SEX-LIMITED EXPRESSION OF GENE LOCI CONTROLLING FLAGELLAR MEMBRANE AGGLUTINATION IN THE CHLAMYDOMONAS MATING REACTION

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ABSTRACT

Mutant strains of *Chlamydomonas reinhardi* that have lost their ability to undergo sexual agglutination *via* their flagellar tips have been induced to undergo zygotic cell fusion and meiosis, using a flagellar-directed antiserum. Genetic analysis of antiserum-mediated crosses involving five nonagglutinating *mt*+ mutant strains reveals the following: (1) None of the mutations is linked to the *mt* locus. (2) All of the mutations are "sex-limited," meaning that they can be carried and transmitted by, but not expressed in, *mt*− cells. (3) Four of the mutations (*imp-2*, *imp-5*, *imp-6*, *imp-7*) are either allelic or closely linked to one another, with *imp-8* defining a second locus.

SEXUAL differentiation is likely to have been among the earliest processes to have evolved in the lower eukaryotes. Although considerable phenotypic diversity is evident in the mating behavior of modern protists (METZ and MONROY 1969), three underlying principles can be recognized: (1) Two mating types (*mt*) are, in general, involved. (2) Cell-surface recognition between gametes of opposite *mt* precedes the fusion of cells and/or nuclei. (3) The capacity to interact sexually with a gamete of opposite *mt* is generally not constitutive, but is induced by some feature(s) of the environment.

In this laboratory we are conducting research to define and characterize those genes that specifically function in the sexual differentiation of the unicellular biflagellate, *Chlamydomonas reinhardi*. The sexual process in this organism is initiated when *mt*+ and *mt*− gametes undergo an agglutination reaction *via* their flagellar tips. Of eight impotent mutants isolated in the *mt*+ strain (GOODENOUGH, HWANG and MARTIN 1976), the five nonagglutinating *imp* strains (*imp-2*, *-5*, *-6*, *-7*, and *-8*) fail to agglutinate sexually with wild-type *mt*− strains (BERGMAN *et al.* 1975) and therefore do not form zygotes. As a consequence, genetic analysis of these strains has to date been indirect and incomplete (GOODENOUGH, HWANG and MARTIN 1976).

We have recently discovered that an antiserum directed against the flagellar surface of *C. reinhardi* elicits mating-structure activation of *imp mt*+ gametes and, therefore, mediates cell fusion between *imp mt*+ and + *mt*− gametes (GOODENOUGH and FOREST 1977; GOODENOUGH and JURIVICH 1977). Here, we report on the genetic analysis of the resultant zygotes.

MATERIALS AND METHODS

Each of the mutant strains, *imp-2*, *imp-5*, *imp-6*, *imp-7*, and *imp-8* was derived from independent UV-mutagenesis experiments (GOODENOUGH, HWANG and MARTIN, 1976), utilizing wild-type strain 137*c-H*, *mt*+ and *mt*⁻ of *C. reinhardi*. As noted previously (GOODENOUGH, HWANG and MARTIN 1976) for crosses involving cells of this strain, meiosis generates the expected four products, but one or more of them sometimes fails to divide mitotically to generate a clone for phenotypic analysis. Subsequent to our 1976 publication, we became aware that an identical pattern of lethality was noted by SMITH and REGERY (1950) in their first published crosses involving the 137*c* strain of *C. reinhardi*. The lethality phenomenon, therefore, appears to be more accentuated in some clones than in others, but is not a recently acquired property of the strain. In the present paper, tetrads were scored only if they were complete or if at least three of the meiotic products could be analyzed, in which case the genotype of the missing fourth product could be deduced. As before (GOODENOUGH, HWANG and MARTIN 1976), no genotype was selectively affected by the lethal process.

Mutant (*imp mt*+) and wild-type (+ *mt*+ and + *mt*⁻) strains were allowed to undergo gametogenesis on agar plates, as previously described (MARTIN and GOODENOUGH 1975). In an antibody-mediated mating, *imp mt*+ and + *mt*⁻ gametes were mixed in the presence of a 1:50 dilution of filter-sterilized antiserum directed against + *mt*+ gametic flagella. Higher antiserum concentrations (1:2 or 1:5) were used for matings involving *imp-7*, since this strain fails to activate at low antiserum concentrations (GOODENOUGH, JURIVICH and BRAUSER, manuscript submitted). After 0.5–1 hr of agglutination, the mating mixture was spread on 4% agar plates containing tris-acetate phosphate (TAP) medium (GORMAN and LEVINE 1965); zygote maturation, germination, and tetrad analysis were performed by standard procedures (LEVINE and EBERSOLD 1960). The crosses summarized in Table 3 were subjected to "total product analysis": a gridwork was drawn on a plate containing 1.5% agar-TAP medium; a single zygote was placed in the center of each gridsquare; the four meiotic products of each zygote were allowed to grow without being separated, so that each gridsquare came to contain a colony composed of four clonal types; the phenotype of each colony was then assessed as described in RESULTS.

Mating type and mating capacity were tested as follows. A clone derived from a single meiotic product was allowed to undergo gametogenesis on plates and was then suspended in two small tubes, each containing 1.0 ml of nitrogen-free minimal medium (SUEOKA, CHIANG and KATES 1967). An equivalent number of + *mt*+ tester gametes was added to one tube, while + *mt*⁻ testers were added to the second tube. Each tube was scored the next morning for the presence or absence of the thick pellicle of zygotes that marks a successful mating (SAGER 1955). The clone was in this way scored as *mt*+ (pellicle with + *mt*⁻ testers), *mt*⁻ (pellicle with + *mt*+ testers) or *imp* (no pellicle in either tube). All the *imp* clones exhibited normal growth and motility, but failed to agglutinate sexually.

RESULTS

Zygotes produced by antibody matings: Zygotes that form during an antibody mating are indistinguishable from zygotes formed "naturally" (SAGER 1955); they secrete thick walls on plates, form dense pellicles in liquid medium, and exhibit standard patterns of maturation and germination. The *imp* mutations under study, therefore, appear to affect sexual agglutination specifically, since the ensuing events of fusion and zygote maturation are normal once the agglutination defect is bypassed by the antiserum.

Zygote yields in an antibody mating are high, although typically not as high as in natural matings, probably in part because many antibody-mediated agglutinations occur between cells of like *mt* that are unable to fuse (GOODENOUGH and FOREST 1977). Crosses involving *imp-7 mt*+ constitute an exception; as discussed

elsewhere (GOODENOUGH, JURIVICH and BRAUSER, manuscript submitted), antibody matings with this strain are very difficult to perform, since high antiserum concentrations are required to activate the *imp-7* mating structure, yet high antiserum concentrations also inhibit zygotic cell fusion. The data presented below for *imp-7* are therefore unavoidably less extensive than for the other four *imp* strains.

Tetrad analysis of imp mt$^+$ × + mt$^-$ *crosses*: Assuming nonlinkage between the various *imp* mutations and *mt*, as suggested by earlier results (GOODENOUGH, HWANG and MARTIN 1976), the cross *imp mt*$^+$ × + *mt*$^-$ can be predicted to yield the following genotypic tetrad classes:

PD[1]	*NPD*	*T*
imp mt$^+$	+ *mt*$^+$	+ *mt*$^+$
imp mt$^+$	+ *mt*$^+$	*imp mt*$^+$
+ *mt*$^-$	*imp mt*$^-$	+ *mt*$^-$
+ *mt*$^-$	*imp mt*$^-$	*imp mt*$^-$

[1] PD = parental ditype; NPD = nonparental ditype; T = tetratype.

with *PD* = *NPD* and with *T* tetrads representing the second-division segregation of one or both pairs of alleles. When zygotes produced by antibody-mediated *imp-mt*$^+$ × + *mt*$^-$ matings are subjected to tetrad analysis, the following phenotypic tetrad classes are encountered:

I	*II*	*III*
no agglutination	normal *plus*	normal *plus*
no agglutination	normal *plus*	no agglutination
normal *minus*	normal *minus*	normal *minus*
normal *minus*	normal *minus*	normal *minus*

The numbers of each phenotypic class for each of the *imp* crosses are given in Table 1, Section A.

TABLE 1

Tetrad analysis of imp mt$^+$ × mt$^-$ *antibody-mediated matings*

A. Phenotypic tetrad class (see text)				B. Tetrad distribution					
	I	II	III	Total tetrads	PD:NPD (predict 1:1)	T tetrads Pred.	T tetrads Obs.	χ^2 values*	*P* values (df=2)
imp-2 mt$^+$ × *mt*$^-$	10	7	31	48	10:7	32	31	0.66	0.8–0.7
imp-5 mt$^+$ × *mt*$^-$	3	4	6	13	3:4	8.6	6	2.53	0.3–0.2
imp-6 mt$^+$ × *mt*$^-$	2	1	15	18	2:1	12	15	2.41	0.3–0.2
imp-7 mt$^+$ × *mt*$^-$	1	1	2	4	1:1	2.6	2	0.51	0.8–0.7
imp-8 mt$^+$ × *mt*$^-$	4	4	16	24	4:4	16	16	0.00	>0.99

* Because of the small sample size, a homogeneity test was performed on the pooled data to test the hypothesis that all of the mutations derive from the same population. The data conformed to the hypothesis ($\chi^2 = 5.97$, df = 8, $P = 0.7$–0.5). Using the pooled data, the hypothesis of a 1/6 P, 1/6 NPD, 2/3 T distribution is also confirmed ($\chi^2 = 0.312$), df = 2, $P = 0.9$–0.8).

Class I tetrads are immediately recognized as PD tetrads. Class II tetrads can be classified as NPD's and Class III tetrads as T's by making one assumption, namely, that cells with an *imp mt⁻* genotype have a normal-minus phenotype. If such an assumption is valid, then of the two normal-minus clones in a Class III tetrad, one should be a true + *mt⁻* clone and yield no *imp* progeny in a backcross to + *mt+* wild-type cells, while the other should be in "*imp* carrier" [which we designate (*imp*) *mt⁻*] and should give rise to nonagglutinating progeny in a + *mt+* backcross. Table 2 summarizes the results of several such backcrosses. Looking, for example, at *imp-2*, the Class III zygote designated #1 yielded two normal-minus clones. Each was backcrossed to + *mt+* gametes, and several of the resultant zygotes were subjected to tetrad analysis. For the cross involving the first normal-minus clone, all five tetrads analyzed contained two normal *mt+* and two normal *mt⁻* products; the genotype of this clone therefore appears to be + *mt⁻*. In contrast, for the zygotes obtained using the second clone, the Class-I, -II, and -III pattern of phenotypes again emerged, with the *imp* mutation being expressed only in cells deduced to be *mt+*. Similar results were obtained for two other *imp-2* tetrads and for *imp-5* and *imp-6* tetrads (Table 2). Moreover, similar backcrosses involving the two normal-minus clones in Class II tetrads revealed that both clones are *imp* carriers, again as predicted. It is concluded, therefore, that the *imp* mutations are sex limited in their expression, having no apparent effect on sexual agglutination when they reside in *mt⁻* cells.

In every backcross involving an *imp* carrier, *imp* progeny were found to be generated in the same sex-limited fashion as observed in the original backcrosses. Since no antibody was used in the backcrosses, the sex-limited patterns of phenotypic expression are clearly not antibody induced.

Nonlinkage of imp *and* mt *loci*: The data in Table 1A allow an analysis of the linkage relationship between *mt* and the various *imp loci*. As formulated by

TABLE 2

Results of backcrossing the two normal-minus progeny from class III tetrads to + mt+ *gametes*

Source of the two normal-minus products	Results of backcrossing clonal descendants of the first normal-minus product to + *mt+* gametes			Results of backcrossing clonal descendants of the second normal-minus product to + *mt+* gametes		
	PD	NPD	T	I(PD)	II(NPD)	III(T)
imp-2 mt+ × + *mt⁻* Class III zygote #1	5	0	0	1	2	2
imp-2 mt+ × + *mt⁻* Class III zygote #2	5	0	0	4	0	5
imp-2 mt+ × + *mt⁻* Class III zygote #3	5	0	0	0	3	2
imp-5 mt+ × + *mt⁻* Class III zygote #1	5	0	0	0	4	3
imp-6 mt+ × + *mt⁻* Class III zygote #1	4	0	0	0	1	3

SHULT and LINDERGREN (1958), if in two-factor crosses two genes are not linked to one another nor closely linked to their respective centromeres, then the tetrad distribution should be such that *PD* equals *NPD*, and the number of *T* tetrads is predicted to equal two-thirds of the total tetrads analyzed. As seen in Table 1B, the data conform to this pattern; we therefore conclude that none of the *imp* loci is linked to *mt*.

Pairwise imp *crosses*: The linkage relationships between the various *imp* mutations were determined by the following rationale. If, on the one hand, two *imp* mutations (*imp-a* and *imp-b*) are allelic or very closely linked, then a cross between an *imp* strain and *imp* carrier is written *imp-a* $mt^+ \times$ (*imp-b*) mt^-; all mt^+ progeny will carry either *imp-a* or *imp-b*, and no agglutinable mt^+ progeny will be present among the four meiotic products. If, on the other hand, *imp-a* and *imp-b* are readily separated by recombination and/or independent assortment, then the cross is rewritten as *imp-a* $+ mt^+ \times +$ (*imp-b*) mt^-, and normal $+ + mt^+$ progeny will often be expected.

Using this rationale, pairwise antibody-mediated matings were performed between the original *imp* mt^+ strains and identified (*imp*)mt^- carriers; individual zygotes were manipulated by the total-progeny technique (see MATERIALS AND METHODS); each zygote colony was suspended in N-free medium overnight; and the tubes were scored the next morning for the presence or absence of zygote pellicle. The presence of pellicle signified that normal mt^+ clones had arisen during meiosis and that the two *imp* mutations in question were separable by recombination; the absence of pellicle indicated that recombination had not occurred. To prove that the mt^- products were normal, $+ mt^+$ gametes were, in some cases, added to tubes where pellicle had failed to form, and a thick pellicle became evident within eight hours.

Results of these crosses are given in Table 3. Each mutant gives no pellicle when crossed against itself [*i.e.*, *imp-2* $mt^+ \times$ (*imp-2*) mt^-], so that none of these strains appears to carry two or more segregating mutations. Strains *imp-2*, *imp-5*, *imp-6* and *imp-7*, when crossed to one another, also failed to generate $+ mt^+$ recombinants in all the individual zygotes tested, since no pellicle is

TABLE 3

Results of pairwise antibody matings between imp mt+ *strains and* (imp) mt- *carrier strains*

Original *imp* mt^+ strain	Carrier (*imp*) mt^- strain: *imp-2* Pell	*imp-2* No pell	*imp-5* Pell	*imp-5* No pell	*imp-6* Pell	*imp-6* No pell	*imp-7* Pell	*imp-7* No pell	*imp-8* Pell	*imp-8* No pell
imp-2	0	27	0	26	0	8	*		9	10
imp-5	0	23	0	37	0	3	*		14	8
imp-6	0	47	0	41	0	3	*		16	6
imp-7	0	9	0	1	0	9	*		2	2
imp-8	27	9	4	4	4	5	*		0	1

Zygotes yielding recombinant $+ mt^+$ cells give a pellicle (pell) when tested; zygotes containing no normal mt^+ cells give no pellicle.

* Cross not performed.

TABLE 4

Tetrad analysis of imp-2 $mt^+ \times$ (imp-5) mt^- *zygotes*

Tetrad	No. cells in germinating zygote*	No. cells forming colonies	*mt*+ clones (mate with *mt*− testers)	*mt*− clones (mate with *mt*+ testers)	*imp* clones (do not mate)
1	8	3	0	1	2
2	4	2	0	1	1
3	8	6	0	2	4
4	8	7	0	4	3
5	4	4	0	2	2
6	4	4	0	2	2
7	8	4	0	2	2
8	8	7	0	3	4
9	8	8	0	4	4
10	8	7	0	4	3
11	8	8	0	4	4
12	8	8	0	4	4
13	8	7	0	4	3
14	8	5	0	2	3
15	8	6	0	3	3
16	8	8	0	4	4
17	8	8	0	4	4
18	4	4	0	2	2
19	8	8	0	4	4
20	8	8	0	4	4
21	8	4	0	2	2
22†	6	6	0	6	0
23	8	8	0	4	4
24†	3	3	0	3	0
25†	3	3	0	3	0
26	8	8	0	4	4
27	8	6	0	2	4
28	4	4	0	2	2
29	4	4	0	2	2
30	8	6	0	3	3
31	8	6	0	3	3
32	8	3	0	1	2
33	4	4	0	2	2
34	8	7	0	3	4
35	8	6	0	3	3
36	8	6	0	3	3
37	4	1	0	1	0
38	4	4	0	2	2
39	8	8	0	4	4
40	8	3	0	1	2

* A postmeiotic mitotic division often generates 8-celled tetrads.

† Meiosis in these three zygotes was clearly aberrant, as judged by the odd number of meiotic products.

formed. In contrast, pellicle often forms, *i.e.*, + mt^+ recombinants arise, when any of these strains is crossed to strains carrying the *imp-8* mutation.

In addition to the individual-zygote data summarized in Table 3, a large number of unseparated zygotes from each cross was allowed to germinate, and the aggregate population of clonal products was subjected to the same pellicle-formation test. Again, pellicle was present only when *imp-8* was one of the *imp* markers.

Finally, in the case of an *imp-2* $mt^+ \times$ (*imp-5*) mt^- cross, 40 zygotes were subjected to traditional tetrad analysis to determine the segregation ratios of the emerging phenotypes. The results are presented in Table 4. Again, no normal mt^+ clones emerge from any of the zygotes. Moreover, when the scattered lethal effects are taken into account, the products are seen to appear in a 2 mt^- : 2 *imp* ratio, the expected ratio if the two *imp* markers fail to recombine.

DISCUSSION

As developed in more detail elsewhere (GOODENOUGH 1977), it is clear that the plus gametic phenotype for *C. reinhardi* entails at least two distinct traits, namely, the ability to agglutinate as plus and the ability to fuse as plus. Similarly, minus gametic cells always express both minus agglutinability and minus fusability. In a given tetrad, two meiotic products are inevitably plus and two minus; "intermediate" or "hybrid" progeny are never encountered. Two general models can account for these observations: (1) All genes concerned with mating-type-specific traits may be tightly linked in either the mt^+ or the mt^- locus, with no recombination occurring between the two loci; or (2) The mating-type-specific genes may lie outside the *mt* locus but be subject to some form of *mt*-locus control, that is, be sex limited in their expression.

Two gene loci are identified in the present study as falling into this second, sex-limited category, being unlinked to *mt*, carried by mt^- cells, but expressed only in mt^+ cells. The first locus, for which we propose the designation *sag-1* (sexual agglutination), is marked by the *imp-2*, *imp-5*, *imp-6*, and *imp-7* mutations. Whether these mutations represent alleles of one gene or mark a cluster of genes cannot presently be assessed: complementation tests are required, diploid strains of *C. reinhardi* are inevitably mating-type minus (EBERSOLD 1967), and the *sag-1* mutations are expressed only against a mating-type plus background.

The second gene locus, *sag-2*, is marked by the *imp-8* mutation. The fact that five independent mutagenesis experiments have yielded four mutations at *sag-1* and one mutation at *sag-2* suggests that the total number of gene loci concerned with specifying mt^+ sexual agglutinability in *C. reinhardi* may prove to be small (*cf.*, WARREN, WARREN and COX 1975); by comparison some 50 loci are believed to function during the aggregation phase of the *Polysphondylium violaceum* and the *Dictyostelium discoideum* life cycles (WARREN, WARREN and COX, 1975; WILLIAMS and NEWELL 1976).

Sex-limited gene expression is not restricted to mt^+ gametes; FOREST and TOGASAKI (1975) were the first to describe such a phenomenon in mt^- cells of *C. reinhardi*. They isolated a mutant strain, *gam-1* mt^-, which is temperature sensitive for zygote formation, and they demonstrated that the *gam-1* mutation, although unlinked to *mt*, is expressed only in mt^- gametes. The major effect of the *gam-1* mutation is to cause a temperature-sensitive defect in sexual signaling (FOREST and GOODENOUGH 1977; manuscript submitted); its phenotypic effects are therefore readily distinguished from those of the five *imp* strains.

Sex-limited gene expression has also been demonstrated in yeast (EGEL 1973; MACKAY and MANNEY 1974). MACKAY and MANNEY (1974), for example, report the isolation of *ste* mutations that block mating-factor responsiveness in *Saccharomyces cerevisiae*; these are unlinked to the **a**/α mating-type locus and are expressed only in **a** or in α cells.

For these (and other possible) examples of sex-limited gene expression, two general sorts of models can be proposed, which we present in the Chlamydomonas context. (1) In a transcriptional control model, the mt^+ locus is visualized as producing a product—perhaps analogous to a positive control factor such as CAP protein in *E. coli* (ZUBAY, SCHWARTZ and BECKWITH 1970)—that stimulates the transcription of plus-specific gene loci (*e.g.*, *sag-1* and *sag-2*) but not minus-specific loci (*e.g.*, *gam-1*) during gametic differentiation; the mt^- locus would produce a product with the reciprocal specificities. The Davidson-Britten model (DAVIDSON and BRITTEN 1973) predicts the existence of eukaryotic *integrator* genes that exert coordinate control over unlinked loci (see also ARNST 1976), and the *mt* locus could contain one or more such genes. (2) In a post-transcriptional control model, expression of both plus and minus "subsidiary" genes would occur during an mt^+ gametogenesis, but the mt^+ locus would specify a product—a membrane polypeptide, for example—with which the plus-specific gene products could interact but the minus-specific products could not; the reciprocal situation would apply to an mt^- gametogenesis.

Both kinds of models explain equally well why mutations in *sag-1* and *sag-2* fail to affect an mt^- gametogenesis, and the available data cannot distinguish between the models. In either case, a gene that is sex limited in its expression assumes major significance in *C. reinhardi* differentiation, for its expression must come under direct control of the *mt* locus.

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