A Gamete-specific, Sex-limited Homeodomain Protein in *Chlamydomonas*

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Abstract. During fertilization in *Chlamydomonas*, gametes of opposite mating types interact with each other through sex-specific adhesion molecules on their flagellar surfaces. Flagellar adhesion brings the cell bodies of the gametes into close contact and initiates a signal transduction pathway in preparation for cell–cell fusion. We have identified a cDNA, *gsp1*, whose transcript levels are upregulated during flagellar adhesion. The GSP1 polypeptide is a novel, gamete-specific homeodomain protein, the first to be identified in an alga. Its homeodomain shows significant identity with several higher plant homeodomain proteins. Although encoded by a single copy gene present in cells of both mating types, immunoblot analysis showed that GSP1 was expressed in mating type (mt)+ gametes, but was not detectable in mt− gametes or in vegetative cells of either mating type. Moreover, GSP1 appeared late during gametogenesis, suggesting that it may function during adhesion with mt− gametes or after zygote formation. GSP1 is expressed in *imp11*, mt− mutant gametes, which have a lesion in the *mid* gene involved in sex determination and exhibit many phenotypic characteristics of mt+ gametes. Thus, *gsp1* is negatively regulated by *mid* and is the first molecule to be identified in *Chlamydomonas* that shows sex-limited expression.

Key words: *Chlamydomonas* • homeodomain • sex-limited • fertilization • cell–cell adhesion

Fertilization is the culmination of a set of well-choreographed cellular and molecular events in sexually reproducing eukaryotic organisms. Initial molecular events in fertilization involve adhesive interactions between cell surface molecules on gametes of opposite sexes, leading to species-specific binding and signal transduction. In many organisms, the adhesive interactions between cognate gametes initiate cellular responses in one or both cells, culminating in cell–cell fusion (Yanagimachi, 1988; Snell 1990; reviewed in Myles, 1993; Wassarman, 1995; Snell and White, 1996). Cell fusion itself generates signals that prevent additional cells from fusing with the zygote and is followed by the initiation of a new developmental pathway (Jurgens, 1995; Nothias et al., 1995; Patterton and Wolffe, 1996). While many of the molecular mechanisms regulating differentiation of sex-specific (or mating type–specific) cells, as well as the mechanisms regulating adhesion and fusion of sex cells and the initiation of development in the zygote (diploid) cell, have been elucidated in yeast and other fungi (reviewed in Johnson, 1995; Madhani and Fink, 1998), much remains to be learned about the molecular mechanisms that regulate these processes in other organisms.

Our laboratory studies fertilization in the biflagellated alga, *Chlamydomonas reinhardtii*. In this organism, cells of the two mating types (mt),[1] mt+ and mt−, are haploid and are cultured asexually as vegetative cells. Under appropriate environmental conditions, the vegetative cells differentiate into fertilization-competent gametes that express mating type–specific adhesion molecules, called agglutinins, on their flagella (Adair, 1985). When gametes of opposite mating types are mixed, they adhere to each other via their flagellar agglutinins. Flagellar adhesion induces a signal transduction pathway involving a complex interplay among protein kinases (Zhang et al., 1991; Zhang and Snell, 1993; Kurvari et al., 1996; Zhang et al., 1996), leading to the activation of a membrane-bound adenylylcyclase and resulting in a rapid increase in intracellular cAMP concentration (Pijst et al., 1984; Pasquale and Goodenough, 1987). The increased levels of cAMP activate the cells for cell fusion by inducing several cellular events including recruitment of additional agglutinins from

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1. *Abbreviations used in this paper:* atpC1, ATP synthase subunit C; mt, mating type; ORF, open reading frame.

the plasma membrane of the cell body, release of the extracellular matrix, and activation of cell fusion organelles called mating structures (Goodenough et al., 1985; Goodenough, 1992; Van Den Ende, 1992; Snell, 1993; Wilson et al., 1997; Wilson and Snell, 1998).

To learn more about regulation of fertilization in *Chlamydomonas* we have used subtractive hybridization and differential screening (Kurvari et al., 1995) to identify molecules abundant in adhering gametes but absent in asexual, vegetative cells. Here we describe the identification and characterization of a cDNA for *gsp1* (for gamete-specific plus [mating type] molecule 1). The *gsp1* cDNA encodes the first homeodomain protein to be identified in an alga and exhibits gamete-specific, sex-limited expression.

Materials and Methods

Cells

Chlamydomonas reinhardtii strains *21gr* (mt+), *imp1* (mt+), and *6145C* (mt−) (available from the *Chlamydomonas* Genetics Center, Duke University, Durham, NC) were cultured at 23°C on a 13-h/11-h light/dark cycle as described earlier (Kurvari et al., 1995). The mt− mutant *imp11* was provided by Patrick Ferris and Ursula Goodenough (Washington University, St. Louis, MO). Vegetative cells were induced to become gametes by resuspension in medium without NH_4NO_3, followed by culturing in continuous light at room temperature (Snell, 1980). Adhering gametes were prepared by incubating mt+ gametes with flagella isolated from mt− gametes as described earlier (Kurvari et al., 1995). Cell walls were removed from vegetative cells by incubating a suspension of cells in a crude preparation of the metalloproteinase GLE (Snell, 1982; Kinoshita et al., 1992; Kurvari et al., 1995).

Nucleic Acid Hybridizations

For Northern blot hybridizations, ~1.0 μg of *Chlamydomonas* poly (A)–selected mRNA was size-fractionated on a 1% denaturing formaldehyde agarose gel, transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH), incubated with a nucleotide probe derived from a 1.0-kb HincII fragment from *gsp1* cDNA, and analyzed by autoradiography as described earlier (Kurvari et al., 1995). The nucleotide probe for ATP synthase subunit C (atpC1) (Yu and Selman, 1988) was prepared from a plasmid containing cDNA (provided by Bruce Selman's laboratory, University of Wisconsin, Madison, WI). For Southern blots, ~10 μg of *Chlamydomonas* genomic DNA was digested with EcoRI and ApaI (Life Technologies, Inc., Bethesda, MD) according to the manufacturer's recommendations, fractionated by agarose electrophoresis, transferred to a nylon membrane, hybridized with a random-primed nucleotide probe derived from the linearized *gsp1* cDNA, and analyzed by autoradiography as done previously (Kurvari et al., 1995).

Cloning and Sequencing

A λ ZapII cDNA library containing *Chlamydomonas* cDNAs prepared from mt+ gametes undergoing adhesion with mt− flagella was constructed and differentially screened for clones whose transcripts were upregulated during flagellar adhesion with mt− gametes. As described previously (Kurvari et al., 1995; Kurvari, 1997), ~50,000 plaques from an unamplified gametic cDNA library in λ ZapII were screened using random primer–labeled, subtracted gametic cDNA and vegetative cDNA probes. The gametic cDNA probe was prepared by removal of transcripts common to both vegetative and gametic cells through one round of subtractive hybridization with an excess of biotinylated vegetative mRNA. After an initial round of differential hybridization using the subtracted gametic cDNA and vegetative cDNA probes, *gsp1* was selected based on the property that it hybridized with the subtracted gametic cDNA and did not hybridize with the vegetative cDNA. After three rounds of plaque hybridizations, the *gsp1* λ ZapII recombinant phage clone was in vitro excised as recommended by the manufacturer (Stratagene, San Diego, CA), yielding a recombinant pBluescriptII plasmid containing *gsp1* cDNA. The cDNA clone contained a 3.5-kb insert that was characterized further by restriction endonuclease mapping and nucleotide sequencing. DNA sequencing was performed by manual methods as described earlier (Kurvari et al., 1995; Kurvari et al., 1996) and automated DNA sequencing methods.

Production and Purification of Polyclonal Antibodies

Antipeptide antibodies were purchased from Quality Controlled Biochemicals, Inc. (Hopkinton, MA). In brief, two peptides (CYPEATPSGQPPTHPHQQ and CAEASTDHKRARTNNP) derived from the open reading frame (ORF) in *gsp1* cDNA (positions 108 and 548) were synthesized, verified by mass spectroscopy, coupled to BSA, emulsified with an equal volume of Freund's adjuvant, and both were injected subcutaneously into two New Zealand White rabbits. The immune sera were collected and affinity-purified on a mixed-bed matrix containing a mixture of the two peptides. The antibodies were repurified in our laboratory on affinity columns containing single peptide matrices using methods described earlier (Kurvari et al., 1995; Kurvari and Snell, 1996).

Cell Fractionation and Immunoblotting

For immunoblot analysis of GSP1 in cells and cell fractions, vegetative cells were induced to become gametes as described earlier (Snell, 1980), and whole cells (vegetative cells or gametes) were collected, resuspended in Tris-saline buffer (10 mM Tris, pH 7.6, 20 mM NaCl) containing protease inhibitors (2 mM PMSF, 10 μM leupeptin, 1 μM pepstatin, 1 mM ortho-phenanthroline, 40 μg/ml chymostatin, and 10 μM E-64 [trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane]), and boiled for 4 min in sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, and 0.001% bromophenol blue) for analysis by SDS-PAGE on 9% acrylamide gels as described previously (Kurvari et al., 1995). Flagella and cell bodies were isolated by the pH shock method of Witman et al. (1972).

Electrophoretic transfer of proteins to polyvinylidene membranes (Millipore Corp., Bedford, MA) was carried out as described earlier (Kurvari and Snell, 1996). The blots were blocked with 5% dry milk (Carnation; Nestle Food Co., Glendale, CA) and 1% BSA in 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween-20 (TBS-T), followed by incubation with affinity-purified antibody diluted 1:100 in TBS-T containing 3% dry milk for 1 h. (In the immunoblotting experiments shown here only the antibody against the NH_2-terminal peptide was used. With the exception that it detected a lower molecular weight band that probably is a GSP1 degradation product, similar results were obtained when the COOH-terminal antibody was used.) After a brief wash with TBS-T, the blots were incubated with horseradish peroxidase–conjugated, goat anti–rabbit antibody (1:10,000) in TBS-T containing 3% dry milk for 1 h. The blots were treated as recommended by the supplier for the ECL detection system (Amersham Corp., Arlington Heights, IL) and exposed to x-ray film.

Gametogenesis and Zygote Formation

For analysis of GSP1 expression during gametogenesis, vegetative cells were resuspended in a medium without NH_4NO_3 6 h after the beginning of the light cycle, aerated in continuous light, and ~2×10^6 cells were collected at the indicated times (see Fig. 6, *G0–G24*). Cells were collected by centrifugation at 4°C, resuspended in Tris-saline buffer containing protease inhibitors, and flash-frozen in liquid N_2 until use. The ability of the newly formed gametes to agglutinate with mt− gametes was determined by microscopic evaluation.

Results

gsp1 Transcripts in mt+ Gametes Were Upregulated by Signals Induced during Fertilization

To identify gamete-specific regulatory molecules with a potential role in fertilization we used subtractive and differential hybridization methods to isolate cDNA clones whose transcripts were expressed by adhering *Chlamydomonas* gametes but not by vegetative cells. Using this strategy, we identified a cDNA clone whose expression was upregulated during adhesion of mt+ gametes; the

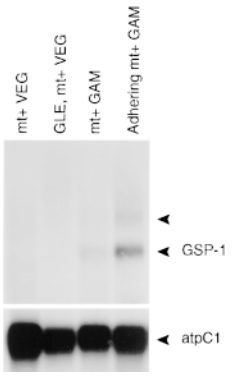

Figure 1. Expression of *gsp1* mRNA. *gsp1* transcript levels in resting and GLE-treated vegetative cells and non-activated and activated gametes. Poly(A)$^+$ RNA was isolated from mt+ vegetative cells (*mt+ VEG*), mt+ vegetative cells whose walls had been removed by incubation with the cell wall releasing enzyme GLE (*GLE, mt+ VEG*), mt+ gametes (*mt+ GAM*), and mt+ gametes incubated with mt− flagella for 4 h as described previously (Kurvari et al., 1995) (*Adhering mt+ GAM*). Arrows indicate the positions of 4.7- and 8.4-kb transcripts. The top panel shows hybridization with a *gsp1* cDNA probe and the bottom panel shows hybridization with a probe derived from the constitutively expressed atpC1 that encodes the ATP synthase subunit C (Yu and Selman, 1988).

molecule is designated *gsp1* (for gamete-specific plus [mating type] molecule 1).

gsp1 cDNA hybridized to a transcript of 4.7 kb in mt+ gametes (Fig. 1, *mt+ GAM*). In adhering gametes, the levels of the 4.7-kb transcript were upregulated severalfold and an 8.4-kb transcript was detected also (Fig. 1, *Adhering mt+ GAM*), although at much lower levels. Transcripts for *gsp1* were not detectable in vegetatively growing mt+ cells (Fig. 1, *mt+ VEG*) or in mt+ vegetative cells induced to undergo cell wall regeneration by incubation with the wall releasing enzyme GLE (Fig. 1, *GLE*, *mt+ VEG*).

Sequence Analysis

Nucleotide sequencing indicated that the *gsp1* cDNA, which was 3.5 kb in length, contained a single ORF of 1,033 amino acids beginning with the ATG codon at nucleotide position 67 through the termination codon at position 3166 (Fig. 2). The ATG codon at position 67 was chosen because of an in-frame stop codon immediately upstream of it, and because of its association with a conventional translation initiation sequence (Kozak, 1988). Codon usage within this ORF was typical of the *Chlamydomonas* bias (LeDizet and Piperno, 1995), and the nucleotide sequence was GC rich (68%). The cDNA contained a 32-bp poly(A) tail at its 3′ end and a putative polyadenylation signal sequence (TTGTTT) at position 3433, similar to those found in other *Chlamydomonas* transcripts (Youngblom et al., 1984; Silflow et al., 1985).

The *gsp1* cDNA predicted a polypeptide of 106 kD with an estimated pI of 6.5. Analysis of the predicted amino acid sequence indicated that the molecule is rich in alanine (20%), glycine (10%), glutamine (9%), histidine (8%), and proline (8%). It contains consensus phosphorylation sites for several protein kinases, a putative site for amidation, and several myristoylation sites (data not shown). GSP1 also contains a potential PEST sequence (Fig. 2, *dashed underline*) from residue 1001 to 1033 with a PEST score of 32.88 (Rogers et al., 1986; Rechsteiner and Rogers, 1996). Analysis of its hydrophilicity properties (Kyte and Doolittle, 1982) suggested that GSP1 does not contain a signal peptide sequence at its NH_2-terminal end and lacks stretches of hydrophobic residues sufficient to form helical transmembrane domains (not shown).

Figure 2. Predicted amino acid sequence of GSP1. The nucleotide sequence along with the predicted amino acid sequence of GSP1 are shown. Several regions of the protein are marked as follows: peptides used to make polyclonal antibodies, *single underline*; H/Q-rich region, *gray shading*; polyPro region, *double underline*; homeodomain, *white letters on black background*; D/E-rich regions, *unshaded boxes*; basic regions, *gray-shaded boxes*; potential PEST sequence, *dashed underline*.

According to the amino acid composition, at least six distinct types of low complexity regions are present in the GSP1 sequence (Fig. 3). One of the most notable is an extended region of high hydrophilicity (amino acids 120–357; Fig. 3, *shaded box*, *H/Q*) enriched in Gln and His (28% of each). This H/Q-rich domain contains imperfect repeats consisting of 3–6 Gln and His. A COOH-terminal hydrophilic sequence consists of four distinct regions: two highly acidic regions (*open box*, *D/E*) next to two regions enriched in positively charged amino acids (*K/R* and *R*). The very COOH-terminal D/E-rich region is particularly long (32 residues), suggesting functional importance. A poly-Pro sequence (*diamond*, *poly-P*) occurs in the middle of

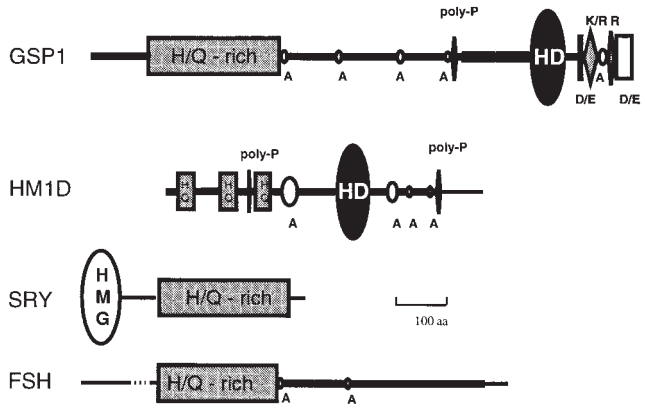

Figure 3. Analysis of GSP1 sequence. Comparison of domain organization of GSP1 with other proteins containing similar low complexity regions. Sequence diagrams are drawn to scale with numbers indicating approximate domain boundaries. SwissProt IDs where available or gi (gene identification) numbers are used as protein identifiers. *HM1D*, homeobox protein OM(1D) from *Drosophila ananassae*; *SRY*, sex-determining protein from mouse; and *FSH*, female sterile homeotic protein from *Drosophila melanogaster*. Dots stand for the regions not shown. Domain coding: *oval*, DNA-binding domain; *black oval* (*HD*), homeodomain; *white oval* (*HMG*), HMG box; *gray rectangle* (*H/Q*), His/Gln-rich region; *white rectangle* (*D/E*), Asp/Glu-rich region; *gray diamond* (*K/R*), Lys/Arg-rich region; *black diamond*, polyproline region; *white circles*, alanine-rich region; *white boxes* between poly-P and HD in GSP1 indicate a possible sequence repeat; *thick line*, Ala/Ser/Gly/Pro rich regions; *thin line*, all other sequences.

GSP1. The rest of the GSP1 sequence, excluding the homeodomain (*HD*; see below), is compositionally biased to a high content of Ala, Gly, Ser, and Pro. Four poly-Ala stretches (no less than 5 Ala in a row) occur between the H/Q and poly-Pro regions (*open circles*, *A*). Additionally, a region with two weak repeats (*open rectangles*) is detected between the poly-Pro region and the homeodomain.

GSP1 Is a Homeodomain Protein

Database search analysis using the BLAST program (Altschul et al., 1997) indicated that GSP1 has regions that share significant sequence similarity with a variety of proteins that contain homeodomains (Fig. 2 and Fig. 4 *A*). The homeodomain is a 60–63-amino acid, DNA-binding domain (McGinnis et al., 1984; Scott and Weiner, 1984; Gehring et al., 1994) that has been found in animals, fungi, and higher plants (Burglin, 1994). Fig. 4 *A* presents an alignment of the homeodomain region of GSP1 with the MATα2 homeodomain from *Saccharomyces cerevisiae*, with the Antennapedia (Antp) homeodomain from *Drosophila melanogaster,* and with homeodomains from several higher plant proteins. With the exception of Antp, all of these are atypical homeodomains and members of the TALE superclass (see below). The GSP1 homeodomain is most similar (37% identity) to the homeodomain of the protein encoded by the *knotted1*-like gene from *Arabidopsis* (Knat1; Fig. 4 *A*) (Lincoln et al., 1994). The GSP1 sequence shows 33% identity with the Maize *knotted1* sequence (Kn1) (Vollbrecht et al., 1991), and 35% with soybean (SBH1) (Ma et al., 1994) and *Arabidopsis* (Bel1) (Reiser et al., 1995) homeodomain sequences. The identity with the MATα2 homeodomain is 19% and with that of Antp, 12%. Within a highly conserved, 12–amino acid region in helix three of the homeodomain (residues 51–62 according to the numbering of the homeodomain in Fig. 4 *A*), GSP1 is 67% identical to the plant proteins. The three well-defined, highly conserved α helices in homeodomains are indicated by open rectangles above the sequences.

Unusual Features of the GSP1 Homeodomain

In addition to the substantive sequence similarity to other homeodomains, the GSP1 homeodomain contains several unique features. One is the near absence of positively charged residues preceding the first helix. Instead, GSP1 contains a hydrophobic, Leu-rich sequence at this site. A second conspicuous feature is the occupation of some sites in GSP1 by residues that rarely occur in these sites in other homeodomain sequences (Burglin, 1994). These include Ala861 (residue 20 in the homeodomain, Fig. 4 *A*), which in most other homeodomains is occupied by aromatic or large aliphatic residues; Trp896 (residue 55 in the homeodomain), which is exclusively Arg, Gln, or Ala in other sequences; Tyr881 (residue 40 in the homeodomain) instead of small hydrophilic residues; and Leu852 (residue 11 in the homeodomain) at a site usually occupied by charged residues. These substitutions are rationalized when the GSP1 sequence is mapped onto the known structure of the MATα*1* homeodomain (Kraulis, 1991; Li et al., 1995) as shown in Fig. 4 *B*. Indeed, residues in positions 861 and 896 are in contact with each other in the hydrophobic core of the molecule and participate in the positioning of helices 1 and 3. In other homeodomain proteins, residue 20 of the homeodomain, the site aligned with 861, is occupied by a bulky residue that interacts with the aliphatic part of a side chain, usually Gln or Arg. In GSP1 the substitutions are compensatory. The bulky Trp in site 896 interacts with the small Ala861. Another pair of unique residues (Leu852 and Tyr881) are also predicted to be in structural proximity. In other homeodomains these sites are on the exposed surface of the molecule and are occupied by hydrophilic residues. These hydrophobic residues in the GSP1 homeodomain might interact with other parts of the GSP1 sequence or with other proteins.

Unlike typical homeodomains, such as those of *Antp* and *MATα1*, which have 60 amino acids in their homeodomains (Burglin, 1994), the GSP1 homeodomain has 63 amino acids, making it a new member of the TALE group of homeodomains (Bertolino et al., 1995; Burglin, 1997). Members of this superclass, which also are found in plants, fungi, and animals, have three extra amino acids between helices 1 and 2. Many members of the TALE group also have well-defined sequence similarities outside the homeodomain (Bharathan et al., 1997; Burglin, 1997; Burglin, 1998). These domains, including the MEINOX domain (Burglin, 1997; Burglin, 1998), were not apparent in GSP1. Clustogram analysis of the similarity of the GSP1 homeodomain to the homeodomains of other TALE proteins

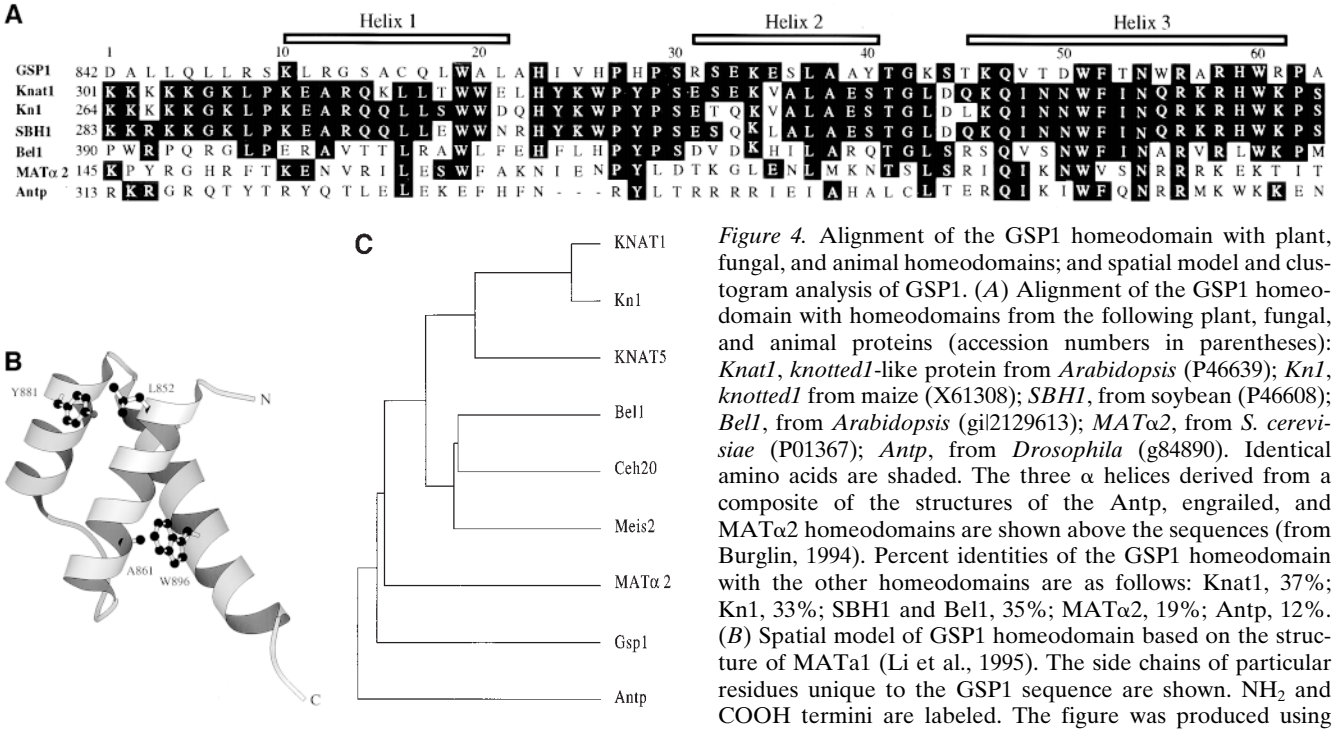

Figure 4. Alignment of the GSP1 homeodomain with plant, fungal, and animal homeodomains; and spatial model and clustogram analysis of GSP1. (*A*) Alignment of the GSP1 homeodomain with homeodomains from the following plant, fungal, and animal proteins (accession numbers in parentheses): *Knat1*, *knotted1*-like protein from *Arabidopsis* (P46639); *Kn1*, *knotted1* from maize (X61308); *SBH1*, from soybean (P46608); *Bel1*, from *Arabidopsis* (gi|2129613); *MATα2*, from *S. cerevisiae* (P01367); *Antp*, from *Drosophila* (g84890). Identical amino acids are shaded. The three α helices derived from a composite of the structures of the Antp, engrailed, and MATα2 homeodomains are shown above the sequences (from Burglin, 1994). Percent identities of the GSP1 homeodomain with the other homeodomains are as follows: Knat1, 37%; Kn1, 33%; SBH1 and Bel1, 35%; MATα2, 19%; Antp, 12%. (*B*) Spatial model of GSP1 homeodomain based on the structure of MATa1 (Li et al., 1995). The side chains of particular residues unique to the GSP1 sequence are shown. NH_2 and COOH termini are labeled. The figure was produced using MOLSCRIPT (Kraulis, 1991). (*C*) Clustogram of GSP1 and the following plant, fungal, and animal homeodomain sequences (accession numbers in parentheses): KNAT1, Kn1, KNAT5 from *Arabidopsis* (X92394), Bel1, Ceh20 from *C. elegans* (U01303), Meis2 from mouse (U57343), MATα2, and Antp. The clustogram was generated by the Pileup Program in the University of Wisconsin Genetics Computer Program software package. Sequences are grouped by similarity, and thus the clustogram does not necessarily reflect evolutionary relationships.

and *Antp* (Fig. 4 *C*) indicated that the divergence between GSP1 and the TALE sequences was greater than the divergence of the other TALE sequences among themselves. This method of analysis groups sequences simply by similarity, and the grouping does not necessarily reflect any evolutionary relationships.

Compositional Similarities between GSP1 and DNA-binding Proteins/Transcription Factors

The database searches described above were carried out using parameters that would exclude proteins that had only compositional similarity to GSP1, and under these conditions only other homeodomain proteins were detected in the searches. On the other hand, when we carried out BLAST searches without filtering out the low complexity regions, we detected compositional similarity to many transcription factors and other DNA-binding proteins (three of which are shown in Fig. 3). Some of these proteins contained homeodomain sequences as well. For example, the protein encoded by the *Drosophila* homeobox gene OM(1D) (HM1D in Fig. 3), involved in eye development, contains a His/Gln-rich region upstream of the homeodomain, a poly-Pro region, and Ala-rich regions (Tanda and Corces, 1991).

In addition to its similarity to homeodomain transcription factors, GSP1 shares low complexity regions with two DNA-binding proteins (shown in Fig. 3) that contain HMG-box motifs as well as His/Gln-rich regions: sex-determining protein from mouse (SRY; Goodfellow and Lovell-Badge, 1993), which is an HMG-box transcription factor, and a female sterile homeotic protein from *Drosophila* (FSH; Digan et al., 1986).

GSP1 Is Encoded by a Single Copy Gene Unlinked to the Mating Type Locus

Southern blot hybridizations of *Chlamydomonas* genomic DNA showed that a nucleotide probe derived from *gsp1* cDNA hybridized to sequences in genomic DNA from mt+ and mt− gametes digested with ApaI and EcoRI (not shown). Moreover, the sizes of the hybridizing DNA fragments in mt+ and mt− gametes were similar. By use of RFLP mapping, the gene for *gsp1* maps to the right arm of linkage group II (Kathir, P., C. Silflow, and P. Lefebvre, personal communication). Thus, the gene for *gsp1* is unlinked to the mating type locus.

α-GSP1 Antibodies Identified an ~140-kD Protein Detectable Only in mt+ Gametes

To further characterize GSP1 we used polyclonal antibodies directed against an NH_2-terminal peptide (YPEATPSGQPPTHPHQQ) and a COOH-terminal peptide (AEASTDHKRARTNNP) derived from the predicted amino acid sequence of the *gsp1* cDNA (singly underlined in Fig. 2). The antibodies were affinity-purified on a mixed-bed affinity matrix containing both peptides, followed by a second round of affinity purification on affinity matrices containing a single peptide species. The immunoblots shown here were done with the antibody against the NH_2-terminal peptide, and similar results were obtained with the sec-

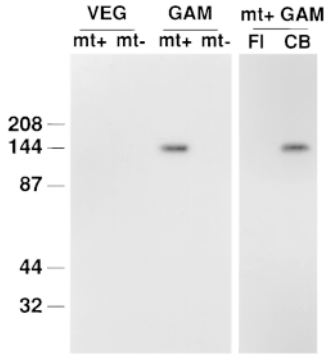

Figure 5. Immunoblots of mt+ and mt− gametes and vegetative cells and mt+ gamete cell fractions. (*Left*) Whole cell extracts of mt+ and mt− gametes and vegetative cells were analyzed by SDS-PAGE and immunoblotting with α-GSP1 antibody. Migration of prestained Kaleidoscope molecular weight standards (BioRad, Richmond, CA) is indicated on the left in this and subsequent figures. (*Right*) Samples of flagella (*Fl*) and cell bodies (*CB*) from mt+ gametes were resolved by SDS-PAGE and analyzed by immunoblotting using affinity-purified α-GSP1 antibody directed against the NH_2-terminal peptide (indicated in Fig. 2) of GSP1.

ond antibody. Analysis by SDS-PAGE and immunoblotting identified an antigen of 140 kD in mt+ gametes. As shown in Fig. 5 (*left*), the 140-kD antigen was detectable in mt+ gametes, and it was not detectable in mt− gametes or vegetative cells of either mating type. Cell fractionation studies showed that GSP1 was present only in the cell bodies and not in the flagella (Fig. 5, *right*). The apparent molecular mass of ~140 kD observed on SDS-PAGE gels was larger than the value estimated from the primary amino acid sequence (106 kD). This discrepancy may be due to the unusual amino acid composition or posttranslational modifications (Graceffa et al., 1992).

GSP1 Is Expressed Late during Chlamydomonas Gametogenesis

To learn more about the timing of the appearance of GSP1 during gametogenesis, mt+ gametes were collected at various times after vegetative cells were transferred to N-free medium, tested for their ability to agglutinate with mt− gametes, and samples were prepared for SDS-PAGE and immunoblotting. As shown in Fig. 6, GSP1 protein was detectable 12 h after the induction of gametogenesis.

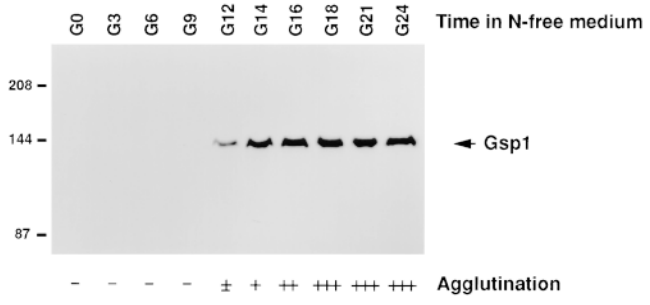

Figure 6. Expression of GSP1 during *Chlamydomonas* gametogenesis. Cells were collected at various times after induction of gametogenesis and analyzed by immunoblotting with α-GSP1 antibody. Estimates of the extent of agglutination shown at the bottom of the figure were based on examination by bright-field microscopy on an inverted microscope and ranged from − (no agglutination) to +++ (>95% agglutination).

More significantly, the appearance of GSP1 was concomitant with the acquisition of other gamete-specific properties including expression of agglutinin molecules as assayed by adhesion to mt− gametes. At this stage, cells are completing the final mitotic division that results in adhesion-competent daughter cells (Snell, 1980; Harris, 1989). These results suggested that GSP1 may play a role late in gametogenesis and may also function during fertilization or after zygote formation.

GSP1 Is Undetectable in Diploid Gametes, But Is Present in imp11 mt− Gametes

The restriction of GSP1 expression to mt+ gametes suggested that it might be regulated by the *mid* (minus dominance) gene (Ferris and Goodenough, 1997), which is located at the mt− locus. *Chlamydomonas* is haploid in both the vegetative and gametic phases of the life cycle, and mating type is determined by the mating type locus, a large region in the left arm of linkage group (VI). Cells carrying the mt− locus differentiate into mt− gametes, and cells carrying the mt+ locus differentiate into mt+ gametes. The presence of the *mid* gene, which is unique to the mt− locus, leads to the turning on of mt− functions and the turning off of mt+ functions (Ferris and Goodenough, 1997). For example, diploid strains of *Chlamydomonas*, which carry both an mt+ locus and an mt− locus, behave as wild-type mt− gametes after sexual differentiation because of the presence of the *mid* gene. Conversely, the *imp11* mt− mutant carries a point mutation in *mid* and behaves phenotypically as an mt+ gamete, except that it is unable to fuse. To determine if expression of GSP1 was under the control of *mid*, gametes from *imp11* and from diploid cells were tested for the presence of GSP1 by immunoblotting with an anti-GSP1 antibody. As shown in Fig. 7, GSP1 was detectable in *imp11* gametes, but it was absent in gametes prepared from diploid cells. This finding was consistent with the notion that GSP1 expression is regulated by *mid*. Control lanes show that GSP1 was detectable in wild-type mt+ gametes and in *imp1* mutant

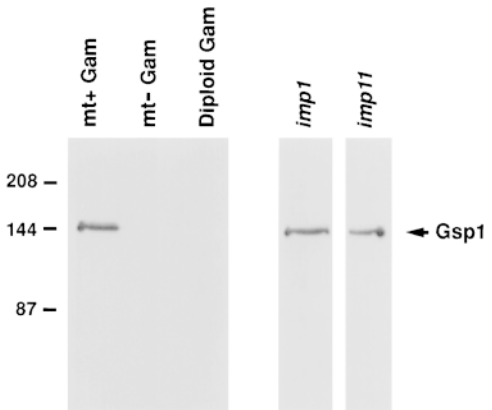

Figure 7. Expression of GSP1 in gametes of the pseudo-plus mutant, *imp11*, but not in diploid gametes. Samples from ~10^5 mt+, mt−, and diploid gametes (*left*) and *imp1* mt+ and *imp11* mutant mt− gametes (*right*) were prepared for SDS-PAGE and immunoblotting with α-GSP1 antibody as described in Materials and Methods.

mt+ gametes, which agglutinate as mt+ gametes but are unable to fuse (Ferris et al., 1996), and GSP1 was not detectable in mt− gametes.

Discussion

In this report, we have described a novel *Chlamydomonas* molecule, GSP1, that was identified by use of subtractive and differential hybridization methods (Dworkin and Dawid, 1980; Travis et al., 1989; Klar et al., 1992; Lelias et al., 1993). The cDNA for *gsp1* contains an ORF that predicts a polypeptide of calculated mass 106 kD. In Northern blot analysis, *gsp1* probes hybridized to a gametic transcript of 4.7 kb, with an additional, minor transcript of 8.4 kb (Fig. 1) observed in adhering gametes. Neither transcript was detected in vegetative cells. Since Southern blot analysis indicated that *Chlamydomonas* contains only a single gene for *gsp1*, the two transcripts probably were generated by differential splicing. On the other hand, both transcripts may not have been translated, since SDS-PAGE and immunoblotting with an antipeptide antibody did not show a band above the 140 kD one. The difference in size between the 3.5-kb cDNA and the 4.7-kb transcript may reflect an unusually long 5′ untranslated region. A full understanding of this difference and an understanding of the significance of the two transcripts will require characterization of the *gsp1* gene.

The Homeodomain of GSP1, the First Identified in an Alga

One noteworthy feature of GSP1 is that it is a homeodomain protein, the first to be identified in an alga. The homeodomain, which is present in many transcription factors, is a 60–63-amino acid DNA-binding motif that contains three α-helical regions. The third helix contains four highly invariant amino acids (W892, F893, N895, and R897) that are part of the DNA-recognition region of homeodomains (reviewed in Burglin, 1994; Gehring et al., 1994). Homeodomain proteins are involved in the genetic control of development (Gehring, 1987; Kornberg, 1993). In animals and plants they are important in pattern formation, and in fungi they are involved in sexual reproductive events, including determination of mating type (Casselton and Olesnicky, 1998). For example, the maize Kn1 molecule, the first homeodomain protein to be identified in a higher plant, regulates cell fate. Alterations in leaf morphology are one phenotype of plants with Kn1 mutations (Vollbrecht et al., 1991). Other plant homeodomains regulate ovule development (Reiser et al., 1995). In *S. cerevisiae*, homeodomain proteins (including STE12, MATa1, and MATα2) determine whether cells are of the a or α mating type (Johnson, 1995). In other fungi, such as *Ustilago* (corn smut) and *Cryptococcus* (Wickes et al., 1997), homeodomain proteins (including STE12 homologues) are involved in mating type determination and in triggering of pathogenic programs (reviewed in Madhani and Fink, 1998).

Database searches showed that the GSP1 homeodomain is most similar to the members of the KNOX class of homeodomains of higher plants (Kerstetter et al., 1994). The entire GSP1 homeodomain shows ~35% identity with Kn1 and other members of the KNOX family (Fig. 4 *A*). Like the homeodomains of the KNOX proteins, the GSP1 homeodomain contains 63 amino acids. The presence of the three extra amino acids (which are not present in typical homeodomains) between helices 1 and 2 defines GSP1 as a new member of the TALE group of homeodomain proteins. As with the typical homeodomain proteins, TALE homeodomain proteins are found also in plants, animals, and fungi (Burglin, 1997). In addition to similarities within the homeodomain, many of the TALE molecules have regions in common outside of the homeodomain that allow them to be grouped into subclasses (Bharathan et al., 1997; Burglin, 1997; Burglin, 1998). For example, four widely shared domains (PBC, TGIF, MEIS, and IRO) outside of the homeodomain have been found in animal TALE molecules and two (KNOX and BEL) have been detected in plant TALE proteins (Burglin, 1997). These domains outside of the homeodomain are not apparent in GSP1. Even when only the homeodomain is considered, clustogram analysis indicates that GSP1 is more divergent from representative TALE homeodomains than the TALE homeodomains are divergent from each other (Fig. 4 *C*). Although the clustogram method of analysis groups sequences simply by similarity and does not necessarily reflect any evolutionary relationships, future studies on this algal molecule may shed new light on the evolution of homeodomains.

Molecular Features of GSP1

Several features distinguish the GSP1 homeodomain from previously described typical and TALE homeodomains. One is the absence of a cluster of basic amino acids upstream of helix 1. Structural analysis has shown that amino acids in this region of homeodomains make contacts with the minor groove of DNA or the sugar-phosphate backbone (Gehring et al., 1994). Although Lys and Arg are represented in the first 10 amino acids in most homeodomains, in the KNOX homeodomains, 6 of the first 10 amino acids are basic residues. This NH_2-terminal region is reported to play an additional role in the KNOX homeodomain proteins. Conversion of K2, K3, and K4 (numbers correspond to those at the top of Fig. 4 *A*) to Ala in Kn1 blocks cell-to-cell trafficking of Kn1 protein and its mRNA through plasmodesmata, the specialized plasma membrane–lined cytoplasmic pores that maintain cytoplasmic and endomembrane continuity between many cells in the plant (Lucas et al., 1995). Since *Chlamydomonas* is a unicellular organism and does not form plasmodesmata, this portion of the molecule may have been conscripted for other functions that do not require this collection of basic amino acids. Alternatively, it is possible that this short region is not present in GSP1. If an 8–amino acid gap is introduced in GSP1 between L848 and R849, then similarity (6 out of 15 identical amino acids) between GSP1 and KNOX homeodomains is detected upstream of the homeodomain in the ELK region (Vollbrecht et al., 1991; Kerstetter, 1994) of the KNOX proteins (not shown). The significance of this 40% identity over 15 amino acids is unknown.

Compensatory exchanges of amino acids in the hydrophobic core are a second distinguishing feature of the

GSP1 homeodomain. Site 20 in the homeodomain (861 in GSP1) is occupied by an Ala in GSP1, whereas all other homeodomains have aromatic or large aliphatic residues at this site. Mapping the GSP1 sequence onto the known structure of the homeodomain revealed that this substitution, which occurs in the hydrophobic core of the homeodomain, is compensated by a complementary substitution at site 896 in GSP1. In GSP1, a bulky Trp at 896 substitutes for the smaller Arg, Gln, or Ala residues that occupy this site in most other homeodomains.

Noteworthy of the GSP1 sequence outside of the homeodomain are the many regions of low complexity, with amino acid compositions strongly biased against the composition typical for globular proteins. Because several amino acids usually dominate in low complexity regions, the statistical methods for establishing sequence homology between globular proteins are not applicable to low complexity regions; any sequence similarity reflects mainly compositional similarity. As noted in Fig. 3, GSP1 shares low complexity regions with several transcription factors. Gln-rich regions, Ala-rich regions, and acidic regions are found in many transcription factors, including other homeodomain proteins (Burglin, 1994). These regions are thought to be non–DNA-contacting regions involved in transcriptional activation (Ptashne, 1988; Mitchell and Tjian, 1989). Despite low complexity, some of these regions might form structures that interact with other regions in GSP1 or with other proteins. For example, Gln-rich regions are important for regulating the activity of the mammalian transcription factor Sp1 (Courey and Tijian, 1988) and the testis-determining mouse SRY protein (Dubin and Ostrer, 1994), and these regions have been shown to cause protein oligomerization (Stott et al., 1995).

The potential PEST sequence at the COOH terminus of GSP1 also is notable. PEST sequences are involved in the rapid degradation of proteins (Rechsteiner and Rogers, 1996), and in some systems they may be involved in transcriptional regulation (Fisher et al., 1998). The presence of this site in GSP1 suggests that tight control of GSP1 levels may be important in the cellular functions of GSP1.

GSP1 Is Gamete- and Mating Type–specific

Further studies on GSP1 revealed that it has a cell type–specific expression pattern. Northern blot hybridization (Fig. 1) and immunoblotting using α-GSP1 antibodies (Fig. 5) indicated that GSP1 is expressed only in gametes and not in vegetative cells. Furthermore, immunoblotting indicated that GSP1 was present only in mt+ gametes. Unlike the *fus1* gene, which is in the mt+ locus and therefore present only in mt+ cells (Ferris et al., 1996), the gene for *gsp1* is present in cells of both mating types. Nevertheless, the GSP1 protein is expressed only in mt+ gametes. This expression pattern, along with the finding that GSP1 is not expressed in diploid gametes and is expressed in *imp11* mt− mutant gametes, indicates that GSP1 expression is under the control of the *mid* gene. The *mid* gene is necessary and sufficient to convert mt+ cells to cells that agglutinate as mt− cells; and the MID protein is proposed to be a transcription factor that, either alone or with other molecules, controls sexual differentiation (Ferris and Goodenough, 1997). Several other gene loci have been reported to show sex-limited expression patterns (*sag1* and *sag2* in mt+ gametes, and *gam1* in mt− gametes) (Forest and Togasaki, 1975; Goodenough, 1995), but none of these genes or gene products have been identified or characterized. To our knowledge, GSP1 is the first gamete-specific molecule to be cloned in *Chlamydomonas* that exhibits a sex-limited expression pattern and may be negatively regulated by *mid*. Thus, *gsp1* should prove to be a valuable tool to study the molecular mechanisms underlying gametogenesis and fertilization in *Chlamydomonas*.

Possible Roles for GSP1

Some clues about possible roles for GSP1 emerge from consideration of its properties. For example, its late appearance during gametogenesis suggests that it may not be involved in the early steps of gamete differentiation. The protein was detected ~12 h after vegetative cells were resuspended in N-free medium, which is about the time that agglutinin activity was expressed. If GSP1 were involved in cell cycle control, for example, it is likely (but not absolutely necessary) that it would have been detected much earlier during differentiation, before the cells would have undergone the one to three mitotic divisions that finally produce gametes. Several roles are possible for GSP1: it might regulate synthesis of gamete-specific molecules during gametogenesis and during fertilization, and it might function in the zygote.

We have shown that during adhesion and signaling the gamete-specific agglutinin molecules are lost from the flagella; their replacement requires new protein synthesis (Snell and Moore, 1980; Snell, 1993), possibly accompanied by an upregulation of agglutinin gene transcription. Based on these considerations, one role for GSP1 could be in the putative signaling-associated control of agglutinin transcripts, possibly through signaling-induced posttranslational modifications of GSP1. Signaling-sensitive transcriptional regulation of adhesion molecules and of other molecules involved in adhesion/fusion would permit cells to adhere to and signal with new partners if their first (or maybe even tenth) previous partners fused with another cell. This mechanism could be used even in a feedback system that would lead to the upregulation of *gsp1* transcript levels that we observed during adhesion (Fig. 1). Such regulatory pathways involving homeodomain proteins are not without precedent. For example, in addition to controlling expression of other molecules, the homeodomain Antp regulates its own expression (Winslow et al., 1989); and in mouse, homeodomain proteins are proposed to regulate the synthesis of the cell–cell adhesion molecule N-CAM (Zhang and Emmons, 1995; Wang et al., 1996).

GSP1 also could function after cell fusion, as is the case for the homeodomain proteins, MATa1 (a typical homeodomain protein) and MATα2, in *S. cerevisiae* (reviewed in Johnson, 1995). MATa1, present only in a cells, and MATα2, present only in α cells, are involved in expression of a cell and α cell genes. After fusion of a and α cells during mating, MATα2 and MATa1 form a heterodimer that represses expression of haploid-specific molecules. The gamete-specific expression of GSP1 and the time of its appearance during differentiation are consistent with a similar role in *Chlamydomonas* zygotes. It will be exciting if,

like the yeast homeodomain proteins (Madhani and Fink, 1998), GSP1 regulates synthesis of many molecules required for mating, cell fusion, and development of the diploid cell. Further insights about the role of this new *Chlamydomonas* homeodomain protein should emerge when *gsp1* mutants become available.

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