Nucleo-Cytoplasmic Interaction During Macronuclear Differentiation in Ciliate Protists: Genetic Basis for Cytoplasmic Control of *SerH* Expression During Macronuclear Development in *Tetrahymena thermophila*

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ABSTRACT

A novel class of mutations affecting the developmental expression of *SerH* cell surface antigen genes of *Tetrahymena thermophila* is described. Unlike previous categories of mutation, the four independently isolated mutations of this class act through the cytoplasm to affect *SerH* genes during macronuclear development. That is, macronuclei which develop under the influence of mutant cytoplasm do not subsequently express H, most likely because the developmental processing of *SerH* genes is affected. The cytoplasmic effect is specific for the *SerH* locus and is independent of which *SerH* allele is present. In place of H, hitherto unknown antigens are expressed. Expression of *SerH* can be rescued during development either by wild-type cytoplasm exchanged between conjugants or by the homozygous wild-type genotype. The mutations segregate independently of the *SerH* genes and identify one, possibly two, bistable genes. Possible models to explain these results are discussed.

ALTHOUGH Mendelian inheritance is well documented in ciliate protists, the inheritance of certain traits defies simple Mendelian explanation. In most of these instances, the traits are inherited in a non-Mendelian manner through the cytoplasmic lineage and involve the "stable differentiation" of the transcriptionally active macronucleus (SONNEBORN 1977). To appreciate this problem, it should be recalled that in ciliates such as Paramecium and Tetrahymena the sexual form of reproduction (conjugation) involves the *temporary* union of two cells. During this union the cells become genetically identical for both micronuclear (germline) and macronuclear genes [see NANNEY (1980) for recent description]. However, despite genetic identity, the cell clones arising from each exconjugant may be dissimilar with respect to phenotype (SONNEBORN 1977; NANNEY 1980). In several instances, such dissimilarity is the result of nucleocytoplasmic interaction during macronuclear development. Specifically, the old macronucleus, through the cytoplasm, influences the development of the new macronuclei in such a way that phenotypic differences between the conjugants are perpetuated in their cytoplasmic lineages. Two examples will illustrate this point.

The classic example is that of mating type determination in *Paramecium tetraurelia* (reviewed by SONNEBORN 1977). In this species, cells express either of two mating types, O or E. The expression of mating type is stable throughout asexual fission; that is, a clone that is O continues to be type O, and a clone that is E continues to be type E. During sexual reproduction (conjugation), despite the resulting genetic identity, descendants of the O conjugant continue to be mating type O, and descendants of the E conjugant continue to be mating type E. Experimental analysis has shown that the mating type is determined by properties of the cytoplasm (as influenced by the old macronucleus) in which new macronuclei develop. Thus, macronuclei that develop in cytoplasm of the O exconjugant differentiate to type O, and macronuclei that develop in cytoplasm of the E exconjugant differentiate to type E. However, when rare or induced cytoplasmic exchange occurs, the macronuclei of the O conjugant usually differentiate to type E, and the newly acquired E phenotype is in turn transmitted cytoplasmically to progeny at the next conjugation.

The second example is mutant d48 of *P. tetraurelia* (EPSTEIN and FORNEY 1984). In this mutant, the inability to express the A cell surface (immobilization) antigen is transmitted at conjugation in a non-Mendelian, cytoplasmic manner similar to the inheritance of O and E mating types. Thus, at conjugation the descendants of A-negative conjugants continue to be A-negative, and the descendants of A-positive conjugants continue to be A-positive. In this mutant, Southern blot analysis of mutant macronuclear DNA using an A gene clone clearly shows that the A gene is not present in the macronucleus of A-negative cells (EPSTEIN and FORNEY 1984; HARUMOTO 1986). In d48 the A gene is developmentally eliminated from the macronucleus.

The exact molecular basis for the cytoplasmic influence over nuclear differentiation is unknown. As sug-

gested by mutant d48, the cytoplasmic influence is almost certainly related to the processing and rearrangement of the genome during macronuclear development. In Tetrahymena the critical role of the cytoplasm in macronuclear development commences at the second postfertilization micronuclear division when precisely timed cytoplasmic signals cause the two anterior-most micronuclei to begin the transformation into macronuclei (RAY 1956; NANNEY 1953; DOERDER and SHABATURA 1980). The developmental events triggered by these signals are profound. Not only does the DNA content increase from two haploid equivalents to 64 (DOERDER and DEBAULT 1975), but the genome is considerably reorganized during the amplification process (reviewed by GOROVSKY 1980). Such reorganization involves the fragmentation of chromosomes into some 5000 pieces (YAO *et al.* 1984), the elimination of 15% of the fragmented material (YAO and GOROVSKY 1974), and the reassembly of the remaining genetic material into several hundred macronuclear linkage groups (ALTSCHULER and YAO 1985; CONOVER and BRUNK 1986). For certain genes, such as for rRNA (YAO, ZHU and YAO 1985) and α-tubulin (CALLAHAN, SHALKE and GOROVSKY 1984) developmental rearrangement is almost certainly required for subsequent expression. For other genes, such as that for histone H4, no rearrangement has been detected (BANNON *et al.* 1984). ORIAS (1981) has proposed that mating type differentiation in *Tetrahymena thermophila* involves both rearrangement and deletion of genetic information. A similar argument can be made for Paramecium in which cytoplasmic factors separate from mating type itself are hypothesized to influence the determination (TAUB 1963; SONNEBORN 1977).

We describe here four mutations similar to d48 affecting the expression of alleles at the *SerH* locus of *T. thermophila*. *SerH* is a member of a dispersed family of polymorphic loci specifying alternative forms of the cell surface immobilization antigen. These antigens include L, expressed below 20°; H, expressed from 20° to 36°; T, expressed above 36°; S (and L) expressed in media supplemented with 0.2 M NaCl; and I expressed when L- or H-expressing cells are grown in the presence of homologous antisera. The four H antigens are proteins ranging in molecular weight from 44,000 to 52,000 (DOERDER and BERKOWITZ 1986) and coat the entire external surface of the cell as shown by immunofluorescence (DOERDER 1981). The normal expression of *SerH* alleles is affected by two categories of Mendelian mutation: (1) dominant and recessive mutations at loci unlinked to *SerH* (DOERDER 1973, 1979, 1986) and (2) regulator genes or sites at or very near *SerH* (DOERDER, BERKOWITZ and SKALICAN-CROWE 1985). In both categories, one or more of the alternative antigens are expressed in place of H.

The mutations described here show that the expression of *SerH* genes is under cytoplasmic control during macronuclear development. This is the first instance in Tetrahymena in which Paramecium-like cytoplasmic regulation of macronuclear differentiation is described. This is also the first instance in the ciliates in which an underlying Mendelian basis for this phenomenon is described.

MATERIALS AND METHODS

Strains: Inbred strains of *T. thermophila* were originally obtained from D. L. NANNEY (University of Illinois at Urbana-Champaign). Their derivation from natural isolates is described in ALLEN and GIBSON (1973). Strains A and B are wild-type strains homozygous for *SerH1* and *SerH3* alleles, respectively. CU399, obtained from PETER BRUNS (Cornell University), is a functional heterokaryon carrying a selectable marker for cycloheximide resistance. It is homozygous for the *SerH3* allele. Mutant strains H1-1 and H1-2 are homozygous for *cis*-acting mutations near the *SerH1* allele (DOERDER, BERKOWITZ and SKALICAN-CROWE 1985). Mutant strains rseA, rseB, rseC and RseD are homozygous for mutations at the *resA*, *rseB*, *rseC*, and *RseD* loci, respectively (DOERDER 1973, 1979, 1986). A*III and B*VII are genomic exclusion strains expressing the *SerH1* and *SerH3* alleles, respectively.

Culture conditions: Cells were grown either in PPY or BP media at 28° unless otherwise stated. PPY (autoclaved) consisted of 1% w/v Difco proteose peptone, 0.15% w/v yeast extract and 0.010 mM $FeCl_3$ in distilled water. BP consisted of a 1:70 dilution in distilled water of PPY inoculated with *Klebsiella pneumoniae*.

Genetic analysis: Procedures for normal crosses have been previously described (ORIAS and BRUNS 1979). In most crosses, 16–32 pairs were isolated; only true exconjugant synclones, as indicated by sexual immaturity, were scored for phenotype. The descendants of an exconjugant pair, collectively called a synclone, consist of four karyonidal lineages each derived from each new macronucleus. Except as noted in RESULTS, synclones were usually uniform with respect to phenotype. In some instances, single cells were isolated from synclones at 10–15 fissions after conjugation and serially transferred for another 40–70 fissions.

Genomic exclusion crosses: Rounds 1 and 2 of genomic exclusion were performed according to standard procedures (ALLEN 1967; WEINDRUCH and DOERDER 1975; ORIAS and BRUNS 1979). Genomic exclusion refers to the exclusion at round 1 of micronuclear genes of defective strains such as A*III (ALLEN 1967; WEINDRUCH and DOERDER 1975) and B*VII (F. P. DOERDER, unpublished data). However, because each round 1 exconjugant retains its old macronucleus (macronuclear development fails), such exclusion is not phenotypically manifest until round 2 when new macronuclei develop and the old macronuclei disintegrate. The cytogenetics of round 1 are shown in Figure 1; those of round 2 are the same as in normal conjugation (RAY 1956). As shown in Figure 1, the micronuclei of round 1 exconjugants are derived by mitosis and endoreplication from a single haploid meiotic product. As a consequence, these micronuclei are homozygous and genetically identical, and, since the old macronucleus of each conjugant is retained, a new micronucleus is essentially "transplanted" into the "*" cell. When the normal round 1 partner is heterozygous, 50% of round 1 exconjugant pairs are homozygous for one allele and 50% are homozygous for the other. Since round 1 exconjugants are sexually mature, their asexual descendants can be re-

mated, either to each other (round 2) or to other strains. Because such remating is cytogenetically normal, round 2 progeny from the same round 1 exconjugant pair will be completely homozygous. In this paper, 1–3 round 2 pairs were isolated from each round 1 pair of exconjugants.

Genetic nomenclature: Because round 1 of genomic exclusion can produce cells in which the micronuclear genotype differs from that reflected by the phenotype of the macronucleus, a standard system of nomenclature has been used in this paper. For example, the symbolism [+/−, (H−)] indicates that the micronucleus is heterozygous for wild-type and mutant alleles and that the macronuclear phenotype is H-negative. The symbolism [+/+, (H+)] indicates that the micronucleus is homozygous for the wild-type allele and the macronucleus is H-positive.

Mutagenesis: Mutagenesis and mutant selection were performed as previously described (DOERDER, BERKOWITZ and SKALICAN-CROWE 1985). Briefly, inbred strain A was exposed to nitrosoguanidine and crossed to CU399 in BP. True exconjugants were selected by their resistance to cycloheximide (25 μg/ml). The heterozygous progeny were screened for H− cells 10–15 fissions after conjugation; such H− cells arise through the random assortment in the macronucleus of dominant and recessive alleles. Cycloheximide resistant H− cells of nonparental mating type were retained as presumptive heterozygous mutants. H− mutants B2092, B2101, B2103 and B2107 represent four independently isolated and inbred mutations. Following the convention that permits distinction between the micronuclear genotype and the macronuclear phenotype (in parentheses), all four mutants may be described as [−/−, (H−)].

Immunological methods: Antisera were prepared against purified or partially purified antigen in New Zealand female rabbits as previously described (DOERDER and BERKOWITZ 1986). Immobilization assays were performed by mixing living cells with diluted antisera and scoring one hour later for the cessation of swimming (DOERDER 1981). Antisera dilutions were chosen so that control cells were completely immobilized after 1 hr. Indirect immunofluorescence assays were performed on living cells as previously described (DOERDER, BERKOWITZ and SKALICAN-CROWE 1985).

RESULTS

Mutant isolation and inbreeding: Mutants B2092, B2101, B2103 and B2107 were independently isolated as unaffected by either anti-H1 (directed at the product of the *SerH1* allele) or anti-H3 (directed against the product of the *SerH3* allele). As a consequence of the mutant selection protocol in which a mutagenized *SerH1* homozygote was crossed to a *SerH3* homozygote (MATERIALS AND METHODS), these H-negative (H−) mutants were expected to be heterozygous either for mutation at *SerH* or for mutation at a gene affecting expression of *SerH* alleles (DOERDER, BERKOWITZ and SKALICAN-CROWE 1985). Because genomic exclusion crosses with the original isolates yielded too few genetically viable progeny, the original isolates were crossed to strain B and the (rare) H− progeny were then crossed to A*III. Since in these crosses *SerH1* segregated from *SerH3* (data not shown; see below), mutation at *SerH1* was ruled out as the cause of the H− phenotype.

This second series of genomic exclusion crosses failed to yield the expected 1:1 Mendelian ratios of H− and H+ (H-positive) phenotypes. In addition, rare round 2 pairs were unexpectedly sectored with respect to phenotype. For example, when crossed to A*III, a B2103 heterozygote yielded 12 H+ sets of round 2 progeny (three round 2 pairs from each round 1 pair) and one sectored set consisting of 2 H− pairs and 1 H+ pair. The ratio 12:1 is statistically significant (binomial probability of 0.0017), and sectoring is inconsistent with genomic exclusion cytogenetics. Similar results were obtained with the other three mutants. Crosses among the round 2 H− homozygotes also yielded unanticipated results. Such crosses (usually) yielded H− progeny, but rare H+ progeny were also observed (*e.g.*, control crosses in Table 5; see also line 1, Table 7).

The combined results suggested both cytoplasmic (non-Mendelian) and nuclear (Mendelian) roles in the expression of *SerH*. Based on mutant d48 of *P. tetraurelia*, the cytoplasm was postulated to affect *SerH* during macronuclear development. Specifically, macronuclei which develop in mutant cytoplasm would fail to subsequently express *SerH* genes. Since both fluid cytoplasm (MCDONALD 1966) and H antigen (DOERDER 1981) are exchanged in both normal conjugation and genomic exclusion (rounds 1 and 2), "dominance" of exchanged wild-type cytoplasm would explain both the absence of expected Mendelian ratios and the deviation from strictly cytoplasmic inheritance as in Paramecium. The postulated nuclear role is to establish and perpetuate the mutant cytoplasmic state. The nuclear mutation would have to be present together with mutant cytoplasm in order for *SerH* expression to be affected. The results presented below support these hypotheses.

It is important to emphasize for the following discussion that all four mutants appear to be homozygous (−/−) for the mutation responsible for the H− phenotype. Such homozygosity is a consequence of genomic exclusion and is consistent with observed homozygosity for *SerH3* (B2092 and B2103), *SerH1* (B2101) and mating type (unlinked to *SerH*; data not shown). For cytogenetic reasons unrelated to its H− phenotype, B2107 is heterozygous for *SerH1* and *SerH3*.

The cytoplasmic role: To test the hypothesis of a cytoplasmic role in *SerH* expression, two genetically identical −/− clones (derived from different round 2 pairs of the same round 1 pair) of B2101 were again crossed to A*III in round 1 of genomic exclusion (Figure 1). As described in MATERIALS AND METHODS, this cross was expected to yield exconjugant clones of identical micronuclear genotype (−/−) but of different phenotype (H+ or H−). This manipulation of micronuclei and macronuclei, together with cytoplasmic exchange during mating was used in direct tests of nucleo-cytoplasmic interaction on both mature

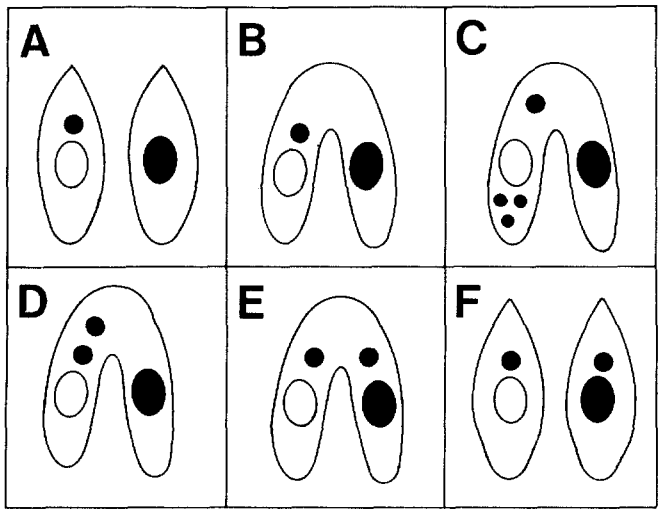

FIGURE 1.—Cytogenetics of round 1 of genomic exclusion. A diploid, H− cell (A, left, clear macronucleus) and a H+ A*III cell (A, right, filled macronucleus) are mated (B). The diploid micronucleus undergoes meiosis (C) to produce four haploid nuclei, three of which disintegrate. After endoreplication, the remaining micronucleus undergoes mitosis (D) to produce two identical haploid nuclei, one of which is transferred to the A*III cell (E). Diploidy, and hence homozygosis, is restored by further endoreplication (DOERDER and SHABATURA 1980). Exconjugants (F) are identical with respect to micronuclear genes, but since macronuclei are retained, they are different with respect to phenotype. Because the "*" cell possesses a functional micronucleus, subsequent mating (round 2) is cytogenetically normal and results in macronuclear development.

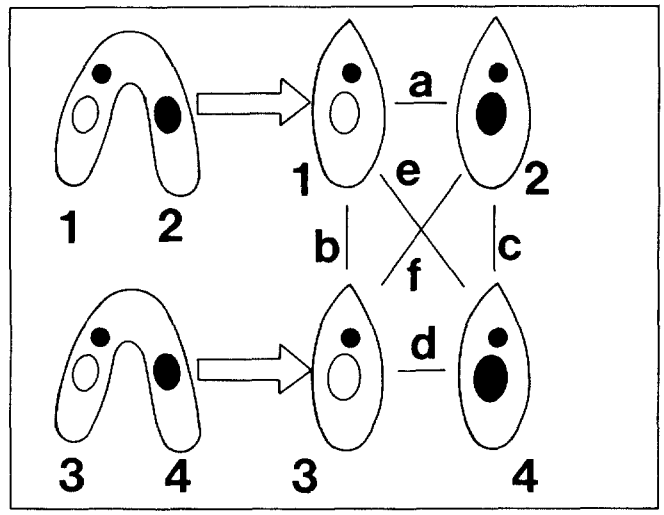

FIGURE 2.—Crosses to demonstrate effect of cytoplasm on *SerH* expression in mutant homozygotes. Genetically identical H− cells [−/−, (H−)] of two different mating types (1 and 3) were crossed to A*III (2 and 4) or to A*III (2) and B*VII (4) to produce round 1 H+ exconjugants with mutant micronuclei [−/−, (H+)]. Clones derived from cells 1, 2, 3 and 4 were then crossed in all possible combinations (a–f).

TABLE 1

H antigen expression in H− and H+ homozygotes of mutant B2101

	[−/−, (H−)] × [−/−, (H+)]				
			No. of progeny		
Cross (Figure 2)	Phenotypes crossed	H:	+	−	+/−[a]
a	H: − × +		32	0	0
c					
d	− × +		47	0	0
e	− × +		30	0	1
f	− × +		48	0	0
b	− × −		0	52	0
2101 × 2101	− × −		0	83	0

All crosses involved cells presumed to be homozygous (−/−) for the mutation resulting in mutant cytoplasm. See text.

[a] Mixture of H+ and H− cells in synclone.

(round 1) and developing (round 2) macronuclei. For example, round 1 exconjugants were unchanged with respect to H− and H+ phenotypes when tested both at 10–15 fissions and at 50–100 fissions after genomic exclusion. Because cytoplasm is exchanged at round 1, this result shows that wild-type cytoplasm has no affect on mature mutant macronuclei.

The round 1 exconjugant clones were then crossed in the combinations shown in Figure 2. The results are presented in Table 1. In each cross with at least one H+ conjugant the progeny were also H+ (crosses a, d, e, and f). However, in crosses between H− cells (cross b), the progeny were phenotypically H−; H− progeny were also observed in the control cross between two B2101 mutants. Since the micronuclei in these crosses were genetically identical (−/−), these results clearly show that H expression in this genotype is determined solely by the cytoplasm in which macronuclei develop. Macronuclei that develop in wild-type cytoplasm express H, but macronuclei that develop in mutant cytoplasm do not. Moreover, since the H− conjugants in H− × H− crosses were exposed to wild-type cytoplasm during the preceding round 1, the appearance of H− progeny shows that exchanged cytoplasm did not affect the mutant cytoplasmic state.

The hypothesis was further tested for all four mutants in crosses among exconjugants derived both from A*III and B*VII. B*VII was chosen so that two H+ cells (cross c) could be crossed. The results shown in Table 2 are virtually identical to those shown in Table 1. Crosses with one or two H+ conjugants yielded H+ progeny, but crosses between H− cells yielded H− progeny. Although this pattern was also evident in crosses involving mutant B2107, the crosses with H+ cells tended to yield more H− progeny than did the other mutants. Because in each set of crosses the micronuclei were of identical −/− genotype, the results demonstrate unequivocally the influence of cytoplasm on macronuclear differentiation.

In the crosses shown in Tables 1 and 2, synclonal uniformity of phenotype was observed in >97% of exconjugant pairs. That is, in most instances, all cells

TABLE 2

H expression in crosses between H− and H+ homozygotes of mutants B2092, B2101, B2103 and B2107

Cross (Figure 2)	[−/−, (H−)] × [−/−, (H+)] No. of progeny with phenotype											
	2092			2101			2103			2107		
H:	+	−	+/−	+	−	+/−	+	−	+/−	+	−	+/−
H− × H+												
a	15	0	1	16	0	0	15	0	0			
d	16	0	0	16	0	0	15	1	0			
e	12	0	0				16	0	0	5	4	0
										1	15	0[a]
f	13	1	2	16	0	0	14	0	0	11	5	0
H+ × H+												
c	14	0	0	16	0	0	12	3	1	1	15	0
										13	0	1[a]
H− × H−												
b	0	32	0	3	6	5	0	48	0	0	47	0
						0[a]						

All crosses involved cells presumed to be homozygous (−/−) for the mutation resulting in mutant cytoplasm. See text.
[a] Repeat cross with different subclones.

descended from a pair were either immobilized by anti-H antisera or were unaffected by it; mixed reactions were observed in fewer than 2.5% of pairs (Table 7). Analysis of mating type distributions (not shown) and growth rates (not shown) provided no evidence for preferential death of either exconjugant or macronuclear lineages. Thus, in most instances, all four developing macronuclei (karyonides) of a conjugating pair were similarly affected by cytoplasm. This conclusion is supported by additional data summarized in Table 7.

The genic component: Although the results presented above clearly demonstrate the importance of the cytoplasm in the determination of *SerH* gene expression, the repeated (but irregular) appearance of exceptional H− and H+ cells in each phenotypic category of crosses suggested the existence of an underlying genetic basis. Specifically, it was hypothesized that the mutant cytoplasmic state could arise and be perpetuated only in the genotypes −/− and +/−.

To demonstrate the presence of a Mendelian gene, mutants were crossed to strains B (*SerH3*) and B3 (*SerH4*). The F_1's were then crossed in all three phenotypic combinations to yield the F_2. Although F_2 genotypic ratios were expected to be the typical 1:2:1 (Table 3), the phenotypic ratios were expected to differ according to the phenotypes crossed (Table 3). Specifically, crosses with an H+ conjugant were expected to yield largely H+ progeny, but crosses between two H− cells were expected to yield 25% H+ progeny.

These genetic expectations were fulfilled for mutants B2092, B2101 and B2103 (Table 4; due to poor viability, B2107 was not tested). H+ × H+ and H+ × H− crosses yielded H+ progeny (Table 4; summarized in line 6, Table 7), and, significantly, the H− × H−

TABLE 3

F_2 expectations from crosses between H− mutants and wild-type strain B; [−/−, (H−)] × [+/+, (H+)]; see text

F_2 Genotypes	Phenotypes crossed		
	H+ × H+	H+ × H−	H− × H−
1 +/+	H+	H+	H+
2 +/−	H+	H+	H−
1 −/−	H+	H+	H−

crosses yielded, in most instances, a 1:3 ratio of H+ to H− phenotypes (Table 4). In crosses in which this ratio was not obtained, an excess of 25% of the progeny were H+ (these exceptional crosses are discussed in greater detail below in the section on phenotypic stability). In none of the crosses were H+ progeny absent (*cf.* Tables 1, 2 and 5). An additional 10 crosses between F_1's derived from different mutants, *e.g.*, (B2092 × B) F_1 × (B2101 × B) F_1, also yielded 1:3 ratios (pooled results, 78 H+: 229 H−); one exceptional cross, not included in this total, yielded 21 H+: 7 H−.

Test crosses between H− conjugants confirmed the existence of a Mendelian mutation. One member of each cross was a heterozygous F_1 [+/−, (H−)] as constructed above. The other member was the H− exconjugant from a round 1 genomic exclusion cross between a different H− F_1 and A*III; half were expected to be [−/−, (H−)] and half [+/+, (H−)]. The cross +/+ × +/− was expected to yield a 1:1 ratio of H+ to H− phenotypes, whereas the cross −/− × +/− was expected to yield all H− phenotypes. The results (Table 4) show that these expectations were fulfilled.

The F_2 and test cross results demonstrate conclusively the presence of a nuclear mutation responsible

TABLE 4

Segregation analysis in F_2 and backcrosses to demonstrate Mendelian basis for cytoplasmic effect in mutants B2092, B2101 and B2103

	Phenotypes observed					
	BF_2		$B3F_2$		Backcross	
H:	+	−	+	−	+	−
B2092	28	33	*6*	*19*	*14*	*18*
	4	*28*	*9*	*23*		
	10	*22*	*8*	*21*		
Totals	42	83	23	63		
B2101	*3*	*12*	7	4	*6*	*5*
	5	*17*	10	10	*8*	*12*
			7	*13*	0	32
Totals	8	29	24	27		
B2103	*6*	*26*	*9*	*23*	0	32
	7	*25*			15	16
Totals	13	51	9	23	19	13

Ratios in *italics* conform to 1:3 (F_2) or 1:1 (backcross).

TABLE 5

Complementation analysis among mutants B2092, B2101, B2103 and B2107

	No. of progeny											
	2092			2101			2103			2107		
No. of progeny H:	+	−	+/−	+	−	+/−	+	−	+/−	+	−	+/−
2092	5	74	8	3	56	0	4	63	0	15	14	0
2101	0	16	0	0	64	0	16	60	0	10	21	0
2103	0	48	0	0	157	0	0	62	2	5	9	0
2107	0	11	0	4	12	0	2	4	0	1	11	0

Diagonal: control crosses within the mutant strain.
Above diagonal: crosses between mutant strains.
Below diagonal: crosses between mutants after round 1 of genomic exclusion. See text.

for the establishment and maintenance of mutant cytoplasm. They also show that the homozygous wild-type genotype is sufficient to rescue *SerH* expression.

Complementation and allelism: Complementation crosses among mutants B2092, B2101, B2103 and B2107 (Table 5) yielded mostly H− progeny. Although crosses with B2107 yielded more H+ progeny than did crosses among the other mutants, these results do not necessarily imply two complementation groups. B2107 did not fully complement, and, conceivably, mutations at several different loci could result in the same cytoplasmic defect and therefore not be distinguished. Special crosses were therefore performed to determine allelism. In these crosses, H− heterozygotes (*e.g.*, F_1 from the cross B2092 × B2101) were crossed to the [+/+, (H−)] strains described in the previous section. For allelic mutations, the resulting heterozygous (+/−) progeny should be H− in phenotype. However, for mutations at independently segregating loci, 25% of the progeny should be H+ [+/+, +/+ (H+)]. Among crosses involving B2092, B2101 and B2103 in all possible combinations, no H+ progeny were observed (N = 227). The mutations in these strains must therefore be allelic or very closely linked. For crosses involving mutant B2107, the results were inconclusive. Specifically, although H+ progeny were observed, their frequencies were variable, ranging from 13% (N = 30) to 79% (N = 29); total progeny consisted of 140 H+ and 122 H− synclones distributed among 9 crosses. Because similar frequencies of H+ progeny were observed in homozygous complementation crosses (Table 5), these results do not necessarily indicate noncomplementation. As a further difficulty, we noted that in many of the crosses synclones scored as H+ consisted of both H+ and H− progeny; such mixed reactions are in contrast to the relative lack of mixed reactions observed in other crosses (*e.g.*, see Table 7). Because B2107 differs in serotype from the other mutants (see below), it is likely that it represents a mutation at a different locus, but this conclusion is neither proved nor disproved by the genetic information.

As mentioned previously, the recovery of H3 in mutants B2092 and B2103 during inbreeding suggested that these mutations segregate independently of genes at *SerH*. This conclusion was verified by the ratios of H1 and H3 in the H+ progeny of the above crosses (data not shown).

Relationship to Mendelian mutations: The expectations and results of crosses of B2092, B2101, B2103 and B2107 to members of each category of Mendelian mutation affecting H expression are shown in Table 6. Control crosses were also performed between the mutants and wild-type strains B and B3. With the exception of progeny in the cross to RseD, all progeny were H+, H− or consisted of a mixture of H+/H− cells. Differences in the proportions of these phenotypes suggest differences among the mutants, but no systematic analysis has been attempted. Significantly, the "dominant" effect of wild-type cytoplasm observed in Table 1 and Table 2 was not observed.

Phenotypic stability, exceptional progeny and mixed H+/H− phenotypes: All Mendelian genes so far identified for *T. thermophila* undergo phenotypic assortment when present in heterozygous macronuclei. In this process a macronucleus that contains multiple copies of each allele gives rise through repeated division to macronuclei that contain only one allele or the other. In this way, genetically identical clones become phenotypically different. Although the molecular nature of the assorting unit is unknown, it is almost certainly larger than a single genetic locus (DOERDER, LIEF and DEBAULT 1977). For most genes, assortment is usually detected by the appearance of the recessive phenotype during asexual reproduction; test crosses show that the micronuclear genotype is unaffected.

TABLE 6

Immobilization antigen expression in progeny of crosses between non-Mendelian mutants and wild-type and Mendelian mutants

Mutant expected[a]	Phenotype when crossed to: B H	H1-1 H	H1-2 H	rseA1 H	rseB H	rseC2 H	RseD2 I
2092	+/−	− ≫ +	−	− > +	+/−	+/−	I
2101	+	+	+	+ ≫ −	+ ≫ −	−	I
2103	+/−	− ≫ +	− ≫ +	+ > −	−	+ > −	I ≫ −
2107	+/−	+/−	+/−	−	+ ≫> −	− > +	I

Key: − = H-negative; + = H-positive, / = equal numbers of + and − clones, or mixed reactions, > = unequal numbers of + and − clones. N = 16–32 conjugants in each cross.

[a] Expected is based upon recessive and dominant nature of Mendelian mutant.

Although the genetic complexity of the present mutants precluded large scale assortment experiments, three different types of assortment questions were asked. The first concerns the appearance of exceptional progeny in −/− × −/− crosses. Specifically, since H+ progeny occasionally appeared in crosses within a mutant line (*e.g.*, B2101 × B2101), the question arose as to whether the macronucleus consists entirely of H+ assorting units or whether it consists a minority of H− units that are masked by a majority of H+ units. Relevant information was obtained from the comparison of H expression at 10–15 fissions after conjugation to H expression in subclones at 40–60 fissions (sexual maturity). In all instances (N > 95 subclones), no H− assortees were found. Similarly, among N > 150 H− subclones from these crosses, no H+ assortees were found, nor were H+ cells found in any H− mutant in 10–16 months of culture representing over 400 fissions. Thus, in these instances (mutants B2092, B2101 and B2103), macronuclei appear to consist entirely of either H+ or H− units.

The second question concerns the origin of exceptional exconjugant progeny consisting of mixtures of H+ and H− cells. Such progeny were observed in crosses both between the mutants and previously described regulatory mutants (Table 6) and in the progeny of B2107 allelism tests. Such mixtures could be the result either of phenotypic differences (H+ *vs.* H−) among synclonal karyonides or of assortment of a single macronucleus containing both H− and H+ units. If the former, then the H+ and H− phenotypes should be stable during asexual reproduction. If the latter, a change from H+ to H− or H− to H+ should be observed. The results of one small scale experiment and an examination of H expression at sexual maturity suggest that both explanations apply. In the small scale experiment involving 96 clones monitored for 70 fissions beginning at 15 fissions after conjugation, 35 were stably H−, 68 were stably H+, and 3 assorted from H− to H+ or vice versa. Since assortment alone is insufficient to account for the ratio 35:68 at 15 fissions, these results support both phenotypic differences between karyonides and phenotypic assortment. Assortment was also observed in the progeny of crosses shown in Table 6 transferred to sexual maturity (data not shown).

The third question concerns the assortment of the underlying Mendelian mutation. This question distinguishes between the assortment of H+ and H− units and the assortment of the (micronuclearly) unlinked mutations. It is possible, for example, that assortment in a heterozygous macronucleus consisting entirely of H− units would produce two classes of subclones, one with wild-type cytoplasm and the other with mutant cytoplasm. Such assortees might be indicated by the production of exceptional progeny at conjugation. Evidence for assortees with wild-type cytoplasm is provided by the exceptional F_2 results shown in Table 4. In three out of the 14 crosses, H+ progeny were found in excess of the 1:3 expectation. However, in another set of crosses in which 4 H− and 3 H+ heterozygotes were backcrossed to the H− parent, no exceptions were found; the H− × H− crosses yielded all H− progeny (N = 105), and the H+ × H− crosses yielded mostly H+ progeny (36 H+, 4 H− and 4 mixed). Crosses were also performed with heterozygous H+ subclones to look for assortees that possessed cytoplasm that behaved as mutant cytoplasm. Such clones were indicated in 2 of 13 F_2 crosses (H+ × H− crosses yielding an excess of H− progeny) and in 3 of 9 backcrosses. These clones indicate that even though they have a common genetic origin, the H− phenotype and mutant cytoplasm affecting *SerH* expression are distinct phenomena.

Genetic stability of H− phenotype in −/− genotypes: No H− homozygote that yielded all H− progeny in H− × H− crosses has been observed to produce H+ progeny at subsequent matings. However, like the heterozygotes described in the previous section, H− homozygotes have been observed to yield H+ progeny. For example, in 1st generation H− × H− crosses, only 2% of progeny were H+ (pooled results, N > 300), whereas third generation progeny totaled 30% H+ (N = 114). The frequency of H+ progeny ranged from 82% (N = 11) in a B2101 derivative to 38%

TABLE 7

Summary of crosses involving B2092, B2101 and B2103 according to genotype and phenotype

Genotypes crossed	H:	Phenotypes observed (%)											
		H+ × H+				H+ × H−				H− × H−			
		+	−	+/−		+	−	+/−		+	−	+/−	
1 −/− × −/−		98.8	0.9	0.3	(332)	98.2	0.6	1.2	(327)	3.1	95.6	1.3	(1143)
2 +/+ × −/−						62.7	37.2	0	(276)	6.9	93.1	0	(102)
3 −/− × +/−						76.2	23.8	0	(164)	1.0	99.0	0	(192)
4 +/+ × +/−		100.0	0	0	(68)	56.4	43.6	0	(101)	52.9	47.1	0	(136)
5 +/− × +/−		100.0	0	0	(406)	76.7	22.3	0	(193)	29.9	70.1	0	(730)
6 +/+ × +/+						100.0	0	0	(23)	100.0	0	0	(88)

Sample size in parentheses. Exceptional progeny mentioned in text are not included in this table.

(N = 16) and 0% (N = 16) in two B2103 derivatives. Despite this increase in H+ progeny, no mutant line has been lost through inbreeding, and in general, [−/−, (H−)] clones breed true when crossed to each other. Curiously, the exceptional H+ progeny from such crosses continue to produce H+ progeny at subsequent crosses (N = 211). The significance of such genetic reversion is analyzed in the DISCUSSION.

Effect of temperature: The stability of the inheritance of the H− phenotype was investigated by using temperature perturbation. Neither cold shock (15°) nor heat shock (40°) induced H expression when homozygous cells were returned to 28°, nor did the temperature of macronuclear development influence the H− phenotype. Progeny from crosses B2092 × B2092 and B2101 × B2101 in which conjugants were placed at 20°, 28° and 34° were H− (N = 29–32 in each cross). The time of refeeding prior to pair isolation also had no effect (data not shown).

Antigens expressed in H− cells: In each of the previously described Mendelian mutants in which expression of H antigen is affected, one or more alternative antigens are expressed on the cell surface (DOERDER 1973, 1979; unpublished data). Mutants B2092, B2101, B2103 and B2107 were considered interesting because they were not immobilized by antisera directed against any of the known antigens of *T. thermophila*. The results of studies to be published elsewhere show that B2092, B2101 and B2103 mutants express a hitherto unknown antigen that is different from the one expressed by B2107.

Only the expression of *SerH* genes is affected. All four mutants express the expected alternative antigens when grown under conditions favorable for their expression (see the Introduction).

DISCUSSION

The present results, as summarized in Table 7, show that the cytoplasm plays a crucial role in the expression of genes at the *SerH* locus. Specifically, macronuclei which develop in mutant cytoplasm subsequently can not express H antigen, whereas those that develop in wild-type cytoplasm, as exchanged at conjugation, can express H. The mutant cytoplasmic state is distinct from the H− phenotype and is the result of mutation in at least one nuclear gene.

The genetic defect in the present mutants is most likely the absence of a cytoplasmic factor controlling the developmental processing of *SerH* genes. This hypothesis is consistent both with locus-specific nature of the mutant phenotype and with the lack of effect of exchanged wild-type cytoplasm on mature macronuclei, and is supported by the similarity to mutant d48 of *P. tetraurelia*. In this mutant, the A antigen gene is eliminated from the macronucleus during macronuclear development (EPSTEIN and FORNEY 1984; HARUMOTO 1986). Direct tests of the hypothesis that *SerH* genes are incorrectly processed are now possible with the finding that cDNA clone PC6 (MARTINDALE and BRUNS 1983) corresponds to the *SerH3* allele (R. L. HALLBERG and F. P. DOERDER, unpublished data). Since recent work has shown that the micronuclear and macronuclear versions of PC6 are different (MARTINDALE, MARTINDALE and BRUNS 1986), it is likely that processing of *SerH* is required for its expression. If the processing of *SerH* genes is indeed abnormal, the present mutants could provide a means for the identification of the molecular components of the processing system.

The nature of the cytoplasmic component(s) responsible for normal developmental expression of *SerH* is unknown. Cytoplasm exchanged at round 1 does not cause mature mutant macronuclei to express H, nor does it cause the mutant macronuclei to promote normal macronuclear development at round 2. The exchanged component therefore neither "resets" the old macronucleus nor persists during the interval between rounds 1 and 2. By contrast, HARUMOTO (1986) has shown that microinjection into d48 cells of either wild-type cytoplasm or nucleoplasm does rescue A antigen expression at the next autogamy (self-fertilization) 15 fissions after injection. Microinjection experiments with the present mutants may be useful in determining whether synthesis of the cytoplasmic

component is limited to conjugation. Microinjection also may be useful both in attempts to purify the product(s) from wild-type cytoplasm and in determining whether that of Tetrahymena are related to that of Paramecium.

The Mendelian mutations resulting in mutant cytoplasm segregate from *SerH*, but the number of loci identified by these mutations is unknown. Subtle genetic and serotype differences suggest that present mutants identify at least two loci, but this conclusion is by no means certain. The data also raise the possibility that the present mutants did not arise through application of mutagen, but instead arose in an inherently unstable, perhaps bistable, gene. Such a gene is suggested in the context of two observations. The first is the apparently complete genetic reversion to homozygous wild-type (+/+) or H+ progeny in [−/−, (H−)] × [−/−, (H−)] and [−/−, (H+)] × [−/−, (H+)] crosses (Table 7, line 1). Such reversion is difficult to account for by simple reversion of a point mutation both because point mutations are unlikely with nitrosoguanidine and because all four independently isolated mutants would have to contain similar point mutations. Moreover, in the former crosses the reversion frequency of about 4% is too high to be due to reverse mutation, and in the latter crosses, where nearly 100% of progeny are H+, the cytoplasm clearly plays a crucial role. The second observation is that both NANNEY (1963) and, more recently in our laboratory, D. SMITH (personal communication) have described spontaneous H− mutants that match the description of those reported here. SMITH's mutant is, in fact, genetically similar and antigenically identical to B2092, B2101 and B2103. In addition, in past mutagenesis runs, up to 80% of H− mutants recovered failed to segregate H− progeny in further crosses (F. P. DOERDER, unpublished data). This high frequency of "false negatives" can be explained if the mutants are of the type described here. Indeed, the only reason the present mutants were pursued in greater detail was that we became curious as to why so many putative H− mutants failed to transmit the H− phenotype to sexual progeny.

The genetic bistability of the sort suggested here could be due to many causes ranging from a regulatory protein to a transposable genetic element. Because reversion affects both the micronucleus (which transmits wild-type alleles at subsequent crosses) and the macronucleus (which shows karyonidal uniformity and does not assort H− subclones), the peculiarities of conjugal cytogenetics imply that such uniform reversion occurs *before* the two post-fertilization mitotic divisions which give rise to new macronuclei and new micronuclei. Such reversions could occur in the haploid gametic nuclei, or in the diploid fertilization nuclei. Precedent for this unconventional idea exists in the work of BRYGOO (1977) and BRYGOO *et al.* (1980). Using amicronucleate Paramecium cells as recipients of micronuclei from O and E cells, BRYGOO showed that both cytoplasm and mating type are predetermined by micronuclear nucleo-cytoplasmic interaction prior to fertilization. The molecular nature of such predetermination is unknown, but could involve specific regulatory protein molecules or a transposable genetic element. Although transposable elements have not been positively identified in ciliates, circumstantial evidence for their existence has been reported (CHERRY and BLACKBURN 1986; HERRICK *et al.* 1986).

The compensatory effects both of exchanged wild-type cytoplasm in −/− genotypes and of the nuclear genotype +/+ in mutant cytoplasm show that either is sufficient to rescue *SerH* expression. The ability of both cytoplasm and genotype to rescue has genetic counterparts, for example, in the ability of injected cytoplasm and zygotic nuclei to rescue maternal effect mutants of *Drosophila melanogaster* (BAKER 1973). In many instances, however, maternal effect genes are rescued only by injection into zygotes of wild-type cytoplasm or RNA (ANDERSON and NUSSLEIN-VOLHARD 1984). Both cytoplasmic and zygotic rescue can be explained by a simple model in which a missing or nonfunctional gene product is provided either by injected cytoplasm or by the appropriate nuclear genotype.

A model to explain the nucleo-cytoplasmic interactions exhibited by the present mutants must necessarily be more complex because it must account not only for the effect on *SerH* but also the properties of mutant cytoplasm. In addition, a successful model must account for the high rate of reversion and the relative lack of genetic rescue in heterozygotes (*cf.* lines 1 and 2 of Table 7). Among the possibilities that we have considered are: (1) that the mutational lesion is in a gene which normally produces a cytoplasmic product required for the processing of both itself and *SerH*; (2) that the mutational lesion is not in a gene for a processing factor itself but is instead in a gene regulating such a gene; (3) that the processing factor is a multimer which is functional when homomeric for wild-type product but is nonfunctional when one or more subunits are mutant; and (4) that the processing factor is an RNA molecule synthesized in the developing macronucleus under control of a cytoplasmic factor. We have also considered combinations of these models. Unfortunately, no consistent model has emerged, and we therefore conclude that more information, particularly molecular information, is necessary.

The cytoplasmic aspects of the present mutants are virtually identical to several instances of non-Mendelian inheritance of phenotypic characters in Paramecium. The basic similarity to mutant d48 (EPSTEIN

and FORNEY 1984), mating type and trichocyst discharge (SONNEBORN 1977) suggest that all such instances in which the cytoplasm exerts locus-specific control over macronuclear differentiation have a homologous genetic basis. That is, in each instance, a gene product present in the cytoplasm is required for a critical step in the processing of the target locus during macronuclear development. As noted in the introduction, cytoplasmic control over macronuclear development commences at the second post-fertilization nuclear division when cytoplasmic location determines whether macronuclear development will proceed. Since transcription probably does not begin immediately at the beginning of macronuclear development (for references, see MAYO and ORIAS 1986), further cytoplasmic regulation of early development is perhaps to be expected.

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