# ISOLATION AND GENETIC ANALYSIS OF MUTATIONS AT THE *SERH* IMMOBILIZATION ANTIGEN LOCUS OF *TETRAHYMENA THERMOPHILA*

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ABSTRACT

Multiple alleles at the *SerH* locus specify the major cell surface protein (immobilization antigen) of the ciliate *Tetrahymena thermophila*. Following mutagenesis of *SerH1* homozygotes, two mutations, *H1-1* and *H1-2*, were recovered in heterozygous form. Mutant homozygotes do not express H1 antigen, nor is H1 expressed in $F_1$ progeny of crosses to wild-type strains homozygous for *SerH2* or *SerH3*. *H1-1* and *H1-2* segregate without recombination from these wild-type alleles in expected $F_2$ and testcross Mendelian ratios. *H1-1* and *H1-2* do, however, complement each other to express H1 antigen. Experiments suggest this complementation is due neither to recombination during macronuclear development nor to interallelic complementation of defective *SerH1* gene products. These results suggest that *SerH1* is intact in one mutant, and possibly both, although no such allele has been segregated in testcross progeny ($N = 205$). The hypothesis is presented that complementation between *H1-1* and *H1-2* is due to interaction between allele-specific regulators closely linked to the *SerH1* gene.

ALTERNATIVE forms of the major cell surface protein, the immobilization (i-) antigen, of ciliate protists such as Paramecium and Tetrahymena are coded by genes at unlinked loci (SONNEBORN 1974a,b). In both genera, only the genes at one locus are normally expressed under a given set of environmental conditions; genes at other loci are silent. Such mutual exclusion is a function of the compound macronucleus and appears unlikely to involve rearrangement of genetic material as in the Trypanosomes (FORNEY *et al.* 1983). In *T. thermophila* the H antigens specified by multiple alleles at the *SerH* locus are expressed from 20–36° and are one of a series of at least five differentially expressed i-antigens. The four H antigens, H1, H2, H3 and H4, are immunologically distinct monomeric proteins with molecular weights of 44,000 to 52,000 (F. P. DOERDER and M. S. BERKOWITZ, unpublished results). Mutational analysis has shown that *SerH* genes are regulated by at least four genes which do not themselves specify i-antigen (DOERDER 1973, 1979). However, the exact role of these genes is not known. To understand more fully the genetic control of *SerH* expression, we have isolated mutations that map to the *SerH* locus. This paper describes the genetic characterization of these mutations.

Although *SerH* heterozygotes normally express both alleles, a special kind of serial dominance is observed when the presence of antigen is assayed by immobilization (NANNEY and DUBERT 1960; NANNEY *et al.* 1963). That is, in certain allele combinations, cells are immobilized by antisera to only one specificity. The alternative antigen is detectable either through immobilization at higher concentrations of antisera or by immunofluorescence (DOERDER 1981). The dominance relationships *SerH4* > *SerH1* > *SerH3* = *SerH2* do not yet have a completely satisfactory explanation but appear to be the consequence of unequal allele replication during macronuclear development (DOERDER, LIEF and DEBAULT 1977). Thus, the allele ratio in a *SerH1/SerH3* macronucleus at the end of its development at conjugation is sufficiently in favor of *SerH1* so that cells are immobilized by anti-H1 antisera but not anti-H3 antisera. Unequal allele replication is directly demonstrated by macronuclear assortment. In macronuclear assortment independent genetic units of the macronucleus are randomly distributed at division in such a way that macronuclei come to contain only one type of unit. The proportion of assortees with either type of unit is a function of the allele ratio at the end of macronuclear development (SCHENSTED 1958; DOERDER, LIEF and DEBAULT 1977; DOERDER and DIBLASI 1984). In the *SerH1/SerH3* heterozygote, macronuclei assort to contain either *SerH1* or *SerH3*, with most assortees containing only *SerH1*.

We have taken advantage of the serial dominance at *SerH* to isolate mutations of the *SerH1* allele. Two conceptually similar protocols which differ in detail were used. We reasoned that, if mutation does not alter *SerH* "dominance," then the greater dosage of *SerH1* mutant alleles in a macronucleus should result in an H-negative phenotype that could be isolated by screening mutagenized exconjugant *SerH1/SerH3* cultures either with anti-H1 antiserum alone or with both anti-H1 and anti-H3 antisera. This paper describes the genetics of two mutations isolated in heterozygous form by application of this idea. Genetically, the mutations behave as alleles of *SerH1*, *i.e.*, they segregate from and fail to complement heterologous wild-type alleles to express wild-type H1 antigen. The mutations do, however, complement each other to express H1. This interesting result has important implications concerning both the regulation of H antigen expression and the genetic organization of the macronucleus.

## MATERIALS AND METHODS

*Strains*: Inbred strains A and B of *T. thermophila* were originally obtained from D. L. NANNEY (University of Illinois at Urbana-Champaign). Strain A is homozygous for *SerH1*, and strain B is homozygous for *SerH3*. A*III is a defective strain which when mated to a normal strain replaces its aneuploid micronucleus with a functional, diploid micronucleus from the normal strain (WEINDRUCH and DOERDER 1975). It is frequently used to induce, in two rounds of mating, complete homozygosis. In round 1, the micronucleus is replaced by a homozygous micronucleus derived from a normal, diploid partner; the old macronucleus is retained. The result is a heterokaryon with the phenotype of the old macronucleus. In round 2, the new micronucleus functions normally and the macronucleus is replaced. CU399 is a functional heterokaryon obtained from P. J. BRUNS (Cornell University, Ithaca, New York); cells are sensitive (through assortment) to cycloheximide, but the micronucleus is homozygous for genes that confer resistance to the drug to sexual progeny. CU399 is homozygous for *SerH3*.

*Culture conditions*: Cells were grown either in PPY (1% (w/v) Difco proteose peptone; 0.15% Difco yeast extract; 0.005 M $FeCl_3$; autoclaved) or in BP (a 6- to 12-hr culture of *Klebsiella pneumoniae* grown in PPY diluted 1:70 with glass-distilled water). Where necessary to minimize the risk of bacterial contamination PPY was supplemented with 1 mg/ml of penicillin and 1 mg/ml of streptomycin (PPYPS). To induce mating PPY cells in late log phase or early stationary phase cells grown in PPY were washed twice with Tris-7.4 buffer (0.01 M Tris-HCl, pH 7.4), suspended in Tris-7.4 and mixed 6–12 hr later; cells grown in BP were fed with fresh BP 24 hr prior to mixing. All cultures and crosses were kept at 28°.

*Antigen assay*: The immobilization assay was performed as previously described (DOERDER 1979). Briefly, living cells (grown in BP or grown in PPY and washed into Tris buffer) were mixed with a dilution of titered antiserum so that controls were completely immobilized after 1 hr. Positive immobilization reactions were scored according to the following scheme: ++, complete immobilization with agglutination; +, immobilization; s, significant slowing of swimming as compared to negative controls. Reactions were also scored as mixed, especially in heterozygotes; for example, ++/n indicated that freely swimming cells were observed in addition to agglutinated cells. Except as described in RESULTS, such reactions when observed among descendants of a pair of exconjugants were taken to indicate the presence of an antigen. A more sensitive immunofluorescence assay on living cells was also used (DOERDER 1981).

*Antisera*: Antisera were prepared in female New Zealand white rabbits. Antigen preparations consisted either of freeze-thaw extracts of whole cells (DOERDER 1973) or of partially purified i-antigen. Antiserum titers (the highest dilution causing immobilization of living cells after 1 hr) ranged from 1/20 to 1/80 for antisera to whole-cell extracts and from 1/80 to 1/320 for antisera to partially purified antigen.

*Mutagenesis*: Log phase cells of strain A in PPY were exposed to 10 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for 3-4 hr, washed three to four times with Tris buffer, permitted to recover in BP or PPY sufficient for one to two cell divisions and crossed with the appropriate strain (see below) in order to bring micronuclear mutations into expression in the macronucleus.

*Isolation of* H1-1: Strain A was mutagenized as described above, suspended in BP and crossed (cross B548) to an A*III/strain B heterokaryon. The heterokaryon was homozygous for *SerH3* derived from strain B but expressed *SerH1* in the macronucleus. At five to ten fissions after conjugation, multiple BP cultures were screened for mutations by adding anti-H1 antiserum to samples of each and, after 1 hr, removing with a micropipet the freely swimming cells. The putative mutant, B548-5-13, continued to be unaffected by anti-H1 and anti-H3 antisera and, therefore, was subjected to the genetic analyses described below. B548-5-13 was isolated in October 1978.

*Isolation of* H1-2: Strain A cells were mutagenized in PPY media, washed with sterile Tris buffer and divided into three aliquots of PPYPS sufficient to permit two to four fissions. After recovery, the mutagenized cells and CU399 cells were washed into sterile Tris buffer, mixed and allowed to mate (cross B1686). At the completion of conjugation PPYPS was added to permit growth, and after two to three fissions (to allow time for cycloheximide resistance to be expressed), cycloheximide was added (10 μg/ml) to select only the true exconjugant progeny. After 3 days each of the three cultures was washed with Tris buffer and subdivided among 40–80 tubes containing 5–8 ml of BP with cycloheximide. At ten to 15 fissions after conjugation, tube cultures were screened for mutants by adding immobilizing amounts of both anti-H1 and anti-H3 antisera to each tube. Since cells immobilized in culture tubes sink to the bottom, mutants were isolated by examining the top 1-2 ml of each tube under a dissecting microscope for freely swimming cells. Clone B1686a-1 containing *H1-2* was isolated in June 1984.

## RESULTS

H1-1 *and* H1-2 *were isolated in heterozygous form*: The inbreeding histories of strains containing *H1-1* and *H1-2* are shown in Table 1. *H1-1* as derived from clone B548-5-13 was inbred to homozygosity both through matings between complementary mating types descended from the same exconjugant pair (sib-mating) and through crosses to A*III. Due to poor genetic viability unrelated

TABLE 1

*Inbreeding of* SerH1-1 *and* SerH1-2 *mutations*

| *SerH1-1* | | | *SerH1-2* | | |
|---|---|---|---|---|---|
| Generation | Mating | Phenotype | Generation | Mating | Phenotype |
| 1 | B548-5-13 × strain B | H3 | 1 | B1686a-1 × A*III | H3:Ho |
| 2 | 1 × 1 | H3:Ho | 2 | 1 × 1 | Ho |
| 3 | 2 × 2 | Ho | 3 | 2 × 2 | Ho |
| 4 | 3 × strain B | H3 | | | |
| 5 | 4 × 4 | H3:Ho | | | |
| 6 | 5 × 5 | H3:Ho | | | |
| 7 | 6 × A*III | Ho | | | |
| 8 | 7 × 7 | Ho | | | |
| 9 | 8 × CU399 | H3 | | | |
| 10a | 9 × 9 | H3:Ho | | | |
| 10b | 9 × A*III | Ho | | | |
| 11a | 10a × 10a | H3:Ho | | | |
| 11b | 10b × 10b | Ho | | | |
| 12 | 11a × 11a | Ho | | | |

In most instances each inbreeding involved a mating between two mating types from the previous generation. In this and in subsequent tables, the Ho phenotype means that none of the tested cells were positive for H antigen by immobilization.

to the *H1-1* mutation, crosses were required both to strain B, to improve viability, and to CU399, to rescue the mutation from a nearly infertile clone. During the course of inbreeding, progeny that were thought to be homozygous for *H1-1* sometimes proved to be heterozygous (as evidenced by the reappearance of H3), thus necessitating yet another round of breeding. Presumably, as with *H1-2* described below, macronuclear allelic imbalance (useful in isolating mutations) resulted in the choice of H-negative heterozygotes instead of homozygotes. To recover *H1-2*, isolate B1686a-1 was crossed to A*III, and the homozygous, H-negative progeny were inbred by sib-mating. As a more recently isolated mutation with excellent genetic viability, *H1-2* does not have a long inbreeding history. Table 1 shows that for both mutants the H-negative phenotype breeds true and that no H1 is detected.

The genetic behavior of the mutations recovered from B548-5-13 and B1686a-1 as described below fails to resolve whether they should be assigned to the *SerH* locus or to one or more closely linked loci. When additional mutations become available, assignments to the proper locus may be possible. In the meantime, both for simplicity and to indicate their genetic origin, we have designated the mutations *H1-1* and *H1-2*, respectively. To avoid both cumbersome symbols and awkward phraseology, these symbols are also used here as if the genes they represent are alleles at *SerH*. The phenotype has been designated Ho (absence of H antigen), meaning that anti-H antisera fail to immobilize the cells.

H1-1 *segregates from and does not complement* SerH *alleles*: Data obtained during the course of inbreeding suggested that the gene identified by *H1-1* is

TABLE 2

H1-1 *segregation analysis in $F_2$ and testcross progeny*

| Genotypes crossed | Cross | No. observed | | *G* |
|---|---|---|---|---|
| $F_2$'s (expected: 3 H:1 Ho) | | H1 | Ho | |
| *H1-1/SerH1* × *H1-1/SerH1* | 790 | 30 | 9 | 0.08 |
| | | H2 | Ho | |
| *H1-1/SerH2* × *H1-1/SerH2* | 949 | 19 | 9 | 0.72 |
| | 950 | 22 | 4 | 1.42 |
| | 951 | 19 | 3 | 1.72 |
| | 952 | 15 | 8 | 1.09 |
| | 798 | 29 | 9 | 0.04 |
| | | | | (4.97) |
| Sum | | 104 | 33 | 0.06 |
| | | H3 | Ho | |
| *H1-1/SerH3* × *H1-1/SerH3* | 776 | 19 | 9 | 0.72 |
| | 779 | 16 | 9 | 1.49 |
| | | | | (2.21) |
| Sum | | 35 | 18 | 2.12 |
| Testcross (expected 1 H:1 Ho) | | H2 | Ho | |
| *H1-1/SerH2* × *H1-1/H1-1* | 956 | 11 | 11 | 0.00 |
| | 957 | 14 | 13 | 0.04 |
| | 954 | 8 | 12 | 0.81 |
| | 955 | 10 | 10 | 0.00 |
| | | | | (0.84) |
| Sum | | 43 | 46 | 0.10 |

Heterogeneity *G* statistic is given in parentheses.

an allele at the *SerH* locus. To further examine the genetic relationship between *H1-1* and *SerH*, homozygotes for *H1-1* were crossed to inbred strains homozygous for wild-type alleles *SerH1*, *SerH2* and *SerH3*. The $F_1$, $F_2$ and testcross progeny were scored for the appropriate H antigens; descendants of an exconjugant pair were scored as positive for an H antigen if a positive immobilization reaction was observed (see MATERIALS AND METHODS). The heterozygous $F_1$'s expressed the appropriate wild-type H allele, as expected. As in the inbreeding, no H1 antigen was detected in combinations with *SerH2* and *SerH3* alleles either by immobilization or by immunofluorescence. The phenotypes of $F_2$ and testcross progeny are shown in Table 2. The observed ratios conform to 3 H:1 Ho ($F_2$) and 1 H:1 Ho (testcross) Mendelian expectations, and, in *SerH2* and *SerH3* combinations, no H1 was observed. *H1-1*, therefore, fails to complement wild-type *SerH* alleles and, in 469 opportunities for recombination (380 H2 and H3 $F_2$; 89 testcross), also fails to recombine with them.

In many of the Table 2 crosses, the immobilization reactions of genetically identical cells descended from the same conjugating pair were often mixed, with some cells swimming freely while others were agglutinated. This reaction is typical when assorting clones are assayed with only one antiserum; immobi-

TABLE 3

H1-2 *segregation analysis in $F_2$ and testcross progeny*

| Genotypes | Cross | No. observed | | *G* |
|---|---|---|---|---|
| $F_2$'s (expected: 3 H3:1 Ho) | | | | |
| *H1-2/SerH3* × *H1-2/SerH3* | | H3/Ho | Ho | |
| | 1884 | 31 | 90 | 129.66*** |
| (expected: 1 H3:3 H3/Ho)[a] | | H3 | H3/Ho | |
| | 1884 | 22 | 99 | 3.22 |
| Testcross: (expected:H3 only) | | | | |
| *H1-2/SerH3* × *SerH3/SerH3* | | H3 | Ho | |
| | 1885 | 67 | 14 | |
| (expected: 1 H3:1 H3/Ho)[a] | | H3 | H3/Ho | |
| | 1885 | 45 | 36 | 1.00 |
| Testcross: (expected: 1 H3:1 Ho) | | | | |
| *H1-2/SerH3* × *H1-2/H1-2* | | H3/Ho | Ho | |
| | 1887 | 39 | 82 | 15.62*** |
| | 1888 | 37 | 90 | 22.80*** |
| | | | | (38.43)*** |
| Sum | | 76 | 172 | 38.15*** |

Heterogeneity *G* statistic is given in parentheses.
[a] Synclones entirely positive for H3 separated from those with mixed reactions (see text).
*, ** and *** indicate significance at 0.05, 0.01 and 0.001 levels, respectively.

lized cells presumably express the homologous antigen, whereas the swimming cells presumably express an alternate, noncross-reacting antigen. Depending on the degree of allele imbalance among the assorting cells, a clone that is heterozygous may actually fail to test positive by immobilization for one of the antigens. This mechanism can account for the excess of Ho progeny observed in some crosses, particularly with H3 (see below).

H1-2 *segregates from and does not complement* SerH3: To determine the relationship between *H1-2* and *SerH*, homozygous mutants were crossed to strain B. As with *H1-1*, the $F_1$ cells were immobilized by anti-H3 antiserum and no H1 was detected. But, unlike *H1-1*, a substantial proportion of conjugants produced progeny that were unaffected by anti-H3 antiserum. This was particularly evident in the $F_2$ and testcross progeny (Table 3). For example, in cross 1884 (an $F_2$), 31 exconjugant pairs produced progeny in which as many as 100% of cells were immobilized by anti-H3 antiserum. The other 90 pairs were completely negative for H3. If it is assumed that H3 is detectable in *H1-2/SerH3* heterozygotes, the expectation is 91 H3:30 Ho, the opposite of what was observed. However, the 31 H3-positive progeny were of two types: in 22 all cells were immobilized by anti-H3 and in nine anti-H3 immobilized only a portion of the cells. The 22 H3 pairs conform to the Mendelian expectation of 25% *SerH3* homozygotes. The absence of H3 in the heterozygotes is presumably due to allele imbalance. A similar argument applies to testcross 1885, and allele imbalance can also account for the observed ratios in crosses 1887 and 1888. No H1 was detected in any $F_2$ or testcross progeny.

TABLE 4

*Joint segregation analysis of* H1-1 *and* H1-2

| Genotypes crossed | Observed | | | *G* |
|---|---|---|---|---|
| | Cross | No. Ho | No. H1 | |
| Testcrosses (expected: 1 Ho:1 H1) | | | | |
| *H1-1/H1-2* × *H1-1/H1-1* | 1878 | 31 | 33 | 0.06 |
| | 1879 | 13 | 18 | 0.81 |
| | 1881 | 34 | 29 | 0.40 |
| | | | | (1.27) |
| Sum | | 78 | 80 | 0.03 |
| Testcrosses (expected: 1 Ho:1 H1) | | | | |
| *H1-1/H1-2* × *H1-2/H1-2* | 1850 | 10 | 7 | 0.53 |
| | 1876 | 19 | 11 | 2.16 |
| | 1877 | 28 | 34 | 0.58 |
| | | | | (3.27) |
| Sum | | 57 | 52 | 0.23 |
| $F_2$'s (expected: 1 Ho:1 H1) | | | | |
| *H1-1/H1-2* × *H1-1/H1-2* | 1863 | 21 | 16 | 0.68 |
| | 1865 | 21 | 19 | 0.10 |
| | | | | (0.78) |
| Sum | | 42 | 35 | 0.64 |

Heterogeneity *G* statistic is given in parentheses.

*H1-2*, therefore, fails to complement *SerH3*, and, in 571 opportunities for recombination (242 $F_2$ plus 329 testcross), fails to recombine with *SerH3*.

H1-1 *and* H1-2 *complement to express H1*: To determine the relationship between *H1-1* and *H1-2* mutations, homozygotes were crossed and the heterozygous progeny were tested with anti-H sera. Unlike previous crosses to other *SerH* alleles, the $F_1$'s *were* immobilized by anti-H1 antiserum. The H1 phenotype was observed regardless of which inbreeding generations (Table 1) of *H1-1* and *H1-2* were used, and in crosses in which a heterozygous clone from generation 10a (Table 1) of *H1-1* was used, the observed phenotypic ratio was 1 Ho:1 H1. Table 4 shows the results both of testcrosses between *H1-1/H1-2* and homozygous parents and of crosses between $F_1$'s to obtain $F_2$'s. In all instances the observed segregation conforms to the 1 Ho:1 H1 expectation based on the assumption that the *H1-1/H1-2* heterozygote expresses H1 antigen. *H1-1* and *H1-2*, therefore, complement each other to express H1, but neither complements wild-type alleles.

Segregation and complementation were also examined in a series of special dihybrid, triallelic crosses (Table 5). Because heterozygotes for *H1-1* or *H1-2* and *SerH3* are often H-negative, H3-positive and H-negative phenotypes were pooled (H3/Ho) for analysis. With the exception of cross 1854, the observed ratios of H3/Ho to H1 conform to Mendelian expectation. The appearance of only one H1 pair in cross 1854 had led us briefly to consider the possibility that one of the mutants was actually homozygous for mutations at two unlinked

TABLE 5

*Segregation analysis with three* SerH *alleles*

| Genotypes crossed | Observed | | | $G$ |
|---|---|---|---|---|
| | | Phenotypes | | |
| | Cross | No. H3/Ho | No. H1 | |
| Special (expected: 3 H3/Ho:1 H1) | | | | |
| *H1-1/H1-2* × *H1-1/SerH3* | 1854 | 30 | 1 | 11.20*** |
| | 1880 | 50 | 10 | 2.43 |
| | | | | (13.62)* |
| Sum | | 80 | 11 | 9.43** |
| Special (expected: 3 H3/Ho:1 H1) | | | | |
| *H1-1/H1-2* × *H1-2/SerH3* | 1883 | 67 | 21 | 0.06 |
| Special (expected: 3 H3/Ho:1 H1) | | | | |
| *H1-1/SerH3* × *H1-2/SerH3* | 1882 | 35 | 16 | 1.05 |
| Special (expected: 1 H3/Ho:1 H1) | | | | |
| *H1-2/H1-2* × *H1-1/SerH3* | 1851 | 15 | 14 | 0.03 |

Heterogeneity $G$ statistic is given in parentheses.
*, ** and *** indicate significance at 0.05, 0.01 and 0.001 levels, respectively.

loci. However, a duplicate cross (1880) using different parental strains conformed to the expected 3:1 ratio. This result together with the data in Tables 4 and 5 caused the idea to be abandoned.

H1-1 *and* H1-2 *are linked in the micronucleus*: The complementation between *H1-1* and *H1-2* suggests that the mutations are in different sites. At least one mutant, possibly both, could have an intact *SerH1* gene. Recombination, therefore, should produce a functional, transmissible wild-type gene. To look for such recombinants, *H1-1/H1-2* heterozygotes were crossed both to A*III and to strain B (Table 6). Among 205 opportunities for recombination, H1 antigen was detected in only one exconjugant pair (cross 1886). However, progeny tests revealed that the H1 phenotype was not the result of recombination. Rather, the putative recombinant behaved as a *H1-1/H1-2/SerH3* trisomic. Subclones of this pair were largely sterile, but among the progeny that were produced in a cross to strain B, the H1 phenotype was observed in only one of 19 progeny. When subclones of this pair were crossed to strain B, ten of 81 pairs were H1-positive. In both of these crosses, if the H1 phenotype had been due to recombination to produce a functional *SerH1* gene, 50% H1-positive pairs would have been expected. If, however, the H1-positive pairs that were testcrossed were trisomic, 16.7% of their progeny would be expected to express H1. The observed numbers are not significantly different from this expectation.

*Complementation is not due to interaction between defective structural gene products*: We looked for interaction between defective *SerH1* gene products in two ways. First, extracts of *H1-1* and *H1-2* homozygotes were mixed and tested for

TABLE 6

*Tests for recombination between* H1-1 *and* H1-2

| Genotypes crossed | Observed | | |
|---|---|---|---|
| | Cross | Phenotypes | |
| $F_2$'s (all Ho) | | No. Ho | No. H1 |
| *H1-1/H1-2* × A*III | 1872 | 12 | 0 |
| | 1873 | 10 | 0 |
| Testcrosses (H3/Ho) | | No. H3/Ho | No. H1 |
| *H1-1/H1-2* × *SerH3/SerH3* | 1868 | 29 | 0 |
| | 1869 | 27 | 0 |
| | 1870 | 28 | 0 |
| | 1886 | 99 | 1[a] |
| Sum | | 183 | 1 |

[a] The descendants of this pair were probably trisomic and therefore not truly recombinant; see text.

H1 antigen by immunodiffusion with anti-H1 antiserum made monospecific by absorption. No precipitin bands corresponding to H1 were seen. The second test was an *in vivo* assay. DOERDER (1981) found that i-antigen is exchanged during conjugation, so that cells initially expressing only one antigen come to have both on their cell surfaces at the end of conjugation. The exchange occurs in the absence of protein synthesis and does not require the development of new macronuclei. *H1-1* and *H1-2* homozygotes were mated and tested by immunofluorescence for H1 antigen both during meiosis and during macronuclear development (prior to expression of genes in the new macronuclei). Within this 4- to 7-hr period, conjugants remained negative for H1.

*Complementation does not involve macronuclear recombination*: If *H1-1* and *H1-2* are at different sites, then the expression of H1 could be due to recombination during macronuclear development. To examine this possibility, the stability of the $F_1$ phenotype was examined by macronuclear assortment. In *T. thermophila* all heterozygotes undergo assortment to express one allele or the other (SONNEBORN 1974b; P. J. BRUNS, personal communication). A *SerH1* allele formed by macronuclear recombination should also assort and, therefore, result in stable H1 assortees. We deliberately tried to detect such stable H1 clones by repeatedly selecting and expanding H1-positive subclones during assortment in a *H1-1/H1-2* heterozygote. This strategy was adopted because, without knowing the number of *SerH1* subunits in the newly developed macronucleus, we could not predict the proportion of stable H1 assortees that would appear in the absence of selection. Therefore, at about 13 fissions after conjugation 12 subclones from each of eight *H1-1/H1-2* heterozygotes were serially transferred by single-cell isolation every five to eight fissions for seven such transfers. Among the 86 subclones tested (at about 60 fissions after conjugation), eight were no longer immobilized by anti-H1. Several H1-positive and Ho (control) subclones were then expanded to 12 (sub-) subclones and again serially transferred seven times, to about 105 fissions after conjugation.

The descendants of the Ho clones continued (with few exceptions) to be Ho, whereas among the H1-positive clones, both H1-positive and Ho subclones were observed. Seven H1-positive subclones were again chosen and expanded to 24 (sub-sub-) subclones each for another seven serial transfers, to about 150 fissions after conjugation. None of these seven were stable for H1; H-negative cells were observed in a minimum of two subclones from each set of 24. As a final check for the lack of stability among H1-expressing clones, six sets of 24 (sub-sub-sub-) subclones derived from H1-positive 150-fission-old subclones were tested after two and seven additional serial transfers, and again, all subclones continued to assort H1-negative cells. We conclude that the H1 phenotype is not stable and, therefore, is not due to the formation of a *SerH1* allele by macronuclear recombination.

*Antigen(s) expressed by* SerH1 *mutations*: In the crosses reported above, the phenotypes were usually scored with reference to immobilization by antisera to wild-type H antigens. To have a positive assay, several attempts were made to make antisera to *H1-1* homozygotes. These included both the use of whole cells as well as extracts taken part way through the protocol (F. P. DOERDER and M. S. BERKOWITZ, unpublished observations) used to purify H antigens. The results of immobilization assays with these sera were ambiguous. Antisera would sometimes immobilize homologous cells, and other times it would not. No relationship was seen between immobilization and growth phase or immobilization and clonal age. Positive reactions, when present, ranged from weak to strong. *H1-2* homozygotes were also tested with these antisera. Surprisingly, these mutants were regularly positive and often strongly immobilized. *H1-1/H1-2* heterozygotes, which express H1, were not immobilized by these sera.

Because immobilization assays were routinely performed on cells grown in bacterized medium, whereas the antisera were prepared against cells grown in PPY, immobilization assays were performed on cells grown in PPY and washed into Tris buffer for assay. All *H1-1* and *H1-2* homozygotes were immobilized, and they were strongly positive by fluorescent antibody assay. Fluorescent antibody assays on BP grown cells were also positive but considerably weaker than parallel PPY assays. We conclude that the mutants differ quantitatively with respect to the presence of an i-antigen. Further description of the i-antigens of *H1-1* and *H1-2* mutants will be presented elsewhere.

## DISCUSSION

*Complementation may be due to interaction between allele-specific regulatory genes (sites)*: The most significant result reported here is that two *SerH1*-derived mutations, *H1-1* and *H1-2*, complement each other to express H1 but fail to complement with heterologous wild-type alleles. Such complementation has three possible explanations: (1) recombination between mutations in the macronucleus, (2) interallelic complementation between defective *SerH1* gene products, (3) interaction between regulator gene products. These explanations are not necessarily mutually exclusive, since macronuclear recombination could occur with either of the other two.

1. Macronuclear recombination. Recombination during macronuclear devel-

opment or during subsequent division cycles has been argued theoretically (ORIAS 1973; DOERDER and DIBLASI 1984) but has yet to be unambiguously demonstrated by experiment. A simple argument against recombination as an explanation for the complementation observed here can be made. Why should recombination occur in 100% of *H1-1/H1-2* heterozygotes but neither between a mutant and wild-type nor between two wild-type alleles (*e.g.*, in *SerH3/SerH4* heterozygotes to give a stable hybrid antigen)? A second argument concerns the number of functional *SerH1* genes that could be formed by recombination during development. Macronuclear development begins with a diploid (2C) amount of DNA and ends with a mean of 128C (DOERDER and DEBAULT 1975). The later recombination occurs, the smaller the proportion of *SerH1* genes. At the 16C stage, for example, recombination would result in 6.25% *SerH1* genes. Both theoretical calculations (SCHENSTED 1958) and computer simulations (F. P. DOERDER, unpublished) show that these are probably too few genes to result in the immobilization of 100% of heterozygous cells when tested ten to 13 fissions after conjugation (*i.e.*, the reactions should be mixed, which they are not).

In a direct experimental approach to macronuclear recombination, we were unable to recover stable H1-positive clones in assorting *H1-1/H1-2* heterozygotes, despite repeated selection to almost 200 fissions of H1-positive clones. At 200 fissions theoretical calculations show that more than 90% of subclones should be fully assorted in the absence of selection. We, therefore, conclude that macronuclear recombination between *H1-1* and *H1-2*, although not eliminated as a rare event, does not occur with sufficient regularity to be the cause of complementation.

2. Interallelic complementation between defective *SerH1* gene products. Complementation might arise through mutual compensation of defective polypeptides in heterodimer antigen molecules. This hypothesis is unlikely for two reasons. First, and most importantly, the H i-antigens have the same apparent molecular weights of 44,000–52,000 both by gel filtration chromatography (nonreducing conditions) and sodium dodecyl sulfate electrophoresis (reducing conditions) (F. P. DOERDER and M. S. BERKOWITZ, unpublished results). Paramecium i-antigens, although considerably larger than *T. thermophila* antigens, are also monomers (HANSMA 1976). Second, both *in vitro* and *in vivo* tests for complementation between gene products were negative. Mixed extracts failed to produce H1 antigen detectable by immunodiffusion, and i-antigen exchange during conjugation failed to produce H1 antigen detectable by immunofluorescence.

3. Interaction between regulator gene products. An alternative to the hypothesis that both mutations are within the *SerH1* structural gene is that one mutant, or both, has an intact *SerH1* structural gene and that the mutations are in regulator sites or regulator genes near the *SerH* locus. Thus, in the *trans* heterozygote, the wild-type phenotype could be due to a diffusible regulator molecule. Because complementation does not occur between *H1-1* or *H1-2* and heterologous wild-type alleles, this hypothetical regulator must be allele specific. The idea of allele-specific regulation, in addition to the locus-

specific regulation demonstrated in previous mutational studies, is consistent with evidence for the evolutionary divergence of *SerH* alleles as suggested by (1) the lack of cross-reactivity between wild-type H antigens, (2) their differences in molecular weight and (3) their differences in serial dominance.

Unfortunately, direct evidence for this hypothesis is not available. A diffusible *trans*-acting regulator substance might be expected to produce a positive result on the *in vivo* complementation assay where it would have approximately 4–7 hr to alter gene expression before the macronucleus ceases transcription late in conjugation (WENKERT and ALLIS 1984). This length of time is sufficient for a change in expression from H antigen to T antigen (expressed at 40°) in nonconjugating cells (N. E. WILLIAMS, A. RON and F. P. DOERDER, unpublished observations) but is too short for transitions such as L (expressed below 20°) to H and H to S (expressed in 0.2 M NaCl) (M. KRAUSE, unpublished results; D. SMITH, unpublished results). The 4- to 7-hr interval may also be too short during conjugation.

Proof of the existence of regulatory genes (or sites) at the *SerH* locus will require both the characterization of additional mutations and the cloning and sequencing of the wild-type and mutant genes. Such sequencing would also reveal the extent to which *H1-1* and *H1-2* contain multiple-site mutations. We have shown here that mutational dissection of *SerH* is possible. The preparation of antisera to purified antigen (F. P. DOERDER and M. S. BERKOWITZ, unpublished results; N. E. WILLIAMS, A. RON and F. P. DOERDER, unpublished results) should also lead to the molecular characterization of this system.

*H i-antigens may play a role in their own regulation*: One of the reasons for searching for *SerH* mutations was to ask what antigen is expressed in the absence of a functional *H* gene. The expression of another i-antigen in cells homozygous for a defective *SerH* gene would imply a regulatory role for H antigen itself. We reasoned that, if H antigen has an autoregulatory role, its absence should result in the expression of an alternative i-antigen in much the same way that homologous anti-H antisera induces the expression of the I antigen (JUERGENSMEYER 1969). Although we can not be certain that either *H1-1* or *H1-2* is indeed defective in the H1 structural gene, both clearly express an antigen in the absence of detectable H1. Preliminary evidence (F. P. DOERDER, M. KRAUSE and M. S. BERKOWITZ, unpublished data) suggests that this antigen may be one of the L antigens expressed below 20°, but both quantitative and qualitative differences between *H1-1* and *H1-2* in its expression are yet to be resolved.

*Genetic organization of the macronucleus*: As described in the analysis of results shown in Tables 2 and 3, *H1-1/SerH3* and *H1-2/SerH3* heterozygotes are frequently H3-negative by the immobilization assay. This result is expected, given the apparent unequal representation of *SerH1* and *SerH3* alleles in wild-type heterozygotes. That the allele imbalance is retained in the mutations is not surprising given the protocol for their isolation. However, the proportion of H3-negatives is greater with the *H1-2* allele than with *H1-1*, implying a mutation-specific difference. Whether this difference is related either to complementation or to antigen expression is unknown. The difference does, however,

indicate that mutational analysis of gene dosage in the macronucleus may be possible. Such mutants may be important in determining which nucleotide sequences are responsible for allele balance.

The apparent lack of either micronuclear or macronuclear recombination between *H1-1* and *H1-2* has implications concerning macronuclear development. YAO *et al.* (1984) showed that during development micronuclear-specific DNA may be deleted and the remaining DNA fragments rejoined. Recent studies have shown that individual genes may undergo considerable processing (CALLAHAN, SHALKE and GOROVSKY 1984). If, as we have proposed, the mutations described here cover at least two genes, the absence of macronuclear recombinants implies that allelic fragments are not regularly exchanged by the deletion/rejoining mechanism.

An alternative to the concept that genes on the same micronuclear chromosome remain on the same macronuclear DNA fragment is that during macronuclear development they come to be on two different fragments. Thus, double heterozygotes for genes that show micronuclear linkage would assort in the macronucleus into not two, but four, genetic classes of equal frequency (for discussion see DOERDER and DIBLASI 1984). Indeed, all micronuclear linkages examined to date show such independent macronuclear assortment, suggesting that these genes are on different macronuclear fragments. The absence of stable H1 assortees (25% expected for independent assortment), therefore, suggests that *H1-1* and *H1-2* are on the same macronuclear fragment as *SerH*.

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## LITERATURE CITED

CALLAHAN, R. C., G. SHALKE and M. A. GOROVSKY, 1984 Developmental rearrangements associated with a single type of expressed $\alpha$-tubulin gene in Tetrahymena. Cell **36:** 441–445.

DOERDER, F. P., 1973 Regulatory serotype mutations in *Tetrahymena pyriformis*, syngen 1. Genetics **74:** 81–106.

DOERDER, F. P., 1979 Differential expression of immobilization antigen genes in *Tetrahymena thermophila*. I. Genetic and epistatic relationships among recessive mutations which alter expression of i-antigens. Immunogenetics **9:** 551–562.

DOERDER, F. P., 1981 Differential expression of immobilization antigen genes in *Tetrahymena thermophila*. II. Reciprocal and non-reciprocal transfer of i-antigen during conjugation and expression of i-antigen genes during macronuclear development. Cell Diff. **10:** 299–307.

DOERDER, F. P. and L. E. DEBAULT, 1975 Cytofluorimetric analysis of nuclear DNA during meiosis, fertilization and macronuclear development in the ciliate *Tetrahymena pyriformis*, syngen 1. J. Cell Sci. **17:** 471–493.

DOERDER, F. P. and S. L. DIBLASI, 1984 Recombination and assortment in the macronucleus of *Tetrahymena thermophila*: a theoretical study by computer simulation. Genetics **108:** 1035–1045.

DOERDER, F. P., J. H. LIEF and L. E. DEBAULT, 1977 Macronuclear subunits in *Tetrahymena thermophila* are functionally haploid. Science **198:** 946–948.

FORNEY, J. D., L. M. EPSTEIN, L. B. PREER, B. M. RUDMAN, D. WIDMAYER, W. H. KLEIN and J. R. PREER, JR., 1983 Structure and expression of genes for surface proteins in *Paramecium.* Mol. Cell. Biol. **3:** 466–474.

HANSMA, H. G., 1976 The immobilization antigen of *Paramecium aurelia* is a single polypeptide chain. J. Protozool. **22:** 257–259.

JUERGENSMEYER, E. B., 1969 Serotype expression and transformation in *Tetrahymena pyriformis.* J. Protozool. **16:** 344–352.

NANNEY, D. L. and J. M. DUBERT, 1960 The genetics of the H serotype system in variety 1 of *Tetrahymena pyriformis.* Genetics **45:** 1335–1349.

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

ORIAS, E., 1973 Alternative interpretation of the molecular structure and somatic genetics of acid phosphatase-1 in *Tetrahymena thermophila.* Biochem. Genet. **9:** 87–90.

SCHENSTED, I. V., 1958 Appendix: model of the subnuclear segregation in the macronucleus of ciliates. Am. Nat. **92:** 161–170.

SONNEBORN, T. M., 1974a *Paramecium aurelia.* pp. 469–594. In: *Handbook of Genetics,* Vol. 2, Edited by R. C. KING. Plenum Press, New York.

SONNEBORN, T. M., 1974b *Tetrahymena pyriformis.* pp. 433–467. In: *Handbook of Genetics,* Vol. 2, Edited by R. C. KING. Plenum Press, New York.

WEINDRUCH, R. H. and F. P. DOERDER, 1975 Age-dependent micronuclear deterioration in *Tetrahymena pyriformis,* syngen 1. Mech. Ageing Dev. **4:** 263–279.

WENKERT, D. and D. C. ALLIS, 1984 Timing of the appearance of macronuclear specific histone variant hv1 and gene expression in developing new macronuclei of *Tetrahymena thermophila.* J. Cell Biol. **98:** 2107–2117.

YAO, M.-C., J. CHOI, S. YOKOYAMA, C. F. ASTERBERRY and C.-H. YAO, 1984 DNA elimination in Tetrahymena: a developmental process involving extensive breakage and rejoining of DNA at defined sites. Cell **36:** 433–440.

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