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The *iso1* Gene of *Chlamydomonas* Is Involved in Sex Determination

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Sexual differentiation in the heterothallic alga *Chlamydomonas reinhardtii* is controlled by two mating-type loci, mt^+ and mt^-, which behave as a pair of alleles but contain different DNA sequences. A mutation in the *mt minus*-linked *imp11* gene has been shown previously to convert a *minus* gamete into a *pseudo-plus* gamete that expresses all the *plus* gametic traits except the few encoded by the mt^+ locus. Here we describe the *iso1* mutation which is unlinked to the mt^- locus but is expressed only in *minus* gametes (sex-limited expression). A population of *minus* gametes carrying the *iso1* mutation behaves as a mixture of *minus* and *pseudo-plus* gametes: the gametes isoagglutinate but they do not fuse to form zygotes. Further analysis reveals that individual gametes express either *plus* or *minus* traits: a given cell displays one type of agglutinin (flagellar glycoprotein used for sexual adhesion) and one type of mating structure. The *iso1* mutation identifies a gene unlinked to the mating-type locus that is involved in sex determination and the repression of *plus*-specific genes.

INTRODUCTION

The unicellular green alga *Chlamydomonas* has four developmental stages: a haploid vegetative cell which divides mitotically, a haploid G_0 gametic cell, a diploid zygote which divides meiotically at the onset of germination, and an alternative pathway that some diploid cells follow where the cells develop as vegetative diploids. As in multicellular organisms, the transition from one stage to the next is regulated by environmental signals and cell-cell interactions. When a vegetative cell is deprived of nitrogen in the presence of light, the cell cycle is arrested and gamete-specific genes are transcribed (Sager and Granick, 1954; Treier *et al.*, 1989; von Gromoff and Beck, 1993). Gametes can be either mating-type *plus* (mt^+) or mating-type *minus* (mt^-) and express *mt*-specific traits (e.g., mating structures and agglutinins). The extracelluar agglutinins are gamete-specific glycoproteins that are displayed on flagella and allow for specific adhesion of cells of opposite mating type (Adair, 1985; Hunnicutt and Snell, 1991). Flagellar agglutination initiates a signaling cascade which causes the gametes to shed their cell walls and activate gamete-specific organelles called mating structures which mediate *mt*-specific cell fusion (Goodenough and Weiss, 1975; Pasquale and Goodenough, 1987). Within 10 min of gamete cell fusion, the diploid zygote activates the transcription of zygote-specific genes (Ferris and Goodenough, 1987; Woessner and Goodenough, 1989).

In most plants and animals, a specialized chromosome, or portion of a chromosome, is required to specify the two sexes of a species. In *Chlamydomonas*, the *mt* locus of linkage group VI determines the mating type of a given cell: a *plus* gamete has inherited the mt^+ locus and a *minus* gamete the mt^- locus (Gillham, 1969; Ferris and Goodenough, 1994). Genetic and molecular studies have demonstrated that the *mt* loci contain multiple genes (Goodenough *et al.*, 1976; Galloway and Goodenough, 1985; Ferris and Goodenough, 1994). When mt^+/mt^- diploid strains differentiate into gametes, they always mate as *minus* gametes (Ebersold, 1967), indicating that mt^- is dominant to mt^+ [*minus* dominance; Galloway and Goodenough (1985)]. Genes have been identified that are

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not linked to the *mt* loci but are expressed only in *plus* or in *minus* gametes; such genes are said to exhibit "sex-limited" expression (Forest and Togasaki, 1975; Goodenough *et al.*, 1978).

The current study was designed to continue the molecular dissection of sex determination in *Chlamydomonas*. In the process of identifying *minus* mutants that could not mate normally, we have isolated and characterized a sex-limited mutant strain, *iso1*, that has unexpected properties: *iso1* gametes agglutinate with themselves (isoagglutinate) but cannot fuse or form zygotes. Clonal *iso1* gametes express either *plus* or *minus* agglutinin and either a defective *plus* or a normal *minus* mating structure, producing a mixed population of *minus* gametes and *pseudo-plus* gametes. The cellular, genetic and initial molecular charaterization of *iso1* has led us to revise previous models for genetic control of sex determination. These data provide the first evidence that a gene unlinked to the *mt* locus is involved in sex determination and *minus* dominance.

MATERIALS AND METHODS

Transformation

Transformation was performed according to Kindle (1990) with the following modifications. The arginine auxotroph *arg-7 mt*$^-$ (CC-1861; all strains were obtained from the *Chlamydomonas* Genetics Center, Duke University, Durham, NC) was treated with gametic lytic enzyme (GLE) (Kinoshita *et al.*, 1992; prepared from a mating of 10^7 cells/ml) to remove cell walls (6×10^6 cells/ml GLE for 1 h under constant illumination). Vegetative cells (2×10^7) were suspended in 300 μl TAP medium (Harris, 1989), to which was added 100 μl of 20% polyethylene glycol and 2 μg of CsCl-purified, supercoiled pARG7.8 DNA (Debuchy *et al.*, 1989). The cells were mixed with glass beads and vortexed for 15 s. TAP (800 μl) was added, and the cells were plated onto three tris acetate phosphate (TAP) agar plates; 1119 transformants grew as individual colonies. These were transferred to fresh TAP agar plates with sterile toothpicks and screened for the ability to form a thick mat of zygotes called a pellicle (Goodenough *et al.*, 1976). The formal name for the mutant described in this report is *iso1r:pARG7.8*, but will be referred to as *iso1* throughout; its culture collection number is CC-2926.

Flagellar Disc Assay

Flagellar discs were prepared as in Goodenough (1993). Briefly, 5×10^7 plate gametes (Martin and Goodenough, 1975) were subjected to pH shock in 10 mM piperazine-*N*,*N*-'-bis(2-ethantesulfonic acid) (PIPES), 50 μM $CaCl_2$, and the flagella were separated from the cell bodies by centrifugation. The flagella were resuspended in 0.5 ml of the same buffer, allowed to swell osmotically into discs, and stored on ice until assayed.

Gametes were incubated with the discs for about 5 s and then placed on a glass slide and covered with a coverslip. After about 30 s, the preparation was perfused with 2% glutaraldehyde and observed using phase contrast optics with a 63× lens. Isoagglutinating pairs were scored only if there were at least two discs bound to the flagella (either one or more discs on each flagellum or at least two discs on the same flagellum), and no discs were bound at the flagellar tips (the configuration expected if gametes of the same mating type had simply adhered to the exogenous discs). Micrographs were made with a DAGE/MTI model 70 video camera and stored on a 23-inch optical memory disc recorder (Panasonic model QH 3038).

Inhibition of Isoagglutination

Dithiothreitol (DTT; 2 mM), tunicamycin (2 μg/ml final concentration), and cycloheximide (7 μM) were purchased from Sigma (St. Louis, MO). The reagents were added to *iso1* gametes that had been suspended for at least 1 h in nitrogen-free high salt medium (NF-HSM) (Harris, 1989) and were observed to be isoagglutinating vigorously. Gametes were incubated in DTT for 30 min; controls were mixed with tester strains of the opposite mating type. Tunicamycin and cycloheximide were added to the gametes, and agglutination was monitored at various times after adding the reagents.

EDTA Extraction

Ten plates each of *iso1*, *imp11* (CC-1148), and wild-type *mt*$^+$ (CC-125) gametes (10^{10} cells of each strain) were extracted with 350 ml of 15 mM EDTA, 20 mM PIPES, pH 7.4, for 1 h at room temperature (Adair *et al.*, 1982), and aliquots were mixed with wild-type gametes of opposite mating type to determine the degree of agglutinin extraction. The *iso1* cells had stopped isoagglutinating but still agglutinated with wild-type *plus* gametes, indicating that the *plus* agglutinin had been extracted but that some *minus* agglutinin remained on the flagella. The *imp11* and wild-type *plus* gametes still retained some *plus* agglutinin, but the extraction was stopped because some of the *iso1* gametes had begun to lyse (probably due to cell wall loss). The cells were centrifuged and the supernatant was used for the spot assay (Adair *et al.*, 1982) with wild-type *plus* and *minus* gametes.

Gametic Cell Wall Loss

Gametes were suspended in NFHSM at 5×10^7 cells/ml and pipetted up and down until there were no visible clumps of cells; this was designated as time = 0 min. Equal volumes of wild-type *plus* and *minus* gametes were then mixed together; *iso1* gametes were not mixed with any other gametes. Cells from the parent strain alone were used as a negative control. At the indicated times, cell wall loss was monitored by the ability of the detergent Nonidet P-40 (NP-40; Sigma) to release chlorophyll: 0.5-ml aliquots of the cell suspensions were mixed with equal volumes of 0.2% NP-40, microcentrifuged at 13,000 rpm for 5 min at room temperature, and the absorbance of the supernatant was immediately measured at 440 nm (Waffenschmidt *et al.*, 1993). For each strain tested, the point at which chlorophyll release was maximal was designated as 100% cell wall loss and other measurements were converted to percent cell wall loss.

Electron Microscopy

Wild-type *plus* gametes and *iso1* gametes were fixed and prepared for transmission electron microscopy as described in Goodenough *et al.* (1982).

Diploid Production

iso1 was mated with *arg4 mt*$^+$ (CC-1997) and an *iso1 arg4 mt*$^-$ meiotic product was isolated and mated with an *arg7 mt*$^+$ strain (CC-50). After 1 h, the *iso1 arg4 mt*$^-$ × *arg7 mt*$^+$ mating suspension was plated on TAP agar plates and placed under constant illumination to select for complementing vegetative diploid colonies (Ebersold, 1967). Four days later, 20 separate clones of vegetative prototrophic diploids were isolated, placed on fresh TAP plates, and allowed to differentiate into gametes. These were suspended in NFHSM for at least 1 h and observed microscopically. To determine the mating type of each diploid, diploid gametes were mixed with wild-type *plus* and *minus* gametes and observed microscopically.

To test for the ability of *iso1 mt*$^-$ gametes to fuse with other *minus* gametes, *iso1 arg4 mt*$^-$ gametes were incubated with *arg7 mt*$^-$ (CC-1861) gametes overnight in the presence or absence of membrane permeant dibutyryl-cAMP and isobutylmethylxanthine (IBMX)

which were used to enhance agglutination and mating efficiency (Pasquale and Goodenough, 1987). This "mating" was plated onto TAP agar plates, put under constant illumination, and examined for growth for 2 wk. Control matings of *arg4 mt*$^-$ (CC-1998) × *arg7 mt*$^+$, *arg4 mt*$^-$ × *arg4 mt*$^+$, and *arg7 mt*$^-$ × *arg7 mt*$^+$ were done in parallel in the presence and absence of dibutyryl-cAMP and IBMX.

DNA Blot Hybridization Analysis

Progeny from a wild-type *mt*$^+$ × *iso1 mt*$^-$ cross were grown on TAP agar plates, and DNA was extracted from each meiotic progeny strain using the rapid miniprep procedure of Newman *et al.* (1990), with the following modifications: the DNA was extracted once with phenol and once with phenol/chloroform before ethanol precipitation.

For DNA blot hybridization analysis, 5–10 μl of miniprep genomic DNA was digested with both *Sal*I and *Eco*RI, electrophoretically separated on a 0.7% agarose Tris borate EDTA gel, transferred to nitrocellulose and baked at 80°C under vacuum. Hybridization and wash conditions were as described in Church and Gilbert (1984); probes were radiolabeled using a Random Primed Labeling Kit (Boehringer Mannheim, Indianapolis, IN). The template for the probe was a polymerase chain reacton product which extended from the ampicillin resistance gene through the origin of replication sequence of pARG7.8 (see Figure 1). The 5′ primer sequence was CGCACGCGGCGCATCTCG, the 3′ primer was TTTTCGGGGAAATGTGCG. The template DNA was pARG7.8 and the reaction conditions were 30 cycles of 94, 65, 72°C for 1 min each.

Growth Rate Analysis

On replicate plates, 7.5 × 10^4 cells of *iso1 mt*$^-$, *iso1 mt*$^+$, wild-type *mt*$^-$ (CC-124) and wild-type *mt*$^+$ were plated on either six TAP agar plates or six TAP plus arginine agar plates. Every 24 h over the next 3 d, cells from duplicate plates were fixed with 2% formaldehyde and two separate aliquots from each plate were counted and the results averaged.

RESULTS

iso1 Gametes Express Either plus or minus Agglutinins

To identify genes involved in sexual reproduction of *C. reinhardtii*, mutagenesis was performed by nuclear transformation (Tam and Lefebvre, 1993). The parent strain was a *mt*$^-$ arginine auxotroph (*arg7*) and the plasmid used for transformation, pARG7.8 (Figure 1), contained the *C. reinhardtii* genomic DNA encoding argininosuccinate lyase (Debuchy *et al.*, 1989). Transformants were selected by plating all potentially transformed cells on arginine-free medium. Prototrophic colonies (1119) were transferred to additional plates and individually tested for their ability to form a pellicle, a thick mat of zygotes, when mixed with wild-type *plus* gametes. Several putative mating mutants were examined further by mixing mutant gametes of interest with wild-type *plus* gametes and observing the resulting mixture microscopically. Most putative mating mutants had defective flagella and thus could not agglutinate properly. However, one mutant (*iso1*) appeared to swim normally but some cells were grossly deformed in size, shape and flagellar number. Initially, this deformed morphology was believed to be the reason the cells could not mate efficiently. Upon closer inspection of *iso1* (Figure 2a), it became apparent that some *iso1* gametes were agglutinating with each other (*iso*agglutinating). To eliminate the possibility that the strain was not clonal, vegetative *iso1* cells was recloned several times and not only did the isoagglutination seem more prevalent, but the morphological deformities diminished. Isoagglutinating *iso1* gametes continued to isoagglutinate for more than 48 h but no zygotes, which are chloroform resistant, were detected when the cells were transferred to agar plates, treated with chloroform, and allowed to grow. To determine whether isoagglutinating cells were capable of fusing with other *minus* gametes, a constructed *iso1 arg4 mt*$^-$ strain (see MATERIALS AND METHODS) was "mated" with *arg7 mt*$^-$. In three independent experiments, gametes were incubated overnight in the presence or absence of IBMX and dibutyryl-cAMP, which enhances mating efficiency (Pasquale and Goodenough, 1987), and placed on agar medium lacking arginine. While "matings" involving *iso1* failed to produce any prototrophic diploids, control matings (*arg7 mt*$^+$ × *arg4 mt*$^-$) were capable of forming prototrophic diploids. Cells from the *iso1* "mating" were examined microscopically for the presence of quadriflagellated cells; none were identified, although definitive scoring

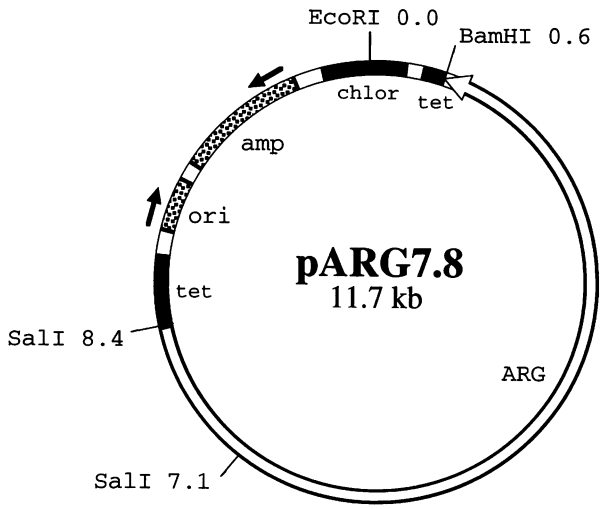

Figure 1. Schematic diagram of plasmid pARG7.8 used for transformation, with the location of restriction sites marked in kilobases (kb) from zero (the *Eco*RI site). The argininosuccinate lyase gene (ARG) is represented by the open arrow; it begins at the *Sal*I (8.4 kb) site and terminates at the *Bam*HI (0.6 kb) site. This gene was cloned into the *Bam*HI and *Sal*I sites of pBR329, which caused disruption of the tetracycline resistance gene (tet). Also present on this plasmid is the gene encoding chloramphenicol resistance (chlor). The origin of replication (ori) and ampicillin resistance gene (amp) are represented by the stippled boxes. The two arrows indicate the location of the polymerase chain reaction primers used for synthesis of the probe used in Figure 4.

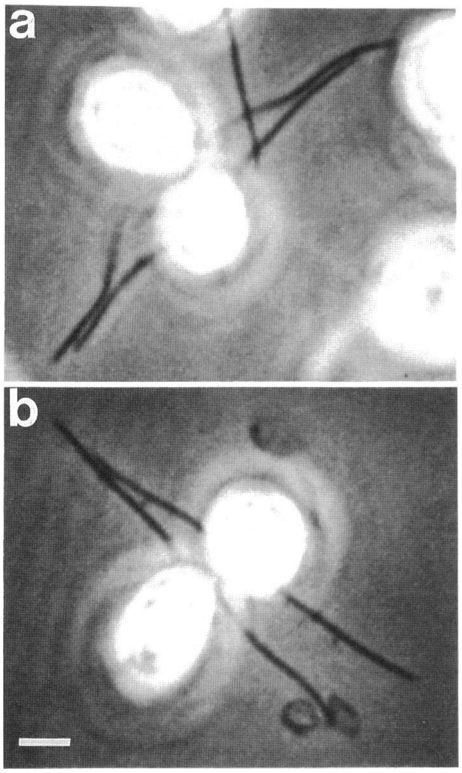

Figure 2. The *iso1* phenotype. Photomicrographs of *iso1* gametes alone (panel a), or incubated with wild-type *plus* flagellar discs (the two dark discs adhering to the flagellum in the bottom right corner of panel b). The gametes in panel b were suspended in NFHSM, incubated with the flagellar discs, and then fixed with glutaraldehyde to facilitate photography. The scale bar in panel b is 5 μm.

of cells was hampered by the deformed morphology of some *iso1* cells.

Four criteria were used to determine that the isoagglutination was due to direct interaction of *plus* and *minus* agglutinins and not a nonspecific flagellar adhesion. 1) Isoagglutination of *iso1* gametes was abolished after 50 min of cycloheximide treatment (Table 1), comparable to the rate for wild-type cells (Snell and Moore, 1980; Cooper *et al.*, 1983). Agglutinin is continuously sloughed from gamete flagella and replenished first from a preexisting intracellular pool and then by a cycloheximide-sensitive synthesis of additional agglutinin (Snell and Moore, 1980; Saito *et al.*, 1985). 2) Isoagglutination of *iso1* gametes is abolished by the mt^+ agglutinin inactivators DTT (Saito and Matsuda, 1986) and tunicamycin (Saito and Matsuda, 1984) with incubation conditions comparable to parallel wild-type mt^+ controls (Table 1). 3) Isoagglutination is gamete-specific; vegetative *iso1* cells do not isoagglutinate, nor do they agglutinate with wild-type gametes or their isolated flagella. Reciprocally, there is no detectable increase in the number of agglutinating cells when *iso1* gametes are mixed with vegetative cells or

Table 1. Inhibition of isoagglutination

Treatment	Effect on gametic agglutination	
	mt^+	*iso1*
DTT, 2 mM	No agglutination	No isoagglutination
Tunicamycin, 2 μg/ml	3-4 h to inhibit agglutination	3-4 h to inhibit isoagglutination
Cycloheximide, 7 μM	45 min to inhibit agglutination	50 min to inhibit isoagglutination

The indicated gametes were incubated with either DTT, tunicamycin, or cycloheximide and aliquots were observed to determine the extent of isoagglutination or agglutination when mixed with tester gametes of the opposite mating type. Cells exposed to DTT were incubated for 30 min before observing.

the isolated flagella from vegetative cells, indicating that *iso1* gametes do not agglutinate with vegetative cells. 4) Finally, the agglutinins from *iso1* gametes were extracted with EDTA and analyzed for the presence of *plus* and *minus* agglutinin activity using a bioassay (Table 2) (Adair *et al.*, 1982). The presence of *plus* agglutinin was confirmed by the specific binding of wild-type *minus* gametes to the spotted *iso1* EDTA extract; the presence of *minus* agglutinin in the same extract was similarly confirmed using *plus* gametes. Therefore, the isoagglutination appears to be a gamete-specific phenomenon due to *plus* and *minus* agglutinin interaction.

To ask whether a single *iso1* gamete within a clonal population expresses both *plus* and *minus* agglutinin, or whether some gametes express only *minus* agglutinin while others express only *plus* agglutinin, isoagglutinating gametes were mixed with purified flagella discs from wild-type *plus* gametes. If an individual *iso1* gamete expresses both *plus* and *minus* agglutinin, then there should be pairs of isoagglutinating cells in which all four flagella bind the exogenous *plus* flagella discs. However, no such cells were seen. Likewise, no gametes were observed to agglutinate with themselves (i.e., one flagellum isoagglutinating with the other flagellum from the same cell). What was observed 35 times, in multiple experiments, were pairs of isoagglutinating cells that had more than one exogenous *plus* flagella disc bound to one or both flagella belonging to only one of the two isoagglutinating cells (Figure 2b). Therefore, it appears that there are two subpopulations of *iso1* gametes; some cells express only *plus* agglutinin while others express only *minus* agglutinin.

Normal agglutinin-agglutinin interaction is rapidly followed by cell wall loss through a second messenger cascade involving cAMP (Pasquale and Goodenough, 1987). Isoagglutinating *iso1* gametes shed their cell walls, although not as quickly as wild-type gametes (Figure 3). The delay was not due to a defect in the

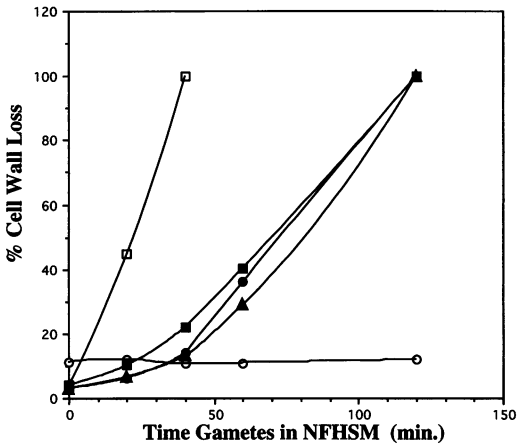

Figure 3. Isoagglutination causes loss of cell wall. Gametes (□, wild-type *plus* and *minus* gametes mixed together; ▲, ●, and ■ *iso1* mt^- gametes alone; ○, *arg7* mt^- parent strain alone) were suspended in NFHSM. At the indicated times, aliquots were incubated with the detergent NP-40. Cells and cellular debris were removed by centrifugation and the amount of chlorophyll released into the medium was spectrophotometrically (440 nm) measured. Results are given as % cell wall loss.

wall-shedding mechanism since *iso1* gametes, like wild-type cells, shed their walls rapidly when given membrane permeant dibututyryl-cAMP and IBMX (Pasquale and Goodenough, 1987). We interpret these results, the data in Table 2, and the fact that *iso1* isoagglutination can be disrupted by pipetting (Forest *et al.*, 1978), to indicate that low levels of *plus* agglutinin are displayed on the *iso1* gametes that behave as *plus* cells, resulting in an attenuated adhesion and cAMP signal and hence slower cell wall loss.

Table 2. Agglutinin extraction

	Source of EDTA extract and tester strain					
	iso1		*imp11*		mt^+	
Fold dilution	mt^+	mt^-	mt^+	mt^-	mt^+	mt^-
0	++++	+++	−	+++	−	++++
1.5	+++	++	nt	nt	nt	nt
2.0	++	+	−	+	−	+
3.0	+	−	nt	nt	nt	nt
4.0	+/−	−	−	+/−	−	−
4.5	+/−	−	nt	nt	nt	nt
8.0	−	−	nt	nt	nt	nt

EDTA was used to extract the flagellar agglutinins from the three strains indicated; *imp11* was used as a comparison since it is an unconditional *pseudo-plus* strain. This extract was spotted onto microscope slides, allowed to dry, and incubated with tester gametes of both mating types. The EDTA extracts were diluted as indicated with water before applying to the slides. The degree of gamete adhesion to the spotted extracts was visually graded on a four point scale with ++++ indicating a high level of cellular adhesion and +/− a low level; − indicated no detectable adhesion; nt indicates dilutions that were not tested.

iso1 Gametes Express Either plus or minus Mating Structures

Chlamydomonas gametes express two *mt*-specific adhesion systems: the flagellar agglutinins described above and an independent set of adhesins, called *plus* and *minus* fringe, localized to the mating structures (Goodenough *et al.*, 1982). The mating structures are located in characteristic sites on the cell (Holmes and Dutcher, 1989) and are structurally distinct: the *plus* mating structure is underlain by a dense doublet zone which nucleates the polymerization of actin in response to an elevation in cytosolic cAMP, whereas doublet zones are absent from *minus* mating structures. When *iso1* gametes were examined by electron microscopy, both *plus* and *minus* mating structures were observed (Figure 4), although never both types on the same cell. Importantly, although the *iso1 plus* mating structures possessed doublet zones which nucleated actin polymerization, they lacked the *plus* fringe (Figure 4, A and B). Such a fringe-less phenotype has been encountered in two other mutant strains (Goodenough *et al.*, 1982): *imp1* mt^+, believed to carry a mutation in a mt^+-linked gene essential for fringe synthesis/localization, and the *pseudo-plus* strain *imp11* mt^- believed to carry a mutation in a *mt minus*-linked gene essential for the expression of *minus* gametic traits. Further consideration of the *pseudo-plus* phenotype is reserved for the DISCUSSION.

Characterization of the iso1 Mutation

If *iso1* cells remain on an agar plate for several weeks, the number of cells that isoagglutinate decreases until the phenotype is completely lost and cannot be recovered by backcrosses. However, cells that have permanently lost the isoagglutinating phenotype have not done so by excising the inserted plasmid DNA since it is still present, as determined by DNA blot hybridization (15 tetrads were examined). On the other hand, if an *iso1* clone exhibits a low percentage (<1%) of isoagglutinating cells, the percentage can be increased by either restreaking vegetative cells and isolating individual colonies or else mating the cells and isolating tetrad progeny. In the best cases, about 50% of the cells are involved in isoagglutination (i.e., a maximum of 25% of the cells behave as *pseudo-plus* gametes). In the process of recloning the strain, the variable number of flagella and deformed cell body phenotypes were reduced to wild-type levels, though some cells did retain a fragmented eyespot. The mating efficiency of *iso1* cells is not as robust as wild-type cells, as demonstrated by the production of a diminished pellicle; the absence of a pellicle during the initial screening appears to have been fortuitous and not persistent.

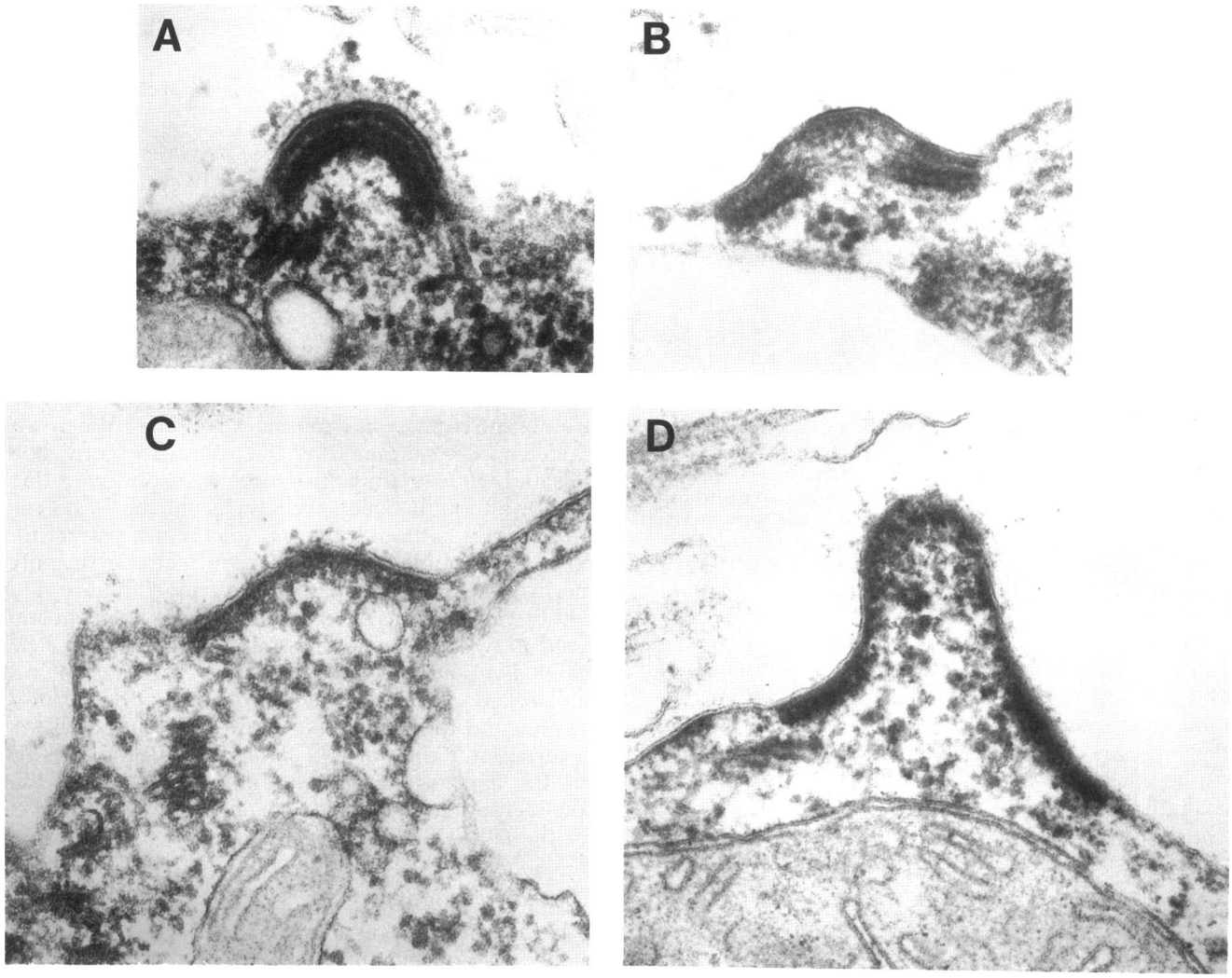

Figure 4. *iso1* displays *plus* and *minus* mating structures. Electron micrographs of mating structures from a wild-type *plus* gamete (panel A), a *pseudo-plus iso1* gamete (panel B), a wild-type *minus* gamete (panel C), and an *iso1* gamete with an activated *minus* mating structure (panel D). *Plus* mating structures are characterized by a dense doublet zone adjacent to the plasma membrane as seen in panels A and B in contrast to the single dense layer seen in panels C and D. Wild-type *plus* gametes display a fringe on their mating structures (panel A), while *pseudo-plus iso1* gametes lack the fringe (panel B). Electron micrographs were taken at 100,000X magnification.

Inasmuch as the isoagglutinating phenotype is unstable, the temperature sensitivity of isoagglutination was tested. *iso1* gametes were produced on agar plates or in liquid medium (Forest *et al.*, 1978) at 10, 15, 25, 30, 32, or 35°C. No effect on the proportion of isoagglutinating cells was detected, indicating that the expression of *plus* and *minus* traits is not temperature-sensitive.

To determine if the *iso1* mutation is recessive or dominant, vegetative diploid cells (Ebersold, 1967) were constructed from the cross *iso1 arg4* mt^- × *arg7* mt^+. Twenty of the resulting vegetative diploid clones were induced to undergo gametogenesis and their phenotypes were determined. As expected (Ebersold, 1967), all 20 isolates agglutinated as *minus* gametes; however, none displayed any isoagglutination, indicating that *iso1* is a recessive mutation.

To verify that the *iso1* phenotype was caused by insertion of the pARG7.8 plasmid, *iso1* was mated with wild-type mt^+ and genomic DNA of 118 meiotic progeny from 32 tetrads was analyzed (Figure 5). Purified genomic DNA was digested with *Sal*I and *Eco*RI, separated by gel electrophoresis, and subjected to DNA blot hybridization with a portion of pARG7.8 used as the probe (see MATERIALS AND METHODS). In the 118 progeny analyzed, two distinctive bands were apparent on the DNA blot and were identical to those seen on blots using DNA from the *iso1* parent. These two bands co-segregated as a unit, indicating that a single insertional event had occurred in

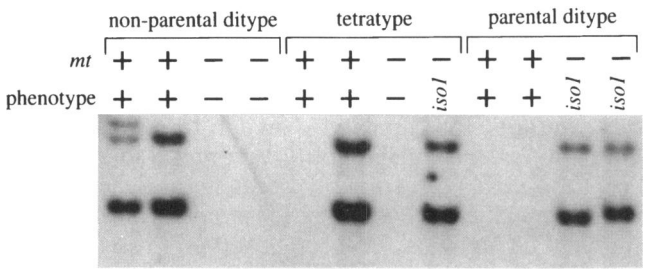

Figure 5. DNA blot hybridization analysis of 3 tetrads resulting from a mating between *iso1* and wild-type *plus* gametes. The progeny were scored for their mating type (+ or −) and whether or not they isoagglutinated ("*iso1*" = isoagglutinating gametes; "+" = agglutinate as wild-type *plus* gametes; "−" = agglutinate as wild-type *minus* gametes). Genomic DNA was isolated from each strain, digested with *Sal*I and *Eco*RI, blotted and probed with a portion of pARG7.8 as indicated in Figure 1 and MATERIALS AND METHODS. The predominant bands are approximately 4 and 7 kb in size.

iso1. Importantly, these bands did *not* co-segregate with the mt^- locus of the *iso1* parent. Although the isoagglutination phenotype was observed in all *minus* gametes which carried the insertion, none of the *plus* gametes carrying the same insertion exhibited any isoagglutination (Figure 5). Therefore, a single insertional mutation has disrupted the wild-type *iso1* gene but its expression is "sex-limited" (Forest and Togasaki, 1975): there is no apparent phenotype in *plus* gametes which carry the same mutation. In a second backcross to wild-type *minus* cells, it was demonstrated that F_1 *plus* cells which carried the pARG7.8 insertion produced F_2 wild-type progeny and isoagglutinating progeny in a 3:1 ratio (21:6; eight zygotes from a single mating). Moreover, the same DNA hybridization banding pattern was observed for the F_2 isoagglutinating cells when their genomic DNA was probed. When *iso1* mt^- was mated with an *arg7* mt^+ strain, arginine prototrophy was inherited 2:2 (20:24; 13 zygotes) and co-segregated with the pair of bands when the genomic DNA was probed on DNA hybridization blots. In addition, isoagglutination was observed in all eight prototrophic mt^- progeny which contained the pair of bands.

A secondary characteristic of *iso1* cells is that they grow slower than wild-type cells on TAP agar plates; this was quantitated by measuring growth rates. However, no difference in growth rate was observed on arginine-supplemented medium. Therefore, the slow growth of *iso1* cells can be attributed to a less efficient expression of the argininosuccinate lyase gene in pARG7.8 transformed *arg-7* cells than in wild-type cells. Since *iso1* gametes produced on arginine supplemented agar plates isoagglutinate as vigorously as gametes grown on nonsupplemented agar plates, the phenotype does not appear to be affected by expression of the transformed argininosuccinate lyase gene.

DISCUSSION

The *iso1* phenotype has provided some new insights into the mechanism used by *Chlamydomonas* for mating-type determination. It has been known for many years that the *mt* locus is critical in *Chlamydomonas* sex determination and regulation of sex-limited gene expression (Goodenough *et al.*, 1976, 1978), and that the mt^- locus is dominant to the mt^+ locus in heterozygous diploids (Ebersold, 1967). The present study is the first to reveal a gene (*iso1*) that is not linked to the *mt* locus but is involved in sex determination, the expression of sex-limited genes, and *minus* dominance, making the *iso1* gene product a key regulatory element for sexual reproduction.

Minus dominance requires two functionally distinct mechanisms: *plus* genes must be repressed while *minus* genes are activated. Previously, the *mid* locus (*minus dominance*) was proposed to contain two genes (Goodenough *et al.*, 1982; Galloway and Goodenough, 1985; Goodenough and Ferris, 1987) responsible for these two aspects of *minus* dominance; m1 was proposed to be the activator of *minus* genes and m2 was proposed to be the repressor of *plus* genes. This model is no longer tenable. From the data presented here, the *iso1* gene appears to be involved in both *minus* activation and *plus* repression. If *iso1* acted only as a *plus* repressor, then gametes that fail to express a functional *iso1* protein would display both *plus*- and *minus*-specific traits, which is not the case (Figure 2b). If the *iso1* gene product instead acted only as a *minus* activator, then gametes that fail to express a functional *iso1* protein would exhibit no gamete-specific traits at all, which is also inconsistent with the data. Therefore, the *iso1* gene product must be involved in both *plus* repression and *minus* activation, the two components of *minus* dominance.

The *mid* locus is marked by the *imp11* mutation which yields an unconditional *pseudo-plus* phenotype: these chromosomally mt^- gametes fail to activate *minus* genes and fail to repress *plus* genes. Since the *imp11* mutation is linked to mt^- and *iso1* is not, the two genes are not alleles. Therefore, both must be taken into account when explaining how sex determination is achieved.

Previous models proposed that *Chlamydomonas* used a yeast-like mechanism [reviewed in Herskowitz (1988)] for sex determination (Galloway and Goodenough, 1985; Goodenough and Ferris, 1987). However, the *iso1* data have led us to a different perspective. Three models of sex determination and *minus* dominance are consistent with our observations of the *iso1* phenotype. These assume, as did previous models (Galloway and Goodenough, 1985; Goodenough and Ferris, 1987), that most *plus*-specific gametic genes are expressed when there is an absence of repression from *minus*-specific genes, and that genes localized to the

mt^+ locus are by definition not expressed in chromosomally mt^- gametes.

The simplest model hypothesizes that the *iso1* gene product is directly involved in sex determination and *minus* dominance and that the function of the *mid* locus is to activate *iso1* expression. In this model, only the *iso1* protein is required to activate *minus* genes and repress *plus* genes in a *minus* gamete. This could be accomplished either by the *iso1* protein acting as both an activator and a repressor, as is the case for a number of eukaryotic transcription factors (Stenlund and Botchan, 1990; Graupner *et al.*, 1989; Levine and Manley, 1989), or else by the *iso1* protein activating the expression of repressor and activator genes. Since the *iso1* gene is present in gametes of both mating types, *iso1* expression would be limited to *minus* gametes because they alone carry the *mid* locus. By this model, the *iso1* mutation would affect upstream sequences necessary for the *mid* gene product to activate *iso1* such that some cells would express *iso1* and differentiate as *minus* gametes whereas other cells would fail to express *iso1* and differentiate as *plus* gametes.

The second model, which we prefer, proposes that the *iso1* gene product interacts with the *mid* gene product (e.g., as a heterodimer; similar to other eukaryotic transcriptional factors) (Maheswaran *et al.*, 1993; Miner and Yamamoto, 1991) to bring about *minus* dominance and *minus* differentiation. As in the previous model, repression of *plus* functions would either be carried out by the postulated heterodimer itself, or the heterodimer would activate expression of repressor activity. In this model, the *iso1* protein might well be constitutively expressed in both mating types but would affect the mating type only in *minus* gametes because only *minus* cells would carry and express *mid*. Other traits might be affected (e.g., the fragmented eyespot) if the *iso1* protein is capable of regulating transcription of additional genes as a part of a regulatory complex that does not include the *mid* protein. The *iso1* mutation would be expected to be phenotypically sex-limited since the *iso1* gene product would have no gametogenic function in *plus* cells. If the mutation in *iso1* gametes produced a disabled *iso1* promoter, then the leaky *iso1* phenotype would be due to the presence (*minus* gametes) or absence (*pseudo-plus* gametes) of the *iso1* gene product. The presence or absence of an *iso1* gene product would in turn determine whether or not there would be a productive interaction of *mid* and *iso1* gene products. If, instead, the mutation has affected the *iso1* coding region, then the postulated heterodimer might be sufficiently unstable or defective in DNA binding that it can only maintain *minus* dominance in a subpopulation of the gametes.

In the third model, the *iso1* gene product would function as a repressor of latent homothallism in *C. reinhardtii*. Thus a *C. reinhardtii* ancestor would be capable of switching from *minus* to *plus* and vice versa, whereas modern *C. reinhardtii* would have lost this capability by acquiring the *iso1* gene. Partial loss-of-function of the *iso1* repressor would generate, in *iso1* mt^- mutants, the capacity to switch to *pseudo-plus*. In contrast, *iso1* mt^+ cells would be unable to switch to (pseudo) *minus*, presumably because they lack one or more genes (*mid*?) necessary for *minus* traits. Interestingly, a red alga has also been observed to switch from heterothallic to homothallic due to mutations expressed in one mating type but not the other (van der Meer *et al.*, 1984; van der Meer, 1986).

Insertional mutagenesis in *Chlamydomonas* typically causes deletions at the site of integration (Tam and Lefebvre, 1993). Since some *iso1* cells appear to express a functionally normal *iso1* gene product (cells that agglutinate and mate as *minus* gametes) while others do not (the *pseudo-plus* gametes), any deletion presumably lies outside the essential coding region of the gene (e.g., within the promoter). When *iso1* cells are maintained on agar plates for weeks, they lose the isoagglutinating phenotype (see RESULTS). We hypothesize that *iso1* mt^- cells that have lost their isoagglutinating phenotype can express the *iso1* gene product at normal levels, either through a suppressor gene that is linked to *iso1* or to the mt^- locus, or a second-site mutation in the *iso1* promoter which allows for more efficient transcription. These alternatives can be evaluated once the *iso1* gene is cloned from wild-type and mutant cells, and the nature of the insertional mutation is understood. Since *iso1* was generated by insertional mutagenesis, the *iso1* gene can be cloned and characterized in detail (Tam and Lefebvre, 1993). We are currently pursuing this next step toward a molecular understanding of sex determination in *Chlamydomonas*.

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