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Mendelian and Non-Mendelian Mutations Affecting Surface Antigen Expression in *Paramecium tetraurelia*

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A screening procedure was devised for the isolation of X-ray-induced mutations affecting the expression of the A immobilization antigen (i-antigen) in *Paramecium tetraurelia*. Two of the mutations isolated by this procedure proved to be in modifier genes. The two genes are unlinked to each other and unlinked to the structural A i-antigen gene. These are the first modifier genes identified in a *Paramecium* sp. that affect surface antigen expression. Another mutation was found to be a deletion of sequences just downstream from the A i-antigen gene. In cells carrying this mutation, the A i-antigen gene lies in close proximity to the end of a macronuclear chromosome. The expression of the A i-antigen is not affected in these cells, demonstrating that downstream sequences are not important for the regulation and expression of the A i-antigen gene. A stable cell line was also recovered which shows non-Mendelian inheritance of a macronuclear deletion of the A i-antigen gene. This mutant does not contain the gene in its macronucleus, but contains a complete copy of the gene in its micronucleus. In the cytoplasm of wild-type animals, the micronuclear gene is included in the developing macronucleus; in the cytoplasm of the mutant, the incorporation of the A i-antigen gene into the macronucleus is inhibited. This is the first evidence that a mechanism is available in ciliates to control the expression of a gene by regulating its incorporation into developing macronuclei.

Every cell in each species of the *Paramecium aurelia* complex contains two diploid micronuclei and a single, highly polyploid macronucleus. Although these nuclei are all derived from a single, diploid fertilization nucleus during conjugation or autogamy, only the macronucleus is transcriptionally active. At least two distinct mechanisms have evolved in *Paramecium* sp. for the stable transmission of alternate modes of macronuclear expression during vegetative growth. One involves the irreversible determination of newly formed macronuclei. The most extensively studied system under this type of control is mating type expression (reviewed in reference 22). In *P. tetraurelia*, developing macronuclei are influenced by the cytoplasm to become determined for the expression of two alternative mating types. The cytoplasmic state, in turn, is controlled by the macronucleus. This macronucleus-cytoplasm control loop results in the maintenance of either mating type through a cytoplasmic line of descent. The changes involved in the determination of the macronucleus and the cytoplasmic factors influencing mating type determination are not known.

A fundamentally different mechanism controls the alternate expression of immobilization antigens (i-antigens) in *P. tetraurelia*. The i-antigens of *Paramecium* sp. (reviewed in references 1, 7, 16, and 19) are large, single-polypeptide glycoproteins, ca. 250,000 to 300,000 daltons in size. They cover the entire surface of every cell, and their function is not known. In stock 51 of *P. tetraurelia*, 11 serologically distinct i-antigens have been observed, A, B, C, D, E, G, H, I, J, N, and Q. Usually no more than one type of i-antigen is expressed by an individual cell, and the i-antigen expressed defines the serotype of that cell. Each serotype has a range of environmental conditions under which it shows maximal stability, but several serotypes may be stably inherited under one set of conditions. Unlike mating types, changes in i-antigen expression can be induced in the absence of nuclear reorganization and are fully reversible. Genetic analyses suggest that each i-antigen is coded by a distinct genetic locus. The control of the expression of these loci is probably at the level of transcription or RNA processing (17). The cytoplasm has also been implicated in the control of the i-antigen genes, but the mechanism is unknown. Expression-linked DNA rearrangements, of the type found in a variety of systems of stable, alternate modes of gene expression (3, 6, 13, 25–27), have not been found for the i-antigen genes (8).

Other than the i-antigen genes themselves, no genes have been previously identified that affect serotype expression. An attempt to select for induced mutations of serotype expression has been reported (18), and although three independent mutations in the structural H i-antigen gene were recovered, a more extensive mutational analysis is needed. In this paper, we present a simple selection scheme for the isolation of X-ray-induced mutations affecting serotype expression. Using this scheme, we recovered cell lines carrying mutations affecting the expression of the A i-antigen gene or affecting sequences near the A i-antigen gene. Two of the mutations were in two unlinked genes involved in the general regulation of i-antigen expression and were the first genes of their type identified. Macronuclear DNA from another mutant line lacked DNA sequences which, in the wild-type genome, flank the 3′ end of the A i-antigen gene. Although this cell line was originally selected due to its inability to express serotype A, cells from this strain can now be induced to express a stable serotype A. What occurred in this strain to allow wild-type phenotypic expression is not known, but these studies demonstrate that sequences controlling gene expression are not included in the 3′ flanking region of this gene.

In addition, a variant line was recovered in which the A i-antigen gene came under the control of a system of cytoplasmically influenced macronuclear determination. A genetic and molecular analysis of this mutant suggests that although the A gene is present in the micronucleus of all cells, it is lost during the formation of the macronucleus in the mutant, but not in the wild-type, cytoplasm. The mode of inheritance of

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the trait is similar to that of the mating type in *P. tetraurelia*. We conclude that the cytoplasmic control of integration into the macronucleus of specific DNA sequences is the basis for at least one example of ciliate nuclear determination.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Bethesda Research Laboratories, Gaithersburg, Md., or New England Biolabs, Inc., Beverly, Mass., and used according to recommendations. DNA polymerase and S1 nuclease were from Bethesda Research Laboratories. [α-^{32}P]dNTPs (10 mCi/ml and >2,000 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, Ill. Nitrocellulose filters for Southern blots were from Schleicher & Schuell, Inc., Keene, N.H. (no. BA 85; 0.45-μm pore size).

***Paramecium* strains.** All *Paramecium* strains were derived from kappa-free stock 51 of *P. tetraurelia* from the Indiana University Collection and were cultured in a 0.15% Cerophyl (Agri-Tech, Kansas City) infusion (21) supplemented with 1 mg of stigmasterol per ml and inoculated with *Klebsiella pneumoniae* 24 to 48 h before use.

***Paramecium* genomic clones.** λSA-1 was obtained from a *Paramecium* genomic library constructed in λ1059 and contains the entire stock 51 A i-antigen gene (8). λSA-2 was obtained from a genomic library constructed in lambda replacement vector EMBL 3, with the 1.7-kilobase (kb) *Hin*dIII-*Eco*RI fragment from λSA-1 as a probe, and was confirmed to contain a region overlapping with λSA-1 by genomic blots (J. Forney, unpublished data). pSA-8.5 and pSA-2.2 consist of the 8.5- and 2.2-kb *Eco*RI fragments of λSA-1, respectively, subcloned in the plasmid vector pUC8 (Bethesda Research Laboratories). The 8.5-kb *Eco*RI fragment was erroneously referred to as an 8.8-kb fragment in our previous paper (8).

Isolation of serotypes. Serotypes were identified as described by Sonneborn (21) with sera from the Sonneborn collection at Indiana University. Serotypes A and D arose spontaneously in wild-type cultures under standard culture conditions at 27°C.

Mutagenesis and screening. Approximately 100,000 well-fed cells expressing serotype A or D were concentrated by centrifugation into a volume of 25 ml. They were then exposed to a 10-krad dose of X-rays at a rate of 1 krad per min with a Torrex 150 X-ray machine (Torr X-ray Corp., Harbor City, Calif.) as a source. After mutagenesis, the cells were starved to induce autogamy, which results in homozygosity at all loci (see below), and grown for 10 fissions at 27°C to overcome phenotypic lag. They were then transferred to 35°C and grown for 4 days at a growth rate of 1 fission per day. This treatment efficiently transforms wild-type cells to serotype A (20). Anti-A serum was added to the cultures, and survivors were isolated and cloned at 27°C. Clones were rescreened by growth at 35°C, as in the primary screen.

Genetic analysis. Laboratory strains of *P. tetraurelia* are typically homozygous clones. Homozygotes are readily obtained by the induction of autogamy, a nuclear reorganization event which occurs within single cells and results in homozygosity at all genetic loci in a newly formed fertilization nucleus. Mitotic products of this fertilization nucleus become either the diploid generative nuclei (micronuclei) or differentiate into the highly polyploid, transcriptionally active macronuclei. Autogamy is inducible by starvation, but cells can be induced to undergo autogamy only if they are members of a vegetative clone which has undergone at least 15 to 20 fissions since the last nuclear reorganization event.

If the two complementary mating types are present, starved cells may pair and undergo conjugation. During this process, genetic material from the micronuclei is exchanged between the two members of a conjugating pair, and the result is that the fertilization nuclei in the two conjugants are heterozygous and identical. Mating between homozygous strains is thus comparable in other organisms to the first cross between completely inbred lines. Although conjugation results in nuclear uniformity between the exconjugants, very little cytoplasm is exchanged. The cytoplasmic identity of the two conjugants is thus maintained in the exconjugants and their vegetative progeny. In this paper, the progeny with cytoplasm derived from the wild-type parent will invariably be called the progeny on the wild-type side of the cross.

To induce mating reactivity, well-fed cells in the early part of the clonal life cycle were concentrated and starved in exhausted Cerophyl (21). They became mating reactive within 8 h, and mating pairs were obtained in the standard way (21). After separation, exconjugant cells were isolated into 1 ml of bacterized Cerophyl and allowed to grow for 10 fissions. New isolations were then made, and daily isolation lines were maintained. Leftover, starved clones were stained for autogamy with acetocarmine-fast green stain (5). When 97 to 100% of the culture showed signs of autogamy (fragmented macronuclei), cells were isolated to initiate the F_2 generation.

To confirm true conjugation, a recessive body shape gene, *tw* (twisty), was always homozygous in one parent of each cross. As described above, normal conjugation results in genotypically identical exconjugants; crosses of $tw^+/tw^+ \times tw/tw$ result in two normally shaped exconjugants, both tw/tw^+ heterozygotes. Any pair of exconjugant clones was discarded if one member showed the twisty body shape after 10 fissions. Normal autogamy of a heterozygote yields the two possible homozygotes in a 1:1 ratio. Pairs of exconjugants were also discarded if the F_2 generation by autogamy did not show a 1:1 ratio of normal- and twisty-shaped progeny.

The cytoplasmic identity of the progeny in these crosses was determined by testing the serotype of a sample of each F_1 clone 10 fissions postconjugation. In each of the crosses, the parents were expressing alternate serotypes at the time of the cross. As long as its serotype is stable under the conditions of the cross, and the genotype acquired at conjugation allows expression of the preconjugation serotype, an F_1 clone will usually continue to express the serotype of its cytoplasmic parent (1). In none of the crosses presented in this paper did conjugation result in the inability of an F_1 clone to express the serotype expressed by its cytoplasmic parent, and testing serotypes was an effective method of determining the cytoplasmic lineage of F_1 clones.

Isolation of *Paramecium* RNA. RNA was prepared by lysing logarithmically growing *P. tetraurelia* cells in guanidine-hydrochloride and purifying it as previously described (17).

Isolation of *Paramecium* DNA. *Paramecium* DNA was isolated by a modification of the procedure described by Forney et al. (8). The cells from 100 ml of culture were pelleted and resuspended in 0.4 ml of their own medium. A 0.8-ml portion of lysing solution (0.01 M Tris, 0.05 M EDTA, 1% sodium dodecyl sulfate, pH 9.5) was added rapidly, and the mixture was incubated at 65°C. After 20 min, it was extracted with phenol at room temperature. DNA was recovered from the aqueous phase, after the sequential addition of 60 μl of 4.5 M sodium acetate and 2.4 ml of absolute ethanol, by winding it onto a 50-μl capillary tube.

TABLE 1. Serotype expression in wild-type and mutant strains

Strain	Maximum % expression of serotype A[a]	Maximum % stability of serotype A[b]	Predominant serotype expressed at 27°C	Other serotypes observed
Wild type	100	100	A	All
d8	100	0	C and E	H and B
d29	30	0	C and E	H
d16	100	100	A	H,B,D, and C
d48	0		D	H,B,D, and C

[a] For each strain, a minimum of 10 independent test tube populations were grown at 35°C at a growth rate of 1 to 2 fissions per day for a minimum of 4 days. The percentages reported represent the percentage of cells in the populations immobilized by anti-A serum.

[b] After transformation to serotype A at 35°C, growth was continued at 27°C and serotyped after a maximum of 5 days.

This procedure typically yielded 20 to 30 μg of DNA. Since the macronuclei are ca. 1,000-ploid, and the micronuclei are diploid, this preparation is, in effect, macronuclear DNA.

DNA for BAL 31 digestions was further purified by equilibrium centrifugation in cesium chloride (4:1 mixture of cell lysate in lysing solution to saturated cesium chloride).

S1 mapping. The method used for S1 mapping was that of Berk and Sharp (2). The 2.2-kb *Eco*RI fragment from pSA-2.2 was isolated from a 0.8% agarose gel and 3′ end labeled with [α-^{32}P]dATP and reverse transcriptase. Hybridization with polyadenylated RNA was for 15 h at 45°C in 80% formamide. S1 reactions were carried out for 60 min at 37°C. Samples were denatured in formamide and electrophoresed on 6% polyacrylamide-urea gels (8 M urea).

Nick translation. Routinely, 1 μg of DNA was nick translated at 15°C to a specific activity of 3×10^7 to 9×10^7 cpm per μg with 50 μCi of [α-^{32}P]dNTP (12).

Southern blots and hybridization reactions. Southern blots were prepared by a modification of the procedure of Southern (24) as described by Forney et al. (8). Filter hybridizations in 10% dextran sulfate–50% formamide were as previously described (8), except that filters were hybridized at 40°C and washed at 72°C.

RESULTS

Phenotypes of the mutants. Four presumptive mutants were isolated by their failure to express serotype A during the primary and secondary screens of the mutagenesis procedure. The strains cultured from these mutants, d8, d16, d29, and d48, have been repeatedly tested by growth in test tubes at 35°C for prolonged periods to determine whether induction of serotype A was possible. In addition, they have been cultured at 13°C, 19°C, room temperature, and 27°C over a period of 6 months and periodically monitored for serotype expression. The relevant observations are summarized in Table 1.

The expression of serotype A has never been induced in strain d48. The predominant serotype in this strain at 27°C is D, which is also a common serotype in wild-type cells. As with wild-type cells, d48 is induced to express serotype H at 13°C, and serotypes B and C have been isolated and stably maintained at 27°C. Other serotypes have not been observed in d48 but are infrequently expressed even in wild-type cells. In conclusion, there is no apparent difference in serotype expression between d48 and wild-type cells other than the lack of expression of serotype A by d48.

Unlike d48, strains d8 and d29 have been induced to express serotype A by growth at 35°C for 4 to 6 days. Although the time at 35°C required for maximal expression of A varied among experiments, the switch to A for both of these mutant strains has invariably taken longer than in parallel tubes of wild-type cells. It has been possible to induce 100% of the cells in a d8 population to express serotype A. A d29 population has never reached more than 30% and rarely exceeds 5% expression of A. Any cells of either of the strains induced to express A at 35°C will transform to another serotype at 27°C within 5 days. Serotypes C and E predominate in both d8 and d29 at 27°C, and often cells are found from either strain which express C and E simultaneously for several fissions. Wild-type cells have

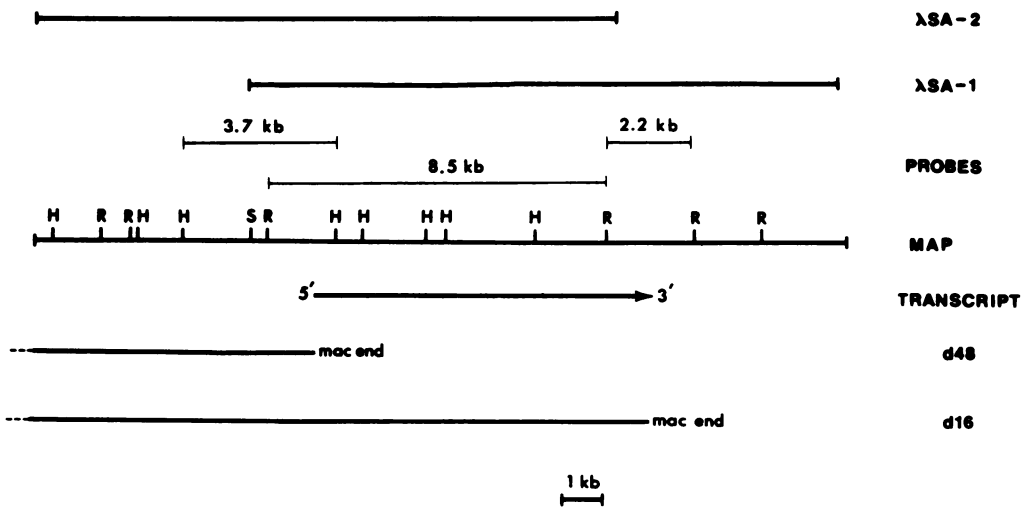

FIG. 1. Restriction map of the cloned A i-antigen gene and its flanking sequences. The genomic sequences contained within the overlapping clones λSA-1 and λSA-2 are indicated by heavy bars above the map. Thin bars above the map represent fragments used as probes in the experiments reported in this paper. The extent of the A i-antigen transcript is represented by the arrow below the map, and its orientation is indicated. The macronuclear sequences homologous to this region contained in strains d48 and d16 are indicated by heavy bars, and the newly located ends of macronuclear chromosomes in these strains are shown. R, *Eco*RI; H, *Hin*dIII; S, *Sal*I.

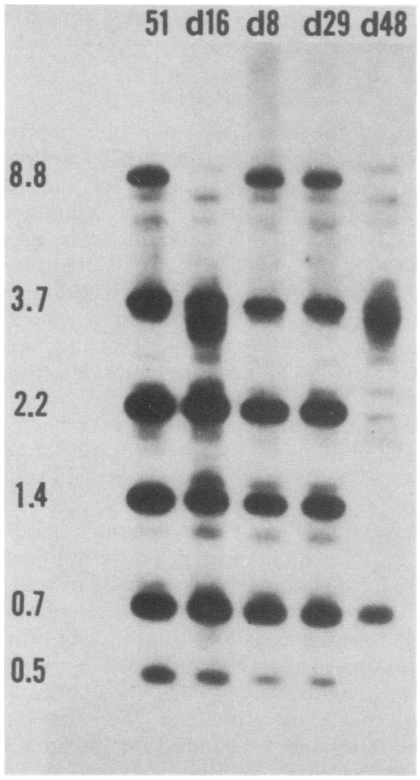

FIG. 2. Hybridization of the 8.5-kb *Eco*RI fragment from λSA-1 to *Hin*dIII-digested genomic DNAs from wild-type stock 51 and the mutant strains. Genomic DNAs (5 μg per lane) from the indicated strains were electrophoresed on a 0.8% agarose gel and blotted to nitrocellulose. The filter was then hybridized to the nick-translated 8.5-kb *Eco*RI fragment from λSA-1 subcloned in pSA-8.5.

never been found which are immobilized by both C and E antiserum for extended periods. Serotype D, which is common in the wild type and predominant in d48, has never been observed in either d8 or d29, but a reliable method of inducing D is not available. Both strains will express serotype H at 13°C. In conclusion, d8 and d29 have the ability to express the A i-antigen, but the regulation of its expression is abnormal. The regulation of other serotypes may also be abnormal.

Strain d16 was originally scored as being noninducible for serotype A. Subsequent tests have shown that d16 cells can be readily induced to express A, and once induced, will stably express A at 27°C. Serotypes H, B, and D have also been isolated and stably maintained. Although d16 is phenotypically indistinguishable from the wild type, data presented below indicate that if d16 reverted to its present form after its isolation, the reversion was not complete at the molecular level.

Molecular analysis of the mutants. Figure 1 shows the restriction map of the macronuclear DNA contained in the overlapping clones λSA-1 and λSA-2. λSA-1 contains the entire 8.4-kb wild-type A i-antigen gene and 5 kb of downstream sequences. λSA-2 contains the 5′ end of the gene and 5.2 kb of upstream sequences. Also shown in Fig. 1 is the extent of the 8.5-kb *Eco*RI fragment from λSA-1 subcloned in pSA-8.5. This fragment contains sequences homologous to every *Hin*dIII fragment entirely within or overlapping the transcriptional unit. This 8.5-kb fragment was isolated from a 0.8% agarose gel of an *Eco*RI digest of pSA-8.5, labeled by nick translation, and used to probe a Southern blot of *Hin*dIII digests of wild-type and mutant macronuclear DNAs (Fig. 2). The first lane shows that DNA from wild-type stock 51 contains each of the expected *Hin*dIII fragments. These include the 0.5-, 0.7-, 1.4-, and 2.2-kb fragments located entirely within the gene, and the 3.7- and 8.8-kb fragments encompassing the 5′ and 3′ ends of the gene, respectively. Fainter signals seen in the wild-type digest are preferentially lost under more stringent wash conditions and are concluded to represent sequences in the genome which share homology to parts of the A i-antigen gene and its flanking sequences.

As predicted from the phenotypic data, all of the *Hin*dIII fragments of the wild-type sequence are found in the genomes of both strains d8 and d29. Although it appears that both d8 and d29 have an intact A i-antigen gene, these results do not rule out the possibility of small deletions or rearrangements, in or surrounding the gene, undetectable by Southern analysis.

Macronuclear DNA from strain d48, which cannot express serotype A, lacks any of the *Hin*dIII fragments of the wild-type A i-antigen gene (we believe that the 0.7-kb *Hin*dIII fragment seen in the d48 digest represents a cross-reacting sequence which comigrates with the 0.7-kb fragment of the A i-antigen gene in the wild-type digest). The 8.5-kb *Eco*RI probe hybridized to the same cross-reacting fragments as in the wild-type digest (indicating the presence of an equivalent

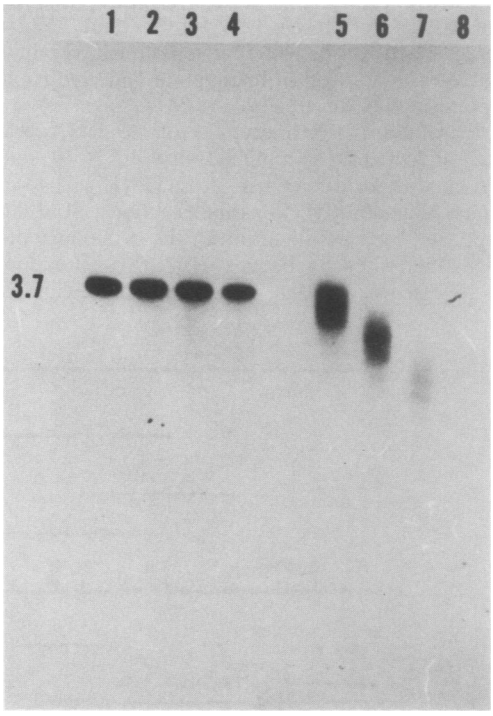

FIG. 3. Preferential digestion of the 3.7-kb *Hin*dIII fragment in strain d48 by BAL 31. Genomic DNAs from wild-type stock 51 (lanes 1 through 4) and mutant strain d48 (lanes 5 through 8) were digested with BAL 31 for various lengths of time before subsequent digestion with *Hin*dIII. The DNAs (5 μg per lane) were then electrophoresed on a 0.8% agarose gel and blotted to nitrocellulose. The filter was hybridized to the nick-translated 3.7-kb *Hin*dIII fragment from λSA-2 containing the 5′ end of the A i-antigen gene. Lanes: 1, 2, 3, and 4, wild-type DNA treated with BAL 31 for 0, 2, 4, and 7 min, respectively; 5, 6, 7, and 8, strain d48 DNA treated with BAL 31 for 0, 2, 4, and 7 min, respectively.

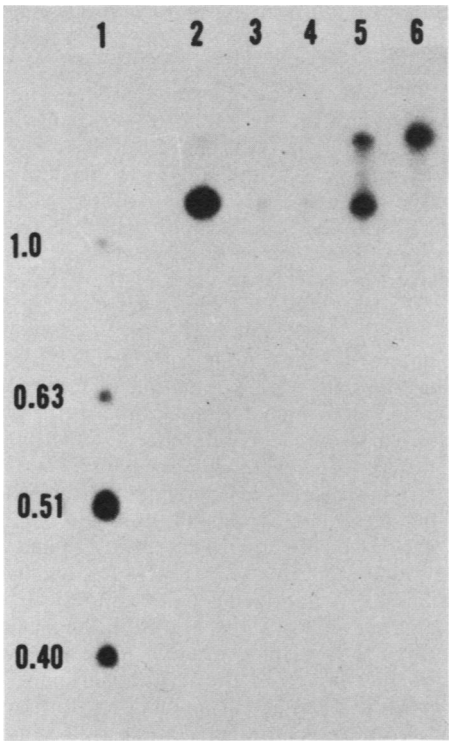

FIG. 4. Protection of the 2.2-kb *Eco*RI fragment from S1 nuclease digestion by wild-type and d16 RNAs. The 2.2-kb *Eco*RI fragment containing the 3′ end of the A i-antigen gene, isolated from pSA-2.2, was 3′ end labeled and hybridized to 5 μg of polyadenylated RNA from either wild-type or d16 cells. After treatment with various amounts of S1 nuclease, the products were run on a 6% denaturing polyacrylamide gel. Lanes: 1, 5′ end-labeled *Hin*fI-digested pBR322 DNA; 2, 2.2-kb *Eco*RI fragment hybridized to d16 RNA and digested with 50 U of S1 nuclease; 3, same as lane 2 but treated with 5 U of S1 nuclease; 4, 2.2-kb *Eco*RI fragment hybridized to wild-type RNA and digested with 5 U of S1 nuclease; 5, same as lane 4 but treated with 50 U of S1 nuclease; 6, untreated 2.2-kb *Eco*RI fragment. The size of the protected fragment in lanes 2, 3, 4, and 5 was calculated to be 1.3 kb.

amount of *Hin*dIII-cut DNA in the d48 lane) as well as to an additional, broad band migrating at 3.7 kb. This same broad band is the only hybridizing *Hin*dIII fragment in a d48 digest probed with the 3.7-kb *Hin*dIII fragment encompassing the 5′ end of the wild-type A i-antigen gene (Fig. 3). These results suggest that all or most of the A i-antigen gene is absent in the macronucleus of d48, but sequences just upstream from the gene in the wild-type genome are present in d48. Hybridization to d48 digests with probes derived from λSA-1, involving sequences even further upstream, verified that sequences upstream from the A i-antigen gene are present in macronuclear DNA from d48 (data not shown).

The breadth of the new 3.7-kb *Hin*dIII fragment in d48 is characteristic of fragments derived from the ends of macronuclear chromosomes which vary in size due to the variable number of C_4A_2 terminal repeats in telomeric regions. The experiment illustrated in Fig. 3 demonstrates that this broad band is preferentially shortened by treatment with the exonuclease BAL 31 before digestion of d48 DNA with *Hin*dIII. Treatment with BAL 31 affects sequences at the ends of chromosomes due to its single- and double-stranded DNA exonuclease activity (9). We conclude that the majority of the A i-antigen gene is absent in d48 macronuclear DNA, and sequences upstream from this gene are located near the end of a macronuclear chromosome in d48 (Fig. 1).

Macronuclear DNA from strain d16 is characterized by the absence of the 8.8-kb *Hin*dIII fragment encompassing the 3′ end of the A i-antigen gene and the appearance of a new, broad band slightly smaller than 3.7 kb. That this band represents a modification to the sequences in the vicinity of the 3′ end of the gene is verified when the 2.2-kb *Eco*RI fragment, specific for the 3′ end of the gene (Fig. 1), is used as a probe. This probe hybridized to the same, broad 3.7-kb band in d16 *Hin*dIII digests (data not shown). The breadth of this band indicated that it also may be located on the end of a macronuclear chromosome. This was verified when it was preferentially shortened by pretreatment with BAL 31 (data not shown). We conclude that an X-ray-induced deletion or rearrangement has placed the 3′ end of the A i-antigen gene in the vicinity of the end of a macronuclear chromosome in d16 macronuclear DNA (Fig. 1). To determine whether transcribed sequences were included in the deletion, the S1 experiment illustrated in Fig. 4 was performed. Polyadenylated RNA from both wild-type and d16 cells protected the 3′ end of the cloned gene from S1 nuclease digestion to the same extent, and thus the transcriptional unit in d16 is intact.

Breeding analysis. Since strain d16 was phenotypically wild type, the progeny in crosses involving d16 were scored

TABLE 2. Inheritance of the mutant traits

Cross no.	Cross[a]	No. of wild-type strains/no. of mutant strains (significance)[b] in F_2 by autogamy	Breakdown of F_2 data					
			Descendants (no.) from wild-type cytoplasm			Descendants (no.) from mutant cytoplasm		
			Wild type	Mutant	Marker segregation (*tw*:*tw*$^+$)	Wild type	Mutant	Marker segregation (*tw*:*tw*$^+$)
1	d16 × 51 (*tw*)	31/35 ($\chi^2 = 0.24$, $P > 0.5$)	17	15	17:15	14	20	16:18
2	d8 × 51 (*tw*)	35/37 ($\chi^2 = 0.06$, $P > 0.5$)	19	17	20:16	16	20	12:24
3	d29 × 51 (*tw*)	27/17 ($\chi^2 = 2.27$, $P > 0.1$)	16	10	17:9	11	7	8:10
4	d48 × 51 (*tw*)	81/32 ($\chi^2 = 21.25$, $P < 0.01$)	68	0	37:31	13	32	24:21
Pair 1[c]			16	0	9:7	0	11	6:5
Pair 2			9	0	6:3	10	0	5:5
Pair 3			24	0	11:13	0	18	11:7
Pair 4			19	0	11:8	3	3	2:4

[a] Mutant traits are characterized as follows: d16, deletion of 3′ flanking sequences of A i-antigen gene; d8 and d29, expression of an unstable A i-antigen; d48, noninducible for A i-antigen expression and absence of A i-antigen gene in macronucleus.

[b] χ^2 values were calculated with an expected ratio of 1:1.

[c] Individual pair data are given to demonstrate the variable patterns of recovery of the mutant trait in strain d48 cytoplasmic progeny.

TABLE 3. Inter-mutant strain crosses

Cross no.	Cross	Characters in F_2 (no. of strains) by autogamy [A i-antigen stability/3′ flanking sequences] or A i-antigen stability alone					
		S/WT	S/16	U/WT	U/16	S	U
5	d8 × d16[a]	7	5	8	5		
6	d29 × d16[b]	12	15	10	10		
7	d29 × d8[c]					18	46

[a] $\chi^2 = 1.08$, $P > 0.5$. Values were calculated with an expected ratio of 1:1:1:1.
[b] $\chi^2 = 1.43$, $P > 0.5$. Values were calculated with an expected ratio of 1:1:1:1.
[c] $\chi^2 = 0.33$, $P > 0.5$. Values were calculated with an expected ratio of 1:3 (S:U).

for the deletion of A i-antigen gene 3′ flanking sequences by Southern analyses similar to that shown in Fig. 2. For crosses involving strain d48, progeny were scored for their ability to produce serotype A at 35°C and often for the presence or absence of a genomic copy of the A i-antigen gene in macronuclear DNA from vegetative clones. In every case where the progeny were scored by both methods, the ability to express the A serotype was correlated to the presence or absence of the A i-antigen gene. The progeny of crosses involving d8 or d29 were scored for their ability to express a stable serotype A. This was done by growing tubed populations of progeny lines at 35°C for 5 days at a one fission per day growth rate. This resulted in the maximal expression of serotype A by wild-type, d8, and d29 cells as described in Table 1. Growth was then continued at 27°C for 4 days at the same growth rate. Whereas wild-type populations maintain a high expression rate of serotype A at the conclusion of this procedure, d29 populations will invariably switch completely away from the expression of serotype A by the end of the 27°C growth, and d8 populations will have dropped to an average of less than 5% expression of A.

The results presented in Tables 2, 3, and 4 are restricted to the F_2 generations because, due to the occurrence of autogamy in starved cells more than 20 fissions old (from a previous nuclear reorganization), it is difficult to maintain an F_1 generation while attempting to score it for traits which are identified by prolonged growth and intermittent periods of starvation. Although this means that the induction of serotype A was not attempted in F_1 lines, the serotypes of these lines were monitored during their growth at 27°C and preparation for the F_2 generation. The occurrence of F_1 lines expressing serotype A indicated a wild-type phenotype since the F_1 lines were under conditions which do not allow expression of serotype A in cells of strain d8, d29, or d48. Thus in certain crosses, the dominant or recessive nature of a mutation was determined, and these results are presented in the text.

Table 2 shows the results of crossing the four mutant strains to wild-type cells of stock 51 carrying the twisty mutation. The mutant traits carried by strains d16, d8, and d29 are inherited as single Mendelian factors, and we conclude that they result from single gene mutations. The continued expression of serotype A by several F_1 lines on the wild-type side of the d8 and d29 crosses indicates that these are both recessive mutations (data not shown). Surprisingly, the results of the d48 cross demonstrate that the macronuclear deletion present in d48 is inherited in a non-Mendelian fashion. The F_2 progeny from cross 4 (Table 2) deviate significantly from the 1:1 ratio expected for a Mendelian trait. The reason for this deviation is evident when the data are broken down into parental cytoplasmic lineages. Whereas mutant and wild-type progeny are derived from the d48 cytoplasmic parents, only wild-type progeny are produced from the wild-type cytoplasmic parents. The cytoplasmic effect is evident on the mutant cytoplasmic side when one looks at the individual pair data from this cross. In some pairs, all the F_2 progeny from the d48 cytoplasmic parent are mutant (pairs 1 and 3), whereas in other pairs, the d48 cytoplasmic progeny are all wild type (pair 2). Segregation in the F_2 progeny from a single d48 cytoplasmic exconjugant can also be found (pair 4). In each of these pairs, the d48 cytoplasmic F_1 lines did not express serotype A, and DNA isolated from the F_1 lines was void of the A i-antigen gene. It should be noted that these four pairs were all obtained by mating wild-type cells to a population of d48 cells cloned from a single isolate from a d48 stock tube. If the differences between the pairs represent differences in the d48 parents, then these differences developed shortly (15 fissions or less) before the cross in vegetative descendents from a single cell.

Tests of linkage among strains d8, d29, and d16. The data presented thus far have shown that strains d8 and d29 carry single gene mutations. Small deletions or rearrangements in or surrounding the A i-antigen gene of either of these strains are not totally excluded by the Southern data, and crosses were performed to determine whether the d8 and d29 mutations were in fact linked to the A i-antigen gene. The d16 mutation, previously defined as a deletion for sequences just downstream from the A i-antigen gene, was used as a marker for the A i-antigen gene. The progeny in these crosses were scored for the production of a stable (S) or unstable (U) A i-antigen, and for the presence (WT) or absence (16) of the 3′ flanking sequences of the A i-antigen gene. If the mutations causing an unstable A i-antigen in strains d8 and d29 are unlinked to the A i-antigen gene, we would expect a 1:1:1:1 ratio of S/WT, S/16, U/WT, and U/16 cells in the F_2 generation. Table 3 shows the results of these crosses (crosses 5 and 6). Although the numbers were small, close linkage of either the d8 or the d29 mutation to the d16 deletion was clearly ruled out. A chi-square analysis comparing the observed data with the expected results, assuming various degrees of linkage, indicated that the minimum recombination frequency between the d18 and d16 mutations was 25%, and the minimum recombination frequency between the d29 and d16 mutations was 30%. To determine whether strains d8 and d29 carry mutations of the same gene or closely linked DNA sequences, these two strains were crossed (Table 3, cross 7). Approximately one-fourth of the F_2 by autogamy expressed a stable A i-antigen, suggesting that d8 and d29 carry mutations of unlinked gene loci even though they have similar phenotypes.

Nature of the d48 mutation. In the crosses presented in Table 2 and a number of subsequent crosses involving strain d48, all F_2 lines derived from the wild-type cytoplasmic parent lacked the d48 mutant trait. If the trait was due to a simple deletion carried in the micronucleus, 50% of the F_2 lines should show the trait. Selective elimination of mutant progeny on the wild-type cytoplasmic side of the crosses cannot account for these results since the F_2 viability averaged 88% in the d48 crosses presented in Table 2.

Two hypotheses have been considered to explain these results. In the first hypothesis, d48 cells do not contain a copy of the A i-antigen gene in their micronuclei or macronuclei. The F_1s resulting from crossing d48 to wild-type cells would initially be hemizygous for the A i-antigen gene, but in F_1 cells carrying wild-type cytoplasm, there is a duplication of the A i-antigen gene to make that sequence diploid. This

TABLE 4. d48 × d16

Pair no. from d48 × d16	Characters in F_2 (no. of strains) by autogamy							
	Descendants from d16 cytoplasm				Descendants from d48 cytoplasm			
	A i-antigen		Character ratio		A i-antigen		Character ratios	
	Inducible	Non-inducible	16:WT[a,b]	*tw*:*tw*$^-$	Inducible	Non-inducible	16:WT[a]	*tw*:*tw*$^+$
1	23	0	3:3	11:12	5	18	4:1	11:12
2	20	0	5:4	9:11	0	22		15:7
3	18	0	4:2	12:6	0	20		8:12
4	21	0	4:2	11:10	0	18		8:10

[a] A sample of A i-antigen-inducible F_2 lines from each pair was analyzed by Southern analysis for the A i-antigen gene and its flanking sequences. The lines were determined to have either the wild-type sequence (WT) or the d16-type deletion (20).

[b] $\chi^2 = 0.93$, $P > 0.1$. The χ^2 value was calculated for an expected 1:1 ratio of 16:WT on the d16 cytoplasmic side of the crosses. When the data for the five A i-antigen-inducible F_2 lines on the d48 cytoplasmic side are included, $\chi^2 = 2.00$, $P > 0.1$.

compensation mechanism does not occur in strain d48 cytoplasm. This model predicts that all the F_2 progeny on the wild-type side of the cross will be wild type and carry an A i-antigen gene and its flanking sequences derived ultimately from the wild-type parent. In the second hypothesis, the micronucleus of d48 is diploid for a complete copy of the A i-antigen gene and its flanking sequences, but these sequences do not become incorporated in the macronucleus in d48 cytoplasm. When crossed to wild-type cells, the wild-type cytoplasm allows the A i-antigen gene from d48 to become incorporated in the macronucleus of both F_1 and F_2 progeny on the wild-type side of the cross. This model predicts that the F_2 progeny on the wild-type side of the cross will be homozygous for the A i-antigen gene and its flanking sequences derived from either the wild type or d48 parent in a 1:1 ratio.

It is possible to decide between these two hypotheses by crossing strain d48 to strain d16, which carries a deletion in the 3′ flanking sequences of the A i-antigen gene in both its micronuclei and macronuclei. The appearance in the progeny of sequences missing in the d16 parent would be expected if the micronucleus of d48 contains these sequences as predicted by the second hypothesis. The first hypothesis predicts all F_2 progeny on the d16 side to be d16 mutants. Table 4 gives the results of crossing d48 with d16. The pattern of inheritance of the d48 trait is the same as when d48 was crossed with wild-type stock 51. As predicted by the second hypothesis, a wild-type and a d16 Southern pattern were found to segregate in an acceptable 1:1 ratio on the d16 side of the cross. The wild-type copy of the A i-antigen gene and its downstream sequences in the F_2 progeny, containing sequences totally absent in the d16 parent, must have come from the d48 parent. We conclude that cells of stock d48 contain a wild-type copy of the A i-antigen gene and its downstream sequences in their micronuclei, but these sequences are not incorporated in the transcriptionally active macronuclei.

DISCUSSION

Serotype expression. In this paper, a method for the selection of mutations of serotype regulation and expression was presented. An analysis of two of the mutations isolated by this method identified two unlinked gene loci which are important for the normal expression of serotype A and possibly serotype D. The mutation of either of these loci resulted in the almost exclusive expression of serotypes C and E. The nature of these genes and their possible products are not presently known. They were the first identified loci outside of the i-antigen genes which affect serotype expression, and their identification suggests that further loci important for serotype regulation will be found. Thus, it is premature to speculate on mechanisms.

A further mutation was recovered in our screen which, in its present form, had no apparent effect on serotype expression. This mutation, carried by strain d16, was defined molecularly as a deletion of the 3′ flanking sequences of the A i-antigen gene. This deletion places the A i-antigen gene in close proximity to the end of a macronuclear chromosome and demonstrates that the 3′ flanking sequences are not important for the expression of the A i-antigen.

In interpreting the results in several of the crosses, we assumed that the micronucleus of d16 carries the same deletion as the macronucleus. The analysis of strain d48 demonstrated that macronuclear genotypes do not always reflect micronuclear genotypes. Although the possibility exists that the d16 mutation is not a simple micronuclear deletion, we prefer the more classical interpretation of the results until evidence is obtained to the contrary.

Macronuclear development. The mutation carried by strain d48 resulted in the cytoplasmic inheritance of a macronuclear deletion. The cells of this strain do not express serotype A because, whereas an intact copy is in the micronucleus, the A i-antigen gene is absent from the transcriptionally active macronucleus. Wild-type cells have an intact A i-antigen gene in their macronuclei whether or not they are expressing serotype A (8). The d48 mutation represents a defect in the normal development of the macronucleus. The inheritance of this defect is analogous to mating type inheritance in *P. tetraurelia*, and suggests that the A i-antigen gene has come under the control of a mechanism similar to that used in determining macronuclei for mating type expression. It may well be that mating type determination, and possibly other ciliate macronuclear determinative events (22), also results from the presence or absence of specific DNA sequences in the macronucleus, as proposed by Orias (15) for mating type determination in *Tetrahymena* sp. We present the first evidence that a mechanism is available in ciliates to control the expression of a gene by regulating its incorporation into developing macronuclei.

In crosses involving d48, reversions to wild type in cells carrying d48 cytoplasm were not correlated to switches in mating type (data not shown). This demonstrates that determination of the macronucleus for each of these traits is under separate control. Another example of macronuclear determination has been studied in *Paramecium* sp. by Sonneborn and Schneller (23) and Nyberg (14). They worked with derived stock d113 and wild-type stock 146 of *P. tetraurelia*,

respectively. They report that the inability to discharge trichocysts in these stocks is inherited not as a Mendelian trait, but, as with mating types, is determined in the macronucleus under the influence of cytoplasmic factors. Reversions of the ability to discharge trichocysts were also not correlated to switches in mating types.

The nature and derivation of the cytoplasmic factors involved in this mechanism are unknown, as is the nature of the induced mutation in strain d48. That the mutation is inherited as a non-Mendelian trait does not mean that micronuclear sequences are not involved. P-M hybrid dysgenesis (10) and homolog destabilization (11) in *Drosophila melanogaster* and the paramutations found in the R gene of maize (4) are just a few examples of systems involving nuclear changes that are not inherited as Mendelian traits. Alternatively, the d48 defect could be located strictly in the macronucleus, indicating that ancestral macronuclei contain and code for information necessary for the integration of specific sequences in newly forming macronuclei. To decide between these possibilities, it is necessary to compare the A i-antigen gene and its flanking sequences in the macronuclei with the same gene in the micronuclei in both wild-type and d48 cells. We have not yet been able to obtain a sufficiently purified preparation of micronuclei or micronuclear DNA to do this experiment but are hopeful that this will soon be accomplished.

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