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A Sex Recognition Glycoprotein Is Encoded by the *plus* Mating-Type Gene *fus1* of *Chlamydomonas reinhardtii*

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Sexual fusion between *plus* and *minus* gametes of the unicellular green alga *Chlamydomonas reinhardtii* entails adhesion between *plus*-specific and *minus*-specific "fringe" proteins displayed on the plasma membrane of gametic mating structures. We report the identification of the gene (*fus1*) encoding the *plus* fringe glycoprotein, which resides in a unique domain of the mating-type *plus* (mt^+) locus, and which was identified by transposon insertions in three fusion-defective mutant strains. Transformation with $fus1^+$ restores fringe and fusion competence to these mutants and to the pseudo-*plus* mutant *imp11* mt^-, defective in *minus* differentiation. The *fus1* gene is remarkable in lacking the codon bias found in all other nuclear genes of *C. reinhardtii*.

INTRODUCTION

The mating-type (*mt*) locus of the unicellular green alga *Chlamydomonas reinhardtii* controls gametic recognition and fusion, zygote development and meiosis, and the sexual transmission of organelle genomes (Goodenough *et al.*, 1995). The locus, which resides in the left arm of linkage group VI, has been cloned recently (Ferris and Goodenough, 1994) and shown to be a large (~1 Mb) region under recombinational suppression and composed of three domains: a centromere-proximal (C) domain and a telomere-proximal (T) domain in which the mating-type *plus* (mt^+) and mating-type *minus* (mt^-) sequences are homologous, and a central rearranged (R) domain in which the mt^+ and mt^- sequences show inversions, deletions/insertions, and nonhomologous regions. An obvious inference from these data is that at least some of the nonhomologous regions contain genes coding for structural and regulatory proteins unique to either mt^+ or mt^- gametes.

Sexual fusion between *C. reinhardtii* gametes entails the interaction of mt^+ and mt^- mating structures, organelles that assemble during gametic differentiation (Martin and Goodenough, 1975) and associate with the apical plasma membrane near the basal bodies. A conspicuous coat of material, termed "fringe," extends from the mating-structure membranes, and the initial fusogenic contact between mating gametes involves fringe–fringe associations (Goodenough *et al.*, 1982). Fringe adhesion fails to occur between *plus* mating structures or between *minus* mating structures (Goodenough and Jurivich, 1978), and mutations that abolish the display of fringe also abolish gametic cell fusion (Goodenough *et al.*, 1982). We have proposed, therefore (Goodenough *et al.*, 1982), that the fringe molecules are sex-specific proteins that interact in a complementary manner to mediate the fusion process.

The *imp1* mutation, which is tightly linked to the mt^+ locus (Goodenough *et al.*, 1976), generates mt^+-mating structures that lack fringe (Goodenough *et al.*, 1982). The mutant can undergo the preliminary agglutination step of the mating reaction, which is mediated by sex-specific agglutinin molecules (Adair *et al.*, 1983), but it cannot undergo cell fusion and, therefore, is said to have an agglutinate-but-not-fuse phenotype. Two other mutant strains, *fus* and *bs37*, also show this phenotype (Matsuda *et al.*, 1978; Forest, 1987), and the latter has been shown to lack mating-structure fringe (Forest, 1987). Both mutations are in an mt^+ background and have been assumed to be allelic to *imp1*,

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although corroborative genetic analysis has not been reported.

We report here the cloning and sequencing of the gene defective in the *imp1*, *bs37*, and *fus* mutant strains. The gene, which we designate *fus1*, is located in a portion of the mt^+ R domain (region c) that is unique to the mt^+ locus, and carries independent transposon insertions in the *imp1*, *bs37*, and *fus* mutant strains. A 4.7-kb genomic segment carrying the *fus1* gene can rescue both the fringe and the fusion defects of the *imp1* mutant in transformation experiments. The clone can also rescue the fusion defect in the *imp11* mutant strain, which also displays an agglutinate-but-not-fuse *plus* phenotype but is genetically mt^- (Goodenough *et al.*, 1982). It has been proposed (Galloway and Goodenough, 1985) that the *imp11* mutation inactivates the expression of the mt^- gametogenesis program and thereby allows the expression of all facets of the mt^+ program except those encoded in the absent mt^+ locus. The ability of the mt^+-linked *fus1* gene to confer fusogenicity on the *imp11* mutant supports this interpretation.

Sequence analysis of *fus1* cDNAs indicates that the gene encodes a single-pass membrane glycoprotein with an N-terminal signal sequence and 14 putative N-glycosylation sites distributed along its length. We propose, therefore, that *fus1* represents the structural gene for *plus* fringe.

Particularly interesting is the finding that the *fus1* gene fails to display the strong codon bias found in the ~100 other nuclear genes of *C. reinhardtii* sequenced to date (LeDizet and Piperno, 1995). One interpretation of this observation is that the gene has been subjected to unique mutational pressures that override the forces maintaining codon bias. We propose that these pressures may play a role in the process of speciation.

MATERIALS AND METHODS

General Methods

Chlamydomonas strains were propagated on tris-acetate-phosphate (TAP) medium supplemented with 100 μg/ml arginine or 4 μg/ml nicotinamide as needed. Gametes were prepared by resuspending cells in nitrogen-free high-salt minimal (HSM) medium (Harris, 1989). Genetic crosses were performed using standard protocols (Harris, 1989). *Chlamydomonas* transformations were performed by vortexing enzymatically de-walled cells in the presence of DNA, polyethylene glycol, and glass beads (Kindle, 1990) as modified in Ferris (1995).

The Fus phenotype is scored by mixing gametes of the mt^+ strain to be tested with wild-type mt^- (CC-621) gametes. Strains with a Fus^+ phenotype produce a thick skin of zygote pellicle when allowed to mate overnight (Goodenough *et al.*, 1976). Strains that fail to form pellicle are checked for flagellar agglutination to ensure that they were truly gametic. A few Fus^+ strains are described as fusing with reduced efficiency. Such strains make less pellicle than other strains, and unfused gametes are still observed to be agglutinating even after an overnight mating. Mutant strains that are nonfusing by the pellicle assay may still make rare zygotes (too few to make a visible pellicle) as a result of either leakiness or reversion. To screen for rare zygotes, a mating mixture is plated on HSM medium (1.5% agar), incubated overnight under illumination, placed in darkness for 5 d, exposed to chloroform vapors for 30 s to kill unmated gametes, and then illuminated to allow the chloroform-resistant zygotes to form colonies.

Chlamydomonas genomic DNA was prepared essentially as described in Weeks *et al.* (1986), and total RNA purification was essentially as described in Kirk and Kirk (1985). Vegetative RNA was isolated from cells grown in liquid TAP medium, at a density of 2×10^6 cells/ml. Southern blots were prepared by electrophoresing restriction-digested genomic DNA on agarose gels containing Tris-borate buffer and transferring the DNA to nitrocellulose (Wahl *et al.*, 1979). Northern blots were prepared by transferring RNA to nitrocellulose after electrophoresis in formaldehyde–agarose gels (Maniatis *et al.*, 1982). Blots were hybridized as described previously (Church and Gilbert, 1984) using probes ^{32}P-labeled by nick translation (Maniatis *et al.*, 1982).

Cells were prepared for electron microscopy as described in Goodenough *et al.* (1982). Before fixation, gametes were treated with gametic lytic enzyme (GLE) (Kinoshita *et al.*, 1992) to remove their walls so that the mating structure surfaces were not obscured by trapped periplasmic materials. Mating efficiency was quantitated as described in Mesland *et al.* (1980).

Chlamydomonas Strains

The three mt^+ strains with an agglutinate-but-not-fuse phenotype are renamed as alleles of the *fus1* locus in this paper. The following strains were used: wild-type mt^+ (CC-620); wild-type mt^- (CC-124 and CC-621); *fus1-1* mt^+ (CC-1158, originally *imp1*); *fus1-1 arg2* mt^+ (CC-1865); *fus1-2* mt^+ (CC-2062, originally *fus*); *fus1-3* mt^+ (CC-2392, originally *bs37*); *imp11* mt^- (CC-1148); *mat3-1* mt^+ (CC-1933); *arg2 nr-u-2-1* mt^+ (CC-1067); and *nic7 ac29a pf14 act2 msr1* mt^- (CC-1336). The *bs37* strain also has a move-backwards-only (*mbo*) phenotype, which always cosegregates with *mt* (our unpublished observations), suggesting that this strain carries a separate mutation in the *mbo1* locus (Segal *et al.*, 1984).

The diploid strain was constructed by mating CC-1067 to CC-1336, plating the cells on nonsupplemented TAP medium, and allowing them to grow under light. Arg^+ Nic^+ colonies were picked and tested for mating type. *Minus* colonies should be diploid, because of the tight linkage of *nic7* and *mt* and the dominance of mt^- over mt^+. Diploidy was verified further by preparing genomic DNA from the diploid strain and testing for the presence of restriction fragment length polymorphisms (RFLPs) from both *mt* alleles.

Genomic Cloning and Sequencing

The *fus1*$^+$ gene was cloned from a λEMBL3 genomic library as part of a chromosome walk through the mt^+ locus (Ferris and Goodenough, 1994). A 4.7-kb *Eco*RI fragment isolated from the walk was subcloned into the *Eco*RI site of pUC13, a construct referred to as pFus4.7. Nested deletions of pFus4.7 were obtained using the Double Strand Nested Deletion Kit (Pharmacia, Uppsala, Sweden) and sequenced using the Sequenase Kit (United States Biochemical, Cleveland, OH). In addition, various restriction fragments from pFus4.7 and from the phage clones were subcloned into pUC118 or pUC119 for single-strand sequencing. DNA sequences were compiled and analyzed with the Genetics Computer Group sequence analysis software package (University of Wisconsin, Madison, WI) for VAX/VMS computers.

The mutant alleles were cloned as follows. Genomic DNA from *fus1-1* was digested to completion with *Eco*RI and ligated into *Eco*RI-cut λEMBL3. Plaque lifts of the resultant library were hybridized with the 1.6-kb *Hin*dIII/*Xba*I fragment from the genomic *fus1*$^+$ (probe A), and hybridizing phage was purified and restriction-mapped. The cloned 14.1-kb *Eco*RI fragment so derived contains the entire *Tcr1* insertion with flanking *fus1* DNA. The same procedure was followed with *fus1-2* genomic DNA. Because there is an *Eco*RI

site within *Tcr3*, *fus1-2* was characterized from two adjoining *Eco*RI fragments of 4.5 and 14.8 kb, cloned in different phage. (The 4.5-kb fragment was isolated in a chimeric phage containing an additional, unrelated *Eco*RI fragment.) Two λEMBL3 libraries were made using *fus1-3* genomic DNA, one using a complete *Eco*RI digest, the other a complete *Bam*HI digest. The *Eco*RI library yielded a phage containing the 14.4-kb *Eco*RI fragment extending from the right *Eco*RI site on the wild-type map to the *Eco*RI site in *Tcr3*. From the *Bam*HI library, both junction fragments between wild-type DNA and *Tcr3* were isolated (in chimeric phage). Their restriction maps confirmed the presence of only a single *Eco*RI site within *Tcr3*.

Isolation of fus1-3 Revertants

Gametes of *fus1-3* and CC-124 were mated for 1 h and then plated for rare zygotes. Twenty-five chloroform-resistant colonies were picked and analyzed for their ability to self-mate. Twenty produced zygote pellicle, indicating that both mt^+ and mt^- progeny had survived in these zygotes and that at least one (presumably both) of the mt^+ progeny was now Fus^+. In two of the colonies, only mt^- progeny survived, and in three of the colonies, only mt^+ Fus^+ progeny survived, presumably because of the lethality of some meiotic products. These data suggest that rare zygotes form only when the *fus1-3* mutation reverts.

Four of the zygote colonies were cloned to isolate a single mt^+ progeny for further analysis. To determine whether reversion had created an excision footprint, ~300 bp spanning the *fus1-3* integration site was amplified from genomic DNA of the four revertants by polymerase chain reaction (PCR) using Vent DNA polymerase (New England BioLabs, Beverly, MA). The PCR reaction used primers 10 and 11 under the following conditions: 94°C for 5 min, then 25 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The amplified product was eluted from a low-melt agarose gel, ligated into *Hin*cII-cut pUC119, and sequenced.

Isolation of fus1-1 Revertants

Revertants of *fus1-1* were isolated during an experiment designed to transform the *fus1-1* mt^+ (CC-1158) strain to Fus^+ by transformation with pFus4.7 alone, followed by selection for Fus^+ cells (see next section). As a control, the strain was also transformed with pNic7.1 (Ferris, 1995) alone. After vortexing, the 2×10^7 cells were inoculated into 5 ml of liquid TAP and cultured for 24–48 h. They were then pelleted and resuspended in 5 ml of N-free HSM overnight to induce gametogenesis. The next morning, the gametes were mixed with an equivalent number of mt^- gametes (CC-621), mated for 3–4 h, then plated for rare zygotes as above. Sixty colonies were picked from the control transformation and self-mated to determine whether any of the mt^+ progeny were Fus^+. Of these, 53 failed to form pellicle even though there was obvious agglutination. This result is consistent with the previous observation (Goodenough *et al.*, 1976) that the *fus1-1* mutation is leaky and will form zygotes at a low frequency. Two other colonies failed to display self-agglutination and were assumed to lack surviving progeny of both mating types. Five colonies self-mated to produce pellicle, potentially the result of reversion of the *fus1-1* mutation. These were cloned to isolate an mt^+ progeny for further analysis. Excision footprints were analyzed by performing PCR amplification of the 300-bp fragment between primers 12 and 13, using the same protocol as for the *fus1-3* revertants.

Transformation of fus1$^+$ into fus1-1

Transformants were obtained in two ways. In most cases, the *fus1-1 arg2* double mutant (CC-1865) was cotransformed with the plasmid pArg7.8, which complements *arg2*, and DNA spanning the region identified by the transposon insertion sites. In one experiment, the *fus1-1* strain (CC-1158) was transformed with pFus4.7 alone, followed by selection for rare zygotes. Most of the resulting 60 colonies failed to form pellicle after self-mating and were presumed to be generated by the leakiness of the *fus1-1* mutation. Five colonies formed at least some visible pellicle when self-mated, the result of either reversion or transformation. The two best pellicle formers were cloned, and genomic DNA was prepared from an mt^+ Fus^+ progeny. Genomic Southern blot analysis using probe A indicated that these two were transformants, because the probe hybridized to both 14.1- and 4.7-kb *Eco*RI fragments that correspond in size, respectively, to the endogenous *fus1-1* allele and the *fus1*$^+$ transgene.

Transformation of fus1$^+$ into imp11

The *imp11* mutant is completely sterile, so it was not possible to construct strains with specific markers for cotransformation. However, the CC-1148 strain, like most laboratory strains, contains a *nit1* and a *nit2* mutation (Harris, 1989) and is unable to grow on either nitrate or nitrite as sole nitrogen source. The *imp11* strain, therefore, was first transformed with a clone of the *nit2*$^+$ gene (pMN68, kindly provided by R. Schnell; Schnell and Lefebvre, 1993) after growth in liquid TAP medium. Transformants were selected for their ability to grow on a modified Sager and Granick Medium I (modified to contain 0.3 g/l K_2HPO_4 and 1.8 g/l sodium acetate), with 2 mM KNO_2 as nitrogen source rather than NH_4NO_3 (Sager and Granick, 1953). One of these transformants was then cotransformed with pFus4.7 and the *nit1*$^+$ plasmid (pMN24) as the selectable marker (Fernandez *et al.*, 1989). For this experiment, the cells were grown in liquid TAP and then pelleted and resuspended in the selective medium for 4 h before GLE treatment and transformation. Transformants were selected for their ability to grow on modified Sager and Granick Medium I, with 5 mM KNO_3 as nitrogen source. The presence of a functional *fus1*$^+$ gene in a transformant was assayed by determining whether visible pellicle formed after mating overnight with mt^- gametes.

Isolation of fus1 cDNAs

Plaque lifts were made from a λZAPII cDNA library constructed from $polyA^+$ RNA isolated from zygotes 60 min after mating (Armbrust *et al.*, 1993). Approximately 2×10^7 plaques were screened with probe A radiolabeled with ^{32}P using the Random Primed DNA Labelling Kit (Boehringer Mannheim, Indianapolis, IN). The longest cDNA isolated was cDNA4, which was completely sequenced and starts at base 1466 in the genomic sequence. In an effort to get cDNAs extending more 5′ than cDNA4, a 400-bp fragment at the 5′ end of cDNA4 was used to screen an additional 2×10^7 plaques, yielding cDNA6, which starts at base 1152. Finally, the 250-bp *Xba*I/*Apa*I genomic fragment near the 5′ end of cDNA6 was used to screen 6×10^7 plaques, yielding cDNA17 (starts at base 926) and cDNA24 (the precise 5′ end of which was not determined). All four cDNAs contained a polyA tail, which commences after base 4405 in cDNA4, base 4547 in cDNA6, base 4713 in cDNA24, and base 4782 in cDNA17.

After screening the cDNA library exhaustively, we used other methods to attempt to extend the cDNA sequence to the 5′ end of the *fus1* transcript. $PolyA^+$ RNA was purified from total RNA of CC-620 gametes using the BioMag mRNA Purification Kit (PerSeptive Diagnostics, Cambridge, MA). Reverse transcription (RT)-PCR was carried out using the RT-PCR Kit from Stratagene (La Jolla, CA) and Vent DNA polymerase, with primers 2 and 3 and the following conditions: 91°C for 1 min, 54°C for 1 min, and 72°C for 2 min, for 30 cycles. No product was observed with total RNA as template, but a 205-bp product was obtained when $polyA^+$ RNA was used. This product was purified by elution from a low-melt agarose gel, ligated into *Sma*I-cut pUC118, and sequenced.

Attempts at RT-PCR using primers more 5′ to primer 3 were unsuccessful and, therefore, the 5′ RACE System (Life Technologies, Gaithersburg, MD) was used to extend further the *fus1* cDNA sequence. $PolyA^+$ RNA from mt^+ gametes was used as a template

for cDNA synthesis with primer 1. The RACE PCR conditions were 94°C for 1 min, 57°C for 30 sec, and 72°C for 2 min, for 35 cycles, using primer 2, the kit's Anchor primer, and *Taq* DNA polymerase. The product from this initial PCR was a faint 520-bp fragment that was reamplified with nested primers—the kit's Universal Amplification Primer and primer 8. The reamplification yielded an abundant product that was eluted from a low-melt agarose gel, treated with T4 DNA polymerase to generate blunt ends, and ligated into *Hin*cII-cut pUC119 for subsequent sequencing.

Inverse PCR (Zeiner and Gehring, 1994) was also attempted as a method to locate the 5′ end of the *fus1* cDNA. Using 2 μg of polyA$^+$ RNA, first-strand cDNA was synthesized using primer 1. Second-strand synthesis was performed using the Stratagene λZAP cDNA kit following the manufacturer's protocol to the linker-addition step. At that point, the blunt-ended cDNA was circularized by ligation in 100 μl of reaction volume overnight at 12°C. PCR with primers 1 and 3 confirmed the presence of *fus1* cDNA in the ligation mix. PCR with primers 7 and 8, which are directed outward around the ligated circle, was performed using Vent DNA polymerase for 35 cycles of 91°C for 1 min, 54°C for 1 min, and 72°C for 1 min. Because of low yield, an aliquot of the first amplification was reamplified with primers 7 and 8. The product of this reaction was then eluted from a low-melt agarose gel and cloned into *Hin*cII-digested pUC119, and several independent clones were sequenced. All of the analyzed RACE and inverse PCR clones terminated in the region of genomic sequence from 264 to 280 (numbering from the *Eco*RI site). Because we predict the starting methionine is an AUG at 267–269, either the 5′-untranslated region is unusually short or we have not succeeded in generating full-length products.

Primers

Primer 1: GGACAGAGATATCCGCG
Primer 2: AGTGGATAAGGCTGTGGAAA
Primer 3: CAAAGTGATTCCACAAACCC
Primer 7: GTTGCAAGCTCCAGATTTGC
Primer 8: GGGTTTGTGGAATCACT
Primer 10: GGAATGCCCAGGCATCA
Primer 11: TCTCACCAGCCAAGACG
Primer 12: TGGGTGTCTATGCCGTCATT
Primer 13: GAAACGAAAGCGACTCTCCT

RESULTS

Transposon Insertions Create fus1 Mutations

The R domain of the *mt*$^+$ locus contains several nonrepetitive regions (a, b, and c) that are not present in the *mt*$^-$ R domain (Ferris and Goodenough, 1994). These regions might be expected to contain genes for *plus*-specific functions. When a hybridization probe from within region c (Figure 1) was used to probe a Southern blot containing genomic DNA from wild-type *mt*$^+$, from the *mt*$^+$-linked mutant *mat3* [which is not defective in gametogenesis (Armbrust *et al.*, 1995)], and from the *imp1*, *fus*, and *bs37* mutants, distinct RFLPs were detected in the three fusion mutants (Figure 2). Genomic libraries were constructed from the three fusion mutants, and the DNA in the vicinity of the region c probe was identified and restriction-mapped. As shown in Figure 1, each mutant carries a large insertion within the probe sequence. This suggests that all three mutants are alleles of the same locus, which we designate *fus1*. Henceforth *imp1*, *fus*, and *bs37* are referred to as *fus1-1*, *-2*, and *-3*, respectively.

Subsequent analysis indicated that the DNA inserts are transposons. Restriction mapping documented that the same 14.6-kb insert is present in both *fus1-2* and *fus1-3*, but in opposite orientations. When a restriction fragment from within the insert was used as a hybridization probe on genomic Southern blots, multiple fragments were seen (our unpublished observations), as expected for a transposon. Restriction fragments that contain the junctions between the inserts and the wild-type gene were subcloned and sequenced for both mutations. Insertion created a 2-bp

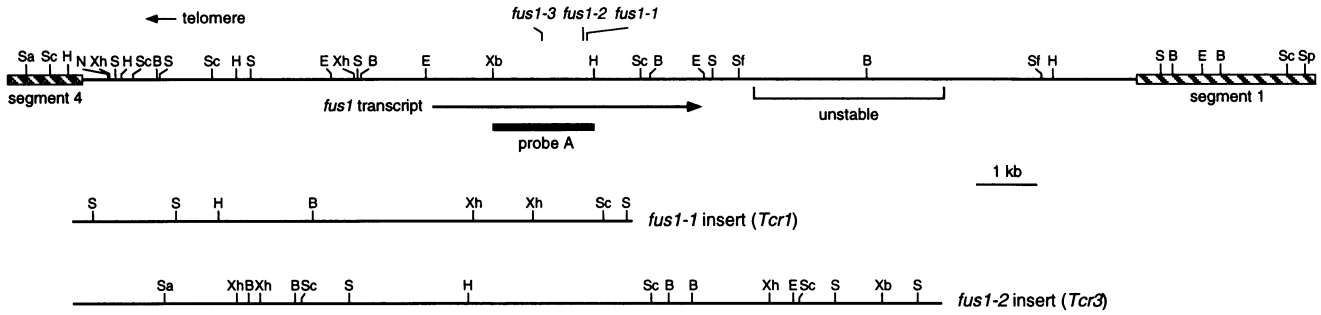

Figure 1. Restriction map of *fus1* and its transposon inserts. (Top) A detailed restriction map of the *mt*$^+$-specific region of the *mt* locus located between segments 4 and 1 (Ferris and Goodenough, 1994). The 1.6-kb *Hin*dIII/*Xba*I fragment labeled "probe A" is the nonrepetitive sequence that defined region c. The locations of the transposon insertions in the three *fus1* mutant alleles are shown above the map, and the location and direction of the *fus1* transcript are drawn below. The 3.2-kb section marked "unstable" is always deleted in phage clones that span the section; consequently, this DNA was never present in the restriction fragments used in *Chlamydomonas* transformation experiments. (The existence of the unstable section was inferred from genomic Southern blot analysis; the segment to the left of its *Bam*HI site has been cloned and restriction-mapped, but the segment to the right has never been cloned and may contain unmapped sites for the restriction enzymes shown on this map.) (Bottom) Restriction maps for the transposons present in *fus1-1* (*Tcr1*) and in *fus1-2* (*Tcr3*). The *fus1-3* allele also has a *Tcr3* insertion, with an identical restriction map but in opposite orientation. The transposons were not mapped with *Not*I, *Sfi*I, or *Spe*I. The restriction enzymes used are *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Not*I (N), *Sac*I (Sc), *Sal*I (Sa), *Sfi*I (Sf), *Sma*I (S), *Spe*I (Sp), *Xba*I (Xb), and *Xho*I (Xh).

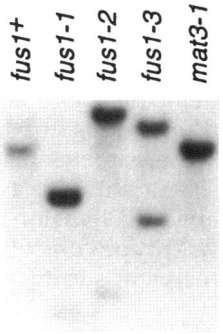

Figure 2. *Fus1* mutants display DNA polymorphisms. Genomic DNA from a wild-type mt^+ strain, strains carrying the three *fus1* alleles, and a *mat3-1* strain was digested with *Sma*I, and a Southern blot was hybridized with labeled probe A. Because the transposons in each of the mutants contain *Sma*I sites, each mutant displays two hybridizing fragments, although the signals from the second fragments in *fus1-1* and *fus1-2* are barely visible in this exposure because of their limited overlap with the probe.

target site duplication in both cases, and the element has a 58-bp inverted repeat containing nine mismatches and three insertions/deletions (Figure 3). This same transposon has also been identified by S.-C. Wang and P.A. Lefebvre (personal communication) and has been named *Tcr3*.

Revertants of the *fus1-3* mutation were recovered and analyzed (see MATERIALS AND METHODS). Genomic Southern blots of four *fus1-3* revertants suggested that the *Tcr3* DNA had been completely excised (our unpublished observations), which confirms that the transposon insertion is responsible for the nonfusing phenotype. The excision footprints were cloned after PCR amplification, and sequencing indicated that in two revertants the wild-type sequence had been restored, whereas the other two displayed an identical 3-bp insertion (Figure 3). (Revertants with the same sequence may represent mitotic clones of a single excision and not separate events.) The revertants containing the insertion (which adds an Asp after Pro_{437}) fuse less efficiently than the true revertants.

The 9.4-kb insert in *fus1−1* is also found in multiple copies in the genome. When the restriction fragments containing the insert/*fus1* junctions were subcloned and sequenced, it was found that an 8-bp target site duplication had occurred (Figure 3) and that the element possesses a terminal 140-bp perfect inverted repeat. This same transposon has also been identified by R.A. Schnell and P.A. Lefebvre (personal communication) and has been named *Tcr1*.

Genomic Southern blots of five revertants of *fus1-1* (see MATERIALS AND METHODS) indicated that the *Tcr1* DNA had been excised in three cases (they may all be mitotic clones); the excision footprints for two of these excisions were cloned after PCR amplification, and both have the wild-type sequence restored (Figure 3). In the other two revertants, ~1.5 and 2.5 kb of (presumably *Tcr1*) DNA was still present. Gametes carrying these apparently incomplete excisions fuse significantly less efficiently than wild-type, but they have not been analyzed further.

The fus1+ Gene Rescues the fus1-1 Mutant

Cotransformation was used to identify the boundaries of the gene disrupted by the transposon insertions. A *fus1-1 arg2* double mutant was cotransformed with the plasmid pArg7.8, which complements *arg2*, and DNA spanning the transposon insertion sites. The DNA used was either the 16.9-kb *Not*I/*Spe*I, 10.6-kb *Not*I/*Sfi*I, or 6.0-kb *Sma*I restriction fragment purified from phage clones, or the pFus4.7 plasmid, which contains the 4.7-kb *Eco*RI fragment (Figure 1). Transformants prototrophic for arginine were tested for their ability to form pellicle when mated with mt^- gametes overnight. All of the fragments tested yielded Fus^+ cotransformants, indicating that the $fus1^+$ gene is contained within the 4.7-kb *Eco*RI fragment. The pFus4.7 construct has only 266 bp of sequence 5′ to the starting

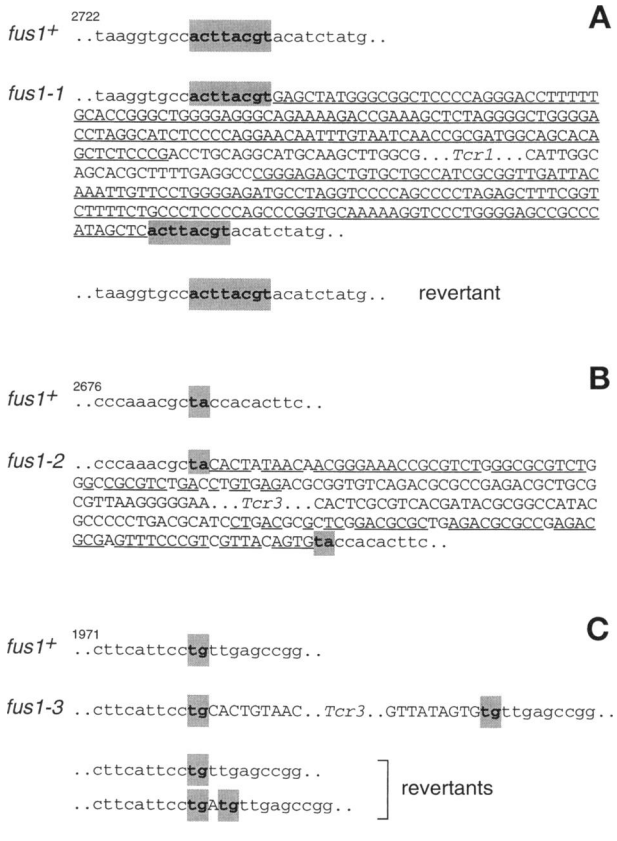

Figure 3. Sequence of the transposon insertions in *fus1* mutations. (A) The *fus1-1* mutation is the result of insertion of the transposon *Tcr1* (upper case) into the indicated $fus1^+$ sequence (lower case), creating an 8-bp target site duplication (shaded). One class of revertant has restored the wild-type sequence. The inverted repeat in *Tcr1* is underlined. (B) The *fus1-2* mutation is the result of insertion of the transposon *Tcr3* into $fus1^+$, creating a 2-bp target site duplication. The inverted repeat of *Tcr3* is underlined; gaps indicate sequence differences between the repeats. (C) The *fus1-3* mutation is the result of a *Tcr3* insertion. The sequences of two classes of revertants are shown.

AUG (see below), which leaves open the issue of whether these transformants have a complete *fus1* promoter and whether *fus1* expression is properly regulated. We have not investigated this issue.

To verify transformation and rule out reversion, one of the *Not*I/*Spe*I-fragment transformants was mated to CC-124 and subjected to tetrad analysis. Figure 4 shows Southern analysis of the parent and two sets of progeny. The endogenous (E) and transformation-introduced (T) copies of *fus1* are readily distinguished in the transformed parent. The two copies assort independently in both tetrads, indicating that the transgene has not inserted into the mt^+ locus and is presumably located in some other chromosome. The mt^+ progeny that fail to inherit the transgene display the agglutinate-but-not-fuse phenotype of *fus1-1*, further confirming that reversion of the original mutation has not occurred. The mt^- progeny that inherit the transgene proceed to carry out an apparently normal mt^- gametogenesis.

Electron microscopy was used to document that pFus4.7 transformants had acquired the ability to produce mating-structure fringe. Figure 5a shows the normal complement of fringe (arrowheads) associated with a wild-type mt^+ mating structure; Figure 5b shows the fringe-defective appearance of a *fus1-1* mating structure. Figure 5c shows a mating structure from a transformed *fus1-1* strain, in which a robust complement of fringe has been restored. Expression of the transgene is apparently variable in this strain in that some gametes display more fringe than others. Consistent with this evaluation, its mating efficiency peaks at 57% cell fusion after 20 min, compared with 96% for wild-type controls and 0% for the original *fus1-1 arg2*-recipient strain.

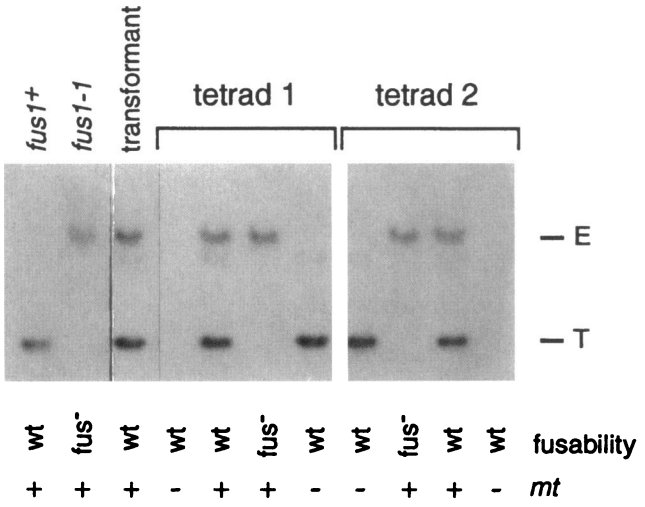

Figure 4. Southern blot analysis of a transformant and meiotic tetrads. A Southern blot was prepared with *Eco*RI-digested genomic DNA from the indicated strains and hybridized with probe A. The lane marked "transformant" is a Fus^+ Arg^+ transformant of CC-1865, transformed with the 16.9-kb *Not*I/*Spe*I fragment. It displays two fragments: the 14.1-kb fragment corresponding to the endogenous (E) *fus1-1* allele and a 4.7-kb fragment corresponding to the $fus1^+$ transforming (T) DNA. The meiotic progeny from two tetrads in a cross between the transformant and CC-124 (which has no hybridizing fragments) are also analyzed. Their mating type (+ or −) and whether they are (wt) or are not (Fus^-) capable of fusing with gametes of the opposite mating type are indicated. A faint band at 11 kb is visible in some lanes. This band cosegregates with the transforming DNA and may indicate that another, incomplete copy of the transforming DNA has also been integrated.

The fus1 Gene also Rescues the imp11 Mutant

The *imp11* mutation confers gametes of an mt^- strain with a "pseudo-*plus*" phenotype: they produce *plus* flagellar agglutinins and mating structures with a *plus* morphology, but the mating structures lack fringe and cannot effect cell fusion with wild-type mt^- gametes (Goodenough *et al.*, 1982). Because the *imp11* mutation is tightly linked to the mt^- locus (Galloway and Goodenough, 1985), it was proposed that the lesion prevents expression of an mt^--specific gene called *mid* (minus dominance) that ordinarily acts to switch on the *minus* gametogenesis program and switch off the *plus* program. It follows that the *imp11* mutant should express *plus* genes but be defective for any genes encoded in the absent mt^+ locus, an obvious example being the $fus1^+$ gene. We therefore created an *imp11 nit1* double mutant (see MATERIALS AND METHODS), cotransformed with the pMN24 and pFus4.7 plasmids, selected transformants that could grow on nitrate as the sole nitrogen source, and tested these for their ability to form zygotes with mt^- gametes. Several fusion-competent clones were verified by Southern analysis as carrying the $fus1^+$ transgene. Genetic analysis demonstrated that the transgene assorts independently of *mt* and, consequently, pseudo-*plus* progeny emerge from the cross (our unpublished observations), documenting that the acquisition of fusion competency is the result of $fus1^+$ transformation.

One *imp11* $fus1^+$-transformed strain was subjected to additional analysis. By electron microscopy, its mating structures were found to carry abundant levels of fringe (compare Figure 5, d and e). However, its mating behavior was quite different from the pFus4.7-transformed *fus1-1* strain described previously. After 20 min of mating, 44% of the cells were found to be closely apposed in pairs, but none of them had fused. Instead, apices remained paired for the next few hours (a transient stage in the normal mating reaction), as if fringe adhesion had occurred but fusion was precluded. By the next morning, many of these pairs were found to have formed zygotes, and genetic analysis of such zygotes demonstrated that they are able to undergo a normal meiosis.

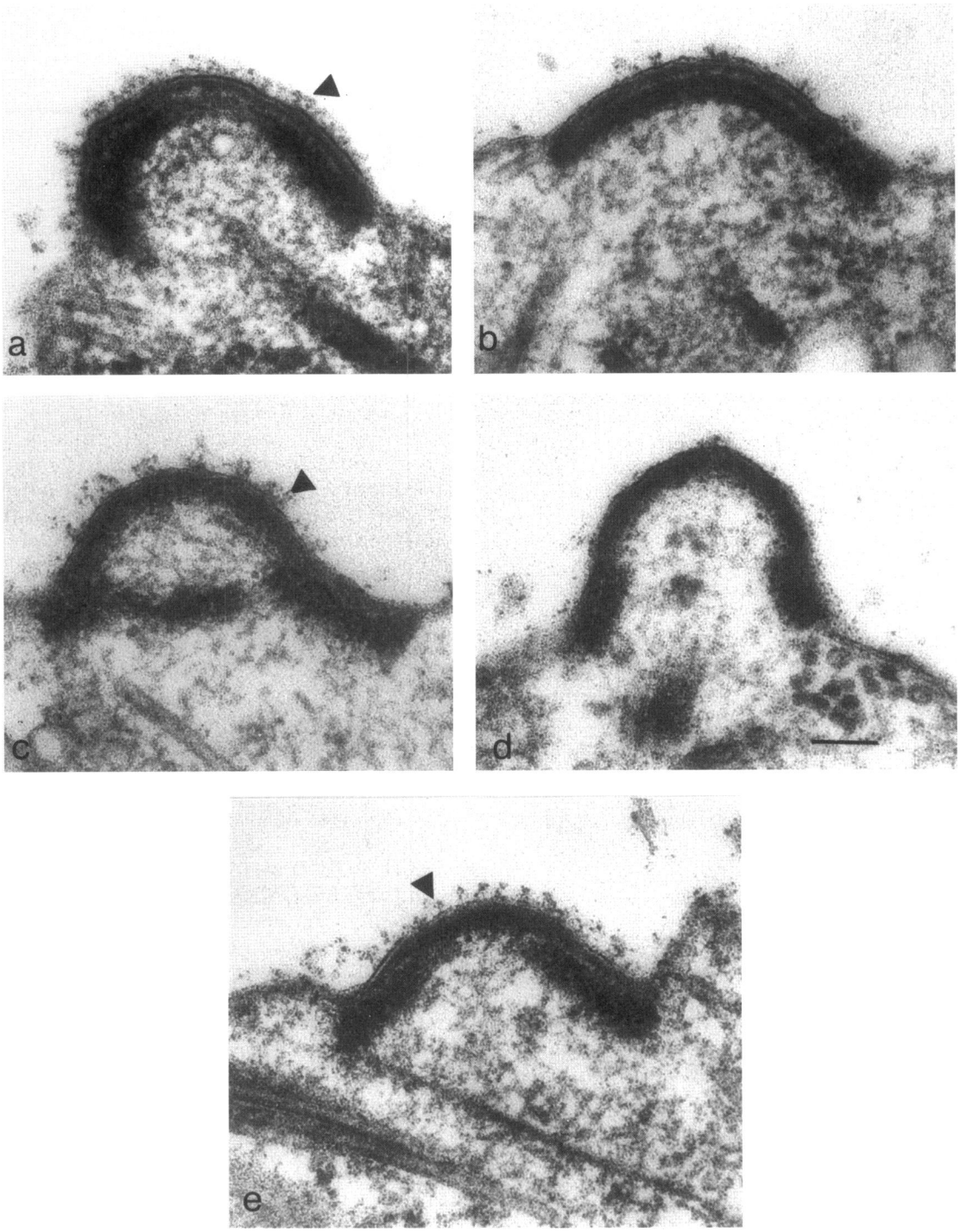

Figure 5. Mating structures of *plus* strains. (a) Wild type; (b) *fus1-1*; (c) *fus1-1* transformed with pFus4.7; (d) *imp11*; (e) *imp11* transformed with pFus4.7. Arrowheads point to fringe. Bar, 100 nm.

To ascertain whether the pairs, in fact, were adhered via their mating structures, the mating mixture was subjected to pH shock (Witman *et al.*, 1972), a procedure that causes the loss of flagella in response to an influx of calcium (Quarmby and Hartzell, 1994). Given this stimulus, the paired cells immediately fused. When the procedure was repeated in the absence of external calcium, the cells failed to fuse.

The fus Gene Encodes a Putative Glycopolypeptide

As detailed in MATERIALS AND METHODS, the 4813-bp *Eco*RI/*Sma*I section of genomic DNA that rescues the nonfusing phenotype of *fus1-1* has been sequenced (GenBank accession number U49864). Probes were selected from this section for exhaustive screening of a cDNA library. Because this screening did not yield a full-length *fus1* cDNA, we used RT-PCR, 5′ RACE, and inverse PCR to extend the cDNA sequence more 5′ to include the putative initiating methionine.

Figure 6 shows schematically the location and sizes of the 13 exons and 12 introns of *fus1*. These introns are unusual in that all except the first and last are no larger than 68 bp, the smallest being 57 bp (Table 1) [the smallest previously reported *Chlamydomonas* introns are 67 and 69 bp (LeDizet and Piperno, 1995)]. Also indicated in Figure 6 are the sites at which four independent *fus1* cDNAs are polyadenylated. None of these sites is preceded by a canonical, TGTAA, polyadenylation signal sequence. In fact, there is no TGTAA sequence in the entire genomic sequence from the *fus1* stop codon to the *Sma*I site (782 bp). Possibly, the lack of an exact match forces random selection of alternate polyadenylation sites.

Figure 7 presents the derived amino acid sequence for *fus1*. The initiating methionine is followed by a short 13-amino-acid sequence with the properties of a signal peptide (von Heijne, 1985). A hydropathy plot predicts a single transmembrane domain near the C terminus (boxed in Figure 7), indicating that the bulk of the molecule extends out from the membrane (compare with Figure 5). The presumed extracellular domain carries 14 putative N-glycosylation sites distributed along the length of the polypeptide.

Expression of the fus1 Gene

When a Northern blot of RNA isolated from various strains and life-cycle stages was hybridized with a labeled restriction fragment carrying only *fus1* coding sequence, a 3.0-kb message was observed in mt^+ gametes but not in mt^- gametes (which do not contain the gene) or in mt^+ vegetative (nongametic) cells (Figure 8). A faint signal is sometimes seen in RNA from zygotes, which could represent mRNA still present in the zygotic cells and/or in residual unmated mt^+ gametes in some zygote preparations. Gametes from an mt^+/mt^- heterozygous diploid (phenotypically *minus*) also express the *fus1* gene.

The fus1 Gene Lacks the Chlamydomonas Codon Bias

The 95 *C. reinhardtii* genes analyzed by LeDizet and Piperno (1995) all display a strong codon bias (Table 2), with the GC content of the coding regions averaging 64.4% (M. LeDizet, personal communication) and with a particular bias for C in the third position (LeDizet and Piperno, 1995). The degree of bias for an individual gene can be quantified as a B value (Long and Gillespie, 1991), which indicates how nonrandomly synonymous codons are used (Figure 9), with a B value of 0 indicating that all synonymous codons are used with equal frequency, and 1.0 indicating that only one codon is used for each amino acid. Bias can also be expressed as a graph generated by the Codonpreference program of the GCG package, which scores codon usage in a particular sequence against a table of *Chlamydomonas* codon preferences (i.e., Table 2). Several examples of the latter are displayed in Figure 10, in which values above the dashed horizontal line indicate the use of preferred codons and tick marks indicate the use of rare codons. β-1 tubulin (Figure 10A) and *ida4* (a component of an inner-arm dynein; Figure 10B) are displayed to illustrate genes with particularly high (B = 0.74) and low (B = 0.316) bias.

The *fus1* cDNA sequence, in contrast, shows no codon bias. Its coding region averages 47.7% GC, and when its codon usage is directly tabulated (Table 2), all possible codon choices are comparably represented, generating a B value (Figure 9) of 0.050, and a dramatically different Codonpreference graph (Figure 10E). Importantly, biased codon usage is found in two other genes located in the *mt* locus,

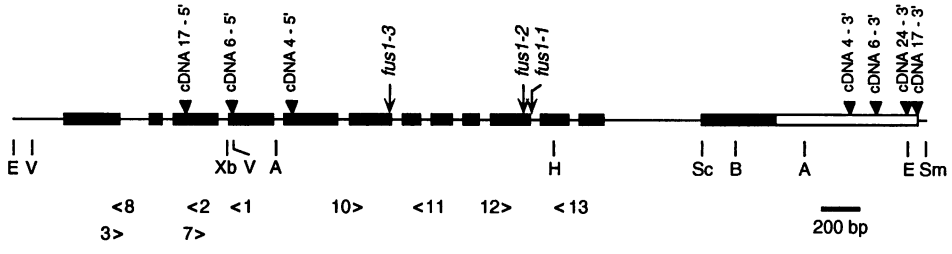

Figure 6. Structure of the *fus1* transcript. The 4813-bp *fus1* genomic sequence is depicted, and the mRNA structure is shown diagrammatically. Filled boxes represent exons; lines between boxes are introns. The open box represents the longest 3′-untranslated region. Triangles point to the 5′ and 3′ ends of indicated cDNA clones (the 5′ end of cDNA24 was not determined). The positions of the transposon insertions in the three *fus1* mutants are indicated, as are restriction sites. Numbered carats mark the approximate locations of oligonucleotide primers, with the point of the carat being the 3′ end. The key for restriction sites is as in Figure 1, with the addition of *Apa*I (A) and *Eco*RV (V).

namely, the *ezy1* gene involved in the uniparental inheritance of chloroplast DNA (Armbrust *et al.*, 1993) and *ezy2* (contained within the 16-kb repeat in Ferris and Goodenough, 1994) (Figures 9 and 10, C and D). Therefore, the absence of codon bias in the *fus1* gene is not a general property of genes subjected to recombination suppression in the *mt* locus.

The *Chlamydomonas* genome is 62% GC (Harris, 1989), suggesting that the codon bias may be the consequence, at least in part, of an overall genomic preference for G+C. Supporting this idea, an analysis of 116 *Chlamydomonas* introns shows them to average 63% GC (S. Fabry and M. Liss, personal communication). The *fus1* introns, by contrast, are 51.6% GC (Table 1); if the large intron 12 is excluded, the introns are 47% GC. Thus, the *fus1* introns, and not just the coding sequence, are different from other *Chlamydomonas* genes, although the possibility exists that the base composition of the small *fus1* introns is constrained by requirements of the splicing machinery. The 3′- and 5′-untranslated regions of *fus1* are also low in %GC (Table 1).

DISCUSSION

The fus1 Gene

The phenotype of the *fus1-1* mutant (Goodenough *et al.*, 1982) originally suggested that a gene necessary for fringe display on the *plus*-mating structure resided in the mt^+ locus. Until this gene was cloned and sequenced, however, it was not known whether it represented the structural gene for the fringe protein or whether it played a more indirect role, such as encoding a transcription factor necessary for the expression of the structural gene. The deduced gene product has the properties expected of a surface glycoprotein: it carries an N-terminal signal sequence, a single putative transmembrane domain with a short, highly charged cytoplasmic domain at the C terminus, and 14 putative N-glycosylation sites along its putative extracellular domain. No significant homologies to the *fus1* protein were found in a search of the databases available using the NCBI BLAST network service.

As might be expected for a gene whose product functions in gamete recognition/fusion, the *fus1* mRNA is present in *plus* gametic cells but not in vegetative cells. Interestingly, the *fus1* mRNA is present in gametes of heterozygous mt^+/mt^- diploids, which are functionally *minus* because of the dominance of the mt^- locus. This suggests that the mechanism that represses *plus*-specific genes in diploids (and presumably also in haploid mt^- cells) does not recognize the *fus1* gene, which is not surprising given that *fus1* is not normally present in a *minus* cell. We have noticed, as have others (Gloeckner and Beck, 1995), that diploid gametes fuse with poor efficiency compared with haploid gametes. We have also observed that haploid mt^- strains carrying a $fus1^+$ transgene fuse inefficiently, and we specu-

Table 1. % GC in the *fus1* gene

Segment	Sequence length (bp)	% GC
5′ upstream[a]	266	42.9
coding region	2472	47.7
intron 1	154	48.7
intron 2	61	44
intron 3	64	48
intron 4	57	53
intron 5	68	50
intron 6	58	47
intron 7	60	38
intron 8	58	50
intron 9	61	49
intron 10	58	45
intron 11	59	42
intron 12	525	58.3
all introns	1283	51.7
3′ UTR[b]	761	54.0

[a] From the *Eco*RI site to the initiating AUG.
[b] 3′ UTR of cDNA17.

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  1  MPIFLILVLLAAVAKSQDCSVVADFKIDFQTSIFIAGNAYNITLTLLDSY
 51  GDPTCVLYDPLLSVSCPSSSSNGRDFCSLQLSPLYNGVYNIKVIPQTLWG
101  GHTVWPPPYSPVAPLPDVYFAGDTAIRITYNGRDIQGSPFPVTVQAEPHI
151  STALSTVNVAVPAKGVASSRFAIAYFYQISDRFTNWIREKSIATQLRVSA
201  YPDADISVQWWQNWWIVLYANSTSAGMYRFQVYFIDDDGSEVPIRILSPG
251  LGLSYDGSFEVLPLALDEAKITASGLPQEVEAGIPVSLTLQAMDIYSNPT
301  RLVDPESYQPFGQQPDNKTLLQVRLVTVDSGALQPNVVAIPTNTTGACSW
351  SITFFTSMDYSVSVTYKESVLHMFSITVRNAQASPSNSTALLPEIGQAGT
401  TLLYVTPRDLWGNIAPLANNDLSIGLTGSTFFHSFIPVEPVRKGDYSVYS
451  LTLTEAGLYVVSIQLHNSSWLEKNITIEASYPSLQRSYVLGFGAGDPYGF
501  APTPLVAGEQYVLRVFIKDLYGNTIQADKVVDLNIVGPGQVLMNMSMLPS
551  GAFEVIYQPIVVGVYAVIANLTTGLLLHRGAVYVQPGTFNPNATTLQVPD
601  YIVAGEASSFKLAFHDSYGNAAASSGEASVVVFKYGGESLSFPLNLSGKF
651  IEELPFRLMHSGLYAFSISVNSVLVKSNNGYLHVVPGSAYALNITELKMT
701  DRLLEVYAYVVDEYLNSLNAAAMTAFAVAIDPPILFHGNVTLSSTGITLQ
751  LDKDPAIENTLQVQLKISNTIFFNCTWKPTAAHATLRRVRSIGPLIAITA
801  VCVCILFASIALVWSFRMLRHKP
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Figure 7. *Fus1* protein sequence. The putative signal sequence is double underlined, the transmembrane sequence is boxed, and the N-glycosylation sites are underlined, with the asparagine residue in bold. The arrow marks the location of an inserted aspartate in some *fus1-3* revertants.

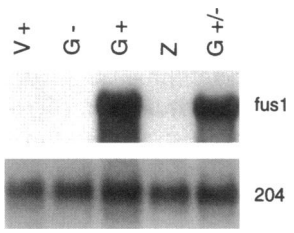

Figure 8. Regulation of *fus1* expression. RNA was prepared from vegetative mt^+ cells (V+), mt^- gametes (G−), mt^+ gametes (G+), zygotes 150 min after mating (Z), and gametes of an mt^+/mt^- diploid (G±). Approximately 10 μg of total RNA was loaded on each lane of a formaldehyde-containing 1% agarose gel, which was subsequently blotted to nitrocellulose and probed with a 1.65-kb *Eco*RV/*Bam*HI fragment (labeled *fus1*) from cDNA17. The same blot was also hybridized with a cDNA called 204 (Ferris and Goodenough, 1987), which is expressed at fairly constant levels in all life-cycle stages, to ensure that all RNA samples were undegraded and had comparable levels of mRNA.

late that the presence of *fus1* protein in both of these cell types may in some way impede *minus* mating structure function.

Sexual recognition in *C. reinhardtii* is effected first by flagellar agglutination and then by fringe–fringe interactions at the plasma membrane (reviewed in Goodenough *et al.*, 1995). The genes encoding the flagellar agglutinins have not yet been cloned, but the proteins have been purified and characterized (Cooper *et al.*, 1983; Collin-Osdoby and Adair, 1985) as hydroxyproline-rich glycoproteins with amino acid compositions very different from the *fus1* protein. Therefore, these two recognition systems clearly represent independent evolutionary ideas.

To our knowledge, this is the first report of a sex-specific adhesion molecule being encoded in a mating-type locus. In yeast, the **a** and α agglutinin proteins, and the **a** and α pheromones and pheromone receptors, are encoded by genes that are expressed in only one mating type but are not linked to MAT (reviewed in Herskowitz, 1989; Lipke and Kurjan, 1992). Genes encoding recognition molecules have been reported in the mating-type loci of two higher fungi: the pheromone/pheromone receptor genes are located in the a1/a2 loci of heterobasidiomycete *Ustilago maydis* (Bölker *et al.*, 1992; Spelling *et al.*, 1994), and analogous gene pairs are located in the B locus of the basidiomycete *Schizophyllum commune* (Wendland *et al.*, 1995).

Table 2. Codon usage in *C. reinhardtii* and in the *fus1* gene

Aa	Codon	Genome	*fus1*	Aa	Codon	Genome	*fus1*	Aa	Codon	Genome	*fus1*
A	GCG	27	11	K	AAG	99	57	S	AGT	2	12
	GCA	5	44		AAA	1	43		AGC	29	32
	GCT	15	25						TCG	30	6
	GCC	53	20						TCA	2	17
				L	TTG	2	22		TCT	8	18
					TTA	0	12		TCC	29	15
C	TGT	4	25		CTG	82	20				
	TGC	96	75		CTA	2	12	T	ACG	23	22
					CTT	4	19		ACA	3	28
D	GAT	13	35		CTC	11	15		ACT	10	30
	GAC	87	65						ACC	64	20
				N	AAT	5	56				
E	GAG	98	56		AAC	95	44	V	GTG	69	29
	GAA	3	44						GTA	1	24
				P	CCG	24	26		GTT	6	34
F	TTT	12	61		CCA	4	42		GTC	25	13
	TTC	88	39		CCT	11	17				
					CCC	62	15	Y	TAT	5	55
G	GGG	3	23						TAC	95	45
	GGA	2	29	Q	CAG	95	55				
	GGT	15	17		CAA	5	45	End	TGA	5	0
	GGC	80	31						TAG	18	100
									TAA	77	0
				R	AGG	3	8				
H	CAT	9	67		AGA	1	17				
	CAC	91	33		CGG	8	21				
					CGA	1	8				
I	ATA	1	18		CGT	10	33				
	ATT	24	51		CGC	76	13				
	ATC	75	31								

The genome column shows the relative frequency of possible codons for each amino acid in all *C. reinhardtii* nuclear sequences in the GenBank database (95 sequences, 36787 codons, LeDizet and Piperno, 1995). The *fus1* column shows the relative frequency of codons in the *fus1* gene (824 codons).

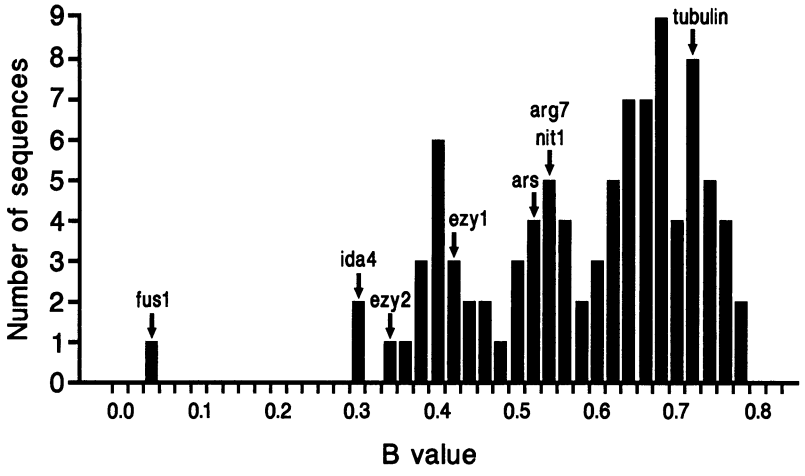

Figure 9. Codon usage bias in nuclear genes of *C. reinhardtii*. The "standardized synonymous codon bias" B (Long and Gillespie, 1991) was evaluated for sequences by LeDizet and Piperno (1995) and M. LeDizet (unpublished observations). A highly biased codon usage is reflected by a high value of B, which varies from 0 to 1. The *fus1* gene shows virtually no bias (B = 0.050). Two genes just outside the R domain are biased (*ezy1* = 0.43; *ezy2* = 0.35), as are two genes whose transcripts are present in low abundance (*arg7* = 0.54; *nit1* = 0.55). A previous report (de Hostos *et al.*, 1989) claiming a low bias in the arylsulfatase (*ars*) gene may be the consequence of sequencing errors (Hallmann and Sumper, 1994); corrected, *ars* = 0.52.

Mutations in the fus1 Gene

Three mutations in the *fus1* gene generate an agglutinate-but-not-fuse phenotype in mt^+ cells. The *fus1-1* mutation is shown here to be the consequence of a *Tcr1* transposon insertion in the 10th intron of the *fus1* gene. Since the *fus1-1* mutant is slightly leaky (Goodenough *et al.*, 1976), the presence of the transposon in an intron may render correct splicing unlikely but occasionally feasible. The partial restoration of fusability in two revertants that appear to have incompletely excised *Tcr1* is consistent with this hypothesis. The *fus1-2* and *-3* mutants, which are both caused by the insertion of *Tcr3* into an exon, behave quite differently. When rare zygotes resulting from a *fus1-2* or *-3* cross are analyzed (the *fus1-2* data are not described here), the mt^+ progeny invariably mate normally. Therefore, it appears that these two strains may be able to form zygotes only if the mutations revert; they either are not leaky or are leaky at a frequency below that of excision of the *Tcr3* transposon. The fact that all three alleles of *fus1* are transposon insertions is consistent with the possibility that the *fus1* gene might be a "hot spot" for transposon insertions.

Fus1 Rescue of the imp11 Fusion Defect

The ability of the $fus1^+$ gene to confer fringe and fusability to the *imp11* mutant strain supports our original interpretation of the *imp11* phenotype. We proposed (Goodenough *et al.*, 1982; Galloway and Goodenough, 1985; Goodenough and Ferris, 1987) that the mt^- *imp11* mutation inactivates a gene necessary to switch on the *minus* gametogenesis program and switch off the *plus* program, a gene that accounts for the dominance of mt^- over mt^+ in diploid *Chlamydomonas* (Ebersold, 1967). By this reasoning, most of the genes in the *plus* gametogenesis pathway would reside outside the mt^+ locus and, thus, would be present in *imp11* gametes, explaining their ability to express most *plus* gametic traits. Any genes resident in the mt^+ locus, however, would be absent in *imp11* gametes. The lack of fringe on *imp11* mating structures, and its restoration when *imp11* cells are transformed with the $fus1^+$ gene, is concordant with this proposal.

In experiments to be reported elsewhere, we have shown that the zygotes resulting from a cross between $fus1^+$-transformed *imp11* and mt^- are able to undergo meiosis and germination normally but fail to transmit their chloroplast DNA to the meiotic progeny in a uniparental manner. Therefore, a second gene product absent from *imp11* gametes, and therefore presumably encoded by the mt^+ locus, plays a key role in the transmission of mt^+ chloroplast DNA. The fact that meiosis proceeds normally when the zygote has no mt^+ chromosome indicates that any *plus* functions necessary for zygote development are encoded elsewhere in the genome and are expressed in the absence of *minus* dominance.

The phenotype of the $fus1^+$-transformed *imp11* strain is not fully wild-type. This transformant establishes fringe–fringe adhesions with its mt^- partners, but fusion is slow and episodic and is aided by manipulations that allow calcium influx at the cell apex. Although it is possible that suboptimal expression of the transgene affects the efficiency of the process, the expression of the same transgene in a *fus1-1* background permits rapid fusion. Therefore, a third gene product may also be encoded in the mt^+ locus, namely, a molecule necessary for efficient gametic fusion. Of interest in this regard is the finding that mammalian sperm display an α/β dimer on their membranes, the β subunit involved in recognizing an egg integrin receptor (Almeida *et al.*, 1995) and the α subunit effecting membrane fusion via a do-

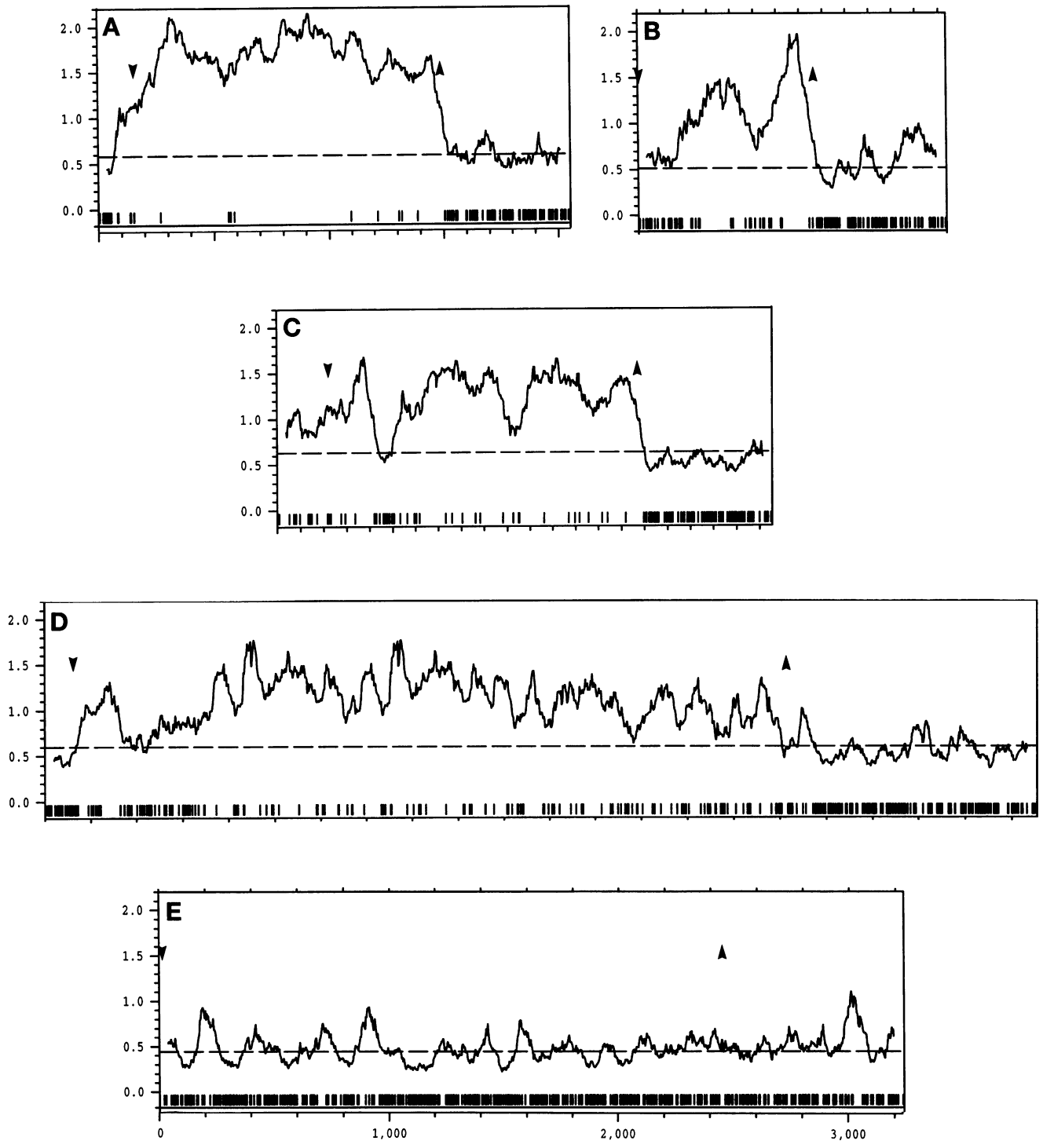

Figure 10. Lack of *Chlamydomonas* codon bias in the *fus1* gene. The graphical output of the GCG program Codonpreference is shown for several *C. reinhardtii* cDNA sequences by using a window of 25 codons and by using the data from LeDizet and Piperno (1995) (Table 2) for the *C. reinhardtii* codon usage table. The start and stop codons of each open reading frame are marked by downward and upward arrowheads, respectively. Tick marks at the bottom of each panel represent rare codons (those used no more than 10% of the time in the *Chlamydomonas* gene set; compare with Table 2). Values above the dashed line indicate use of preferred codons. Note how noncoding sequences (i.e., the 3'-untranslated region) do not show a codon bias. (A) β1 tubulin (Youngblom *et al.*, 1984); (B) *ida4* (LeDizet and Piperno, 1995); (C) *ezy1* (Armbrust *et al.*, 1993); (D) *ezy2* (E.V. Armbrust, unpublished observations); (E) *fus1*.

main homologous to viral fusion peptides (Wolfsberg *et al.*, 1995).

Evolution of Sexual Recognition Genes

Of more than 95 published *Chlamydomonas* cDNA sequences, the *fus1* gene is the first that fails to display strong codon bias (Figures 9 and 10). The *Chlamydomonas* bias, which favors G and particularly C in the third position (Table 2), is found throughout the *Volvocales* (Schmitt *et al.*, 1992), a lineage that extends back to the Cambrian (Larson *et al.*, 1992). The random use of codons in the *fus1* gene, therefore, is remarkable.

The most popular explanation for the maintenance of codon bias is that there is weak selection against codons that are not translated with optimal efficiency (cf. Akashi, 1995). By this logic, the *fus1* gene product would be translated under a unique set of constraints, a possibility we consider unlikely but cannot rule out. The fact that the *fus1* introns are as AT-biased as the *fus1* exons, moreover, suggests that translational selection may not be the critical factor.

A second possibility is that the *fus1* gene has been introduced recently into the *Chlamydomonas* genome from some other source (e.g., a virus), a possibility we also consider unlikely but cannot rule out.

The third possibility is that the *fus1* gene has been subjected to mutational pressures that override the bias-maintaining constraints. For example, mutations that generate nonpreferred codons might be occurring at a higher frequency than the (weak) selective events that ordinarily eliminate them (Akashi, 1995). The fact that the R domain has clearly been subject to numerous mutations at the chromosome level (Ferris and Goodenough, 1994) may extend to the nucleotide level as well. Interestingly, rapid evolution has also been documented in the mammalian male sex-determining gene, *SRY* (Tucker and Lundrigan, 1993; Whitfield *et al.*, 1993). Furthermore, *SRY* exhibits an unusual discrepancy between codon usage and base composition of the surrounding DNA, suggesting that the selective forces governing *SRY* evolution outweigh those determining codon bias (G. McVean and L. Hurst, personal communication). Moreover, dramatic differences in sex-related genes are found when closely related species are compared in molluscs (Swanson and Vacquier, 1995) and echinoderms (Metz and Palumbi, 1996). If sex-related genes, in general, are subject to unique mutational mechanisms, this might well play a role in the evolution of species.

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