. M., 1937 Sex, sex inheritance and sex determination in *Paramecium aurelia*. Proc. Natl. Acad. Sci. U.S. **23:** 378–395. —— 1939 *Paramecium aurelia:* mating types and groups; lethal interactions; determination and inheritance. Am. Naturalist **73:** 390–413. —— 1947 Recent advances in the genetics of Paramecium and Euplotes. Advan. Genet. **1:** 263–358. —— 1954 Patterns of nucleo-cytoplasmic integration in Paramecium. Proc. 9th Intern. Congr. Genet. Caryologia **6** (suppl. vol.): 307–325.

WEILER, E., 1965 Differential activity of allelic gamma-globulin genes in antibody-producing cells. Proc. Natl. Acad. Sci. U.S. **54:** 1765–1772.