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Gametogenesis in the *Chlamydomonas reinhardtii minus* Mating Type Is Controlled by Two Genes, *MID* and *MTD1*

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ABSTRACT

In the unicellular algae *Chlamydomonas reinhardtii*, the *plus* and *minus* mating types are controlled by a complex locus, *MT*, where the dominant *MID* gene in the MT^- locus has been shown to be necessary for expression of *minus*-specific gamete-specific genes in response to nitrogen depletion. We report studies on *MID* expression patterns during gametogenesis and on a second gene unique to the MT^- locus, *MTD1*. Vegetative cells express basal levels of *MID*. An early activation of *MID* transcription after nitrogen removal, and its sequence similarity to plant RWP-RK proteins involved in nitrogen-responsive processes, suggest that Mid conformation/activity may be nitrogen sensitive. A second stage of *MID* upregulation correlates with the acquisition of mating ability in *minus* gametes. Knockdown of *MTD1* by RNAi in *minus* strains results in a failure to differentiate into gametes of either mating type after nitrogen deprivation. We propose that intermediate Mid levels are sufficient to activate *MTD1* transcription and to repress *plus* gamete-specific genes and that *MTD1* expression in turn allows the threshold-level *MID* expression needed to turn on *minus* gamete-specific genes. We further propose that an *MTD1*-equivalent system, utilizing at least one gene product encoded in the MT^+ locus, is operant during *plus* gametogenesis.

CHLAMYDOMONAS reinhardtii is a flagellated unicellular green alga that has two mating types, *plus* and *minus*, determined by the mating type (*MT*) loci (MT^+ and MT^-). The center of this ~1-Mb locus of recombinational suppression carries translocations and inversions and is called the rearranged (R) domain (Ferris and Goodenough 1994). Both housekeeping and sex-limited genes are found in this region (Ferris *et al.* 2002), similar to mating-type loci and sex chromosomes in other organisms (Graves 2006). Six unique regions (*a–f*) are found within the R domain, three (*a–c*) specific to MT^+ and three (*d–f*) specific to MT^-. Four genes have been identified in these regions: *MTA1* (***MT*** locus, region ***a***) in *a*, *FUS1* (***fus****ion*) in *c*, *MTD1* (***MT*** locus, region ***d***) in *d*, and *MID* (***mi****nus* ***d****ominance*) in *f* (Ferris *et al.* 2002). The two MT^--specific genes are the focus of this study.

In response to nitrogen starvation, haploid vegetative Chlamydomonas cells differentiate into gametes. Gametes of opposite mating type are able to agglutinate and fuse to form zygotes (Harris 1989). Occasionally, heterozygous mt^+/mt^- diploids form after mating, resume vegetative growth, and differentiate as gametes with N-starvation. The fact that these diploids always mate as *minus* indicates that *minus* is dominant to *plus* (Harris 1989), a phenomenon found to be controlled by the *MID* gene (Galloway and Goodenough 1985). *MID* encodes a transcription factor in the RWP-RK family that also includes several proteins in higher plants that are suggested to exert their function during nitrogen limitation (Schauser *et al.* 1999, 2005; Borisov *et al.* 2003).

Previous studies revealed that *MID* is necessary and sufficient to convert wild-type *plus* gametes to mate as *minus*: mt^+ cells transformed with the *MID* gene differentiate as *minus* gametes (Ferris and Goodenough 1997), and mt^- cells carrying loss-of-function *MID* mutations (*mid-1* or *mid-2*) differentiate as *plus* gametes (Ferris and Goodenough 1997; Ferris *et al.* 2002). In fact, although the *mid* mutants express *plus* flagellar agglutinins (Ferris and Goodenough 1997; Ferris *et al.* 2002) and *plus* mating structures (Ferris and Goodenough 1997), they are unable to fuse with *minus* gametes due to the lack of *FUS1*, a gene restricted to the MT^+ locus and encoding a glycoprotein required for fusion (Ferris *et al.* 1996; Misamore *et al.* 2003); hence the phenotype of *mid* mutants is designated *pseudo-plus*. The *pseudo-plus* phenotype can be rescued by transforming *mid* mutants with *FUS1* (Ferris *et al.* 1996).

MID has been shown to be involved in the activation/repression of the following genes:

1. *SAD1* (***s****exual* ***ad****hesion*), located within the *MT* locus but just outside the R domain, encodes the *minus* agglutinin. Expression of *SAD1* is inhibited in *mid* mutants (Ferris *et al.* 2005) and restored by transformation with *MID* (data not shown).
2. *SAG1* (***s****exual* ***ag****glutination*), unlinked to *MT*, encodes the *plus* agglutinin. It is expressed in *mid* mutants and

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wild-type *plus* gametes but not in wild-type *minus* gametes (Ferris *et al.* 2005).

3. *GSP1* [***g****amete-****s****pecific* ***p****lus* (*mating type*) *molecule* ***1***], unlinked to *MT*, encodes a *plus* gamete-specific homeodomain protein that functions in the zygote. Expression of *GSP1* occurs in *mid-1* and wild-type *plus* gametes but not in wild-type *minus* gametes nor in mt^+/mt^- diploids (Kurvari *et al.* 1998; Wilson *et al.* 1999).
4. *GSM1* [***g****amete-****s****pecific* **m***inus* (*mating type*) *molecule* ***1***], unlinked to *MT*, encodes a homeodomain partner of Gsp1 in *minus* gametes and shows *MID*-dependent activation in wild-type *minus* cells (J.-H. Lee, H. Lin and U. W. Goodenough, unpublished results).

Previous studies of the *MTD1* gene showed that it encodes a protein with five predicted NXT/S glycosylation sites, three predicted transmembrane regions, and no homologs in the current database (Ferris *et al.* 2002). This protein is not essential to Chlamydomonas: *MID*-transformed mt^+ gametes are able to form viable zygotes with wild-type *plus* gametes where *MTD1* is not present in either cell line (Ferris and Goodenough 1997). Both *MID* and *MTD1* are MT^- localized and only ~20 kb apart (Ferris *et al.* 2002), and both are gamete specific by Northern blotting (Ferris and Goodenough 1997; Ferris *et al.* 2002), suggesting that *MTD1* might be involved in *minus* gametogenesis.

We report here studies on the expression of *MID* and *MTD1* upon nitrogen removal using synchronous cell culture. The results reveal an early (~30 min) upregulation of *MID* expression in response to nitrogen starvation. A second stage of *MID* expression is induced when cells display the gametic phenotype. We propose that this second activation is dependent on Mtd1 function. We also show that knockdown of *MTD1* by RNA interference (RNAi) compromises or prevents *minus* gametogenesis, indicating an essential role for *MTD1* in this process.

MATERIALS AND METHODS

Cells and cell culture: *C. reinhardtii* strains (available from the *Chlamydomonas* Genetics Center, Duke University, Chapel Hill, NC) were maintained on Tris–acetate–phosphate (TAP) plates (Harris 1989). Vegetative cells were cultured in flasks of TAP medium with gentle shaking for 3 days. Gametes were prepared by resuspending at-least-5-day-old cells from TAP plates in nitrogen-free high salt minimal (NFHSM) medium (Martin and Goodenough 1975) for 2–3 hr. Synchronous cells were cultured with aeration in liquid high-salt minimal medium on a 12-hr light/12-hr dark cycle for 3 days (Harris 1989). A portion of cells was saved as the vegetative cell sample while the rest were harvested and resuspended in NFHSM immediately. At the time points indicated, cells were collected by centrifugation and prepared for RNA isolation or SDS–PAGE.

Transformation of Chlamydomonas: Nine copies of FLAG (Castrucci *et al.* 1992) were inserted into the *MID* gene just before the stop codon. FLAG-tagged and nontagged *MID* were transformed into *mid-2* cells by glass-bead transformation (Kindle 1990), using pSI103 (Sizova *et al.* 2001) as a selection marker. Transformants were selected on paromomycin plates and screened by PCR for the *MID* gene. Transformants were further screened for their ability to mate with wild-type *plus* gametes. The *MTD1* RNAi construct was transferred into wild-type *minus* cells using pSI103 as a cotransformant by electroporation (Shimogawara *et al.* 1998).

BLAST and phylogenetic analysis: The C-terminal sequence (aa 96–147) of *C. reinhardtii* Mid, which includes the conserved RWP-RK motif, was used in a protein BLAST against translated nucleotides in the Chlamydomonas JGI (Doe Joint Genome Institute) genome database version 3.0 (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Chlre3&advanced=1) with expected values ≤1*e*–3. Among the 14 proteins obtained from BLAST, 1 of them contained RWP but no RK in the conserved region and was omitted from alignment and phylogenetic analysis. In scaffold 27, 2 RWP-RK proteins were 5 kb away from each other although BLAST recognized only 1 of them (RWP11); the second protein (RWP4) was added to this study. Sequences containing the conserved motif from different proteins were aligned using Clustal X 1.83 (Thompson *et al.* 1997) and the alignment output was prepared using BOXSHADE. The aligned sequences were used to draw a neighbor-joining tree with bootstrap repeats of 1000 using MEGA 3.1 (Kumar *et al.* 2004).

SDS–PAGE and immunoblotting: For antibody preparation, full-size *MID* or *MTD1* cDNA was cloned into pET21a vectors (Novagen) and transformed into bacteria. Recombinant His-tagged proteins were induced by IPTG and purified using a His-affinity purification kit (Novagen) according to the manufacturer's protocol. The purified proteins were used to generate anti-Mid and anti-Mtd1 antibodies in rabbits (Cocalico Biological). Both antibodies were subjected to affinity purification using recombinant a protein-conjugated Sephorase 4B (Amersham Biosciences) column.

For detection of Mid, freshly harvested cells were resuspended in 1× SDS gel-loading buffer (50 mm Tris–HCl, pH 6.8, 100 mm DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 min (Sambrook and Russell 2001). Typically, proteins from 1×10^7 cells were separated by 15% acrylamide SDS–PAGE (Laemmli 1970) at room temperature (RT), 85 V for stacking gel and 120 V for resolving gel. After electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) at 12 V for 1 hr using the semidry method at 4°. Membranes were blocked in 5% milk in TBST (137 mm NaCl, 20 mm Tris–HCl, pH 7.6, 0.05% Tween-20) for 1 hr at RT. Blocked membranes were washed for 5 min with TBST and inoculated with anti-Mid antibody (1:5000 dilution) or anti-FLAG antibody M2 (1:10,000 dilution, Sigma, St. Louis) in TBST containing 3% milk for 1 hr at RT or overnight at 4°. Membranes were rinsed once and washed with TBST three times, 5 min each. Peroxidase-conjugated goat-anti-rabbit or goat-anti-mouse antibodies (1:10,000 dilution each, Bio-Rad, Hercules, CA) were used as secondary antibodies for 1 hr at RT. Membranes were washed as above (Harlow and Lane 1988). Signals were detected using homemade enhanced chemiluminescence reagent (Yang and Widmann 2001).

For detection of Mtd1, the anti-Mtd1 antibody was further purified by preabsorption with acetone-precipitated proteins from wild-type *plus* gametes (Harlow and Lane 1988). Freshly harvested cells were resuspended in buffer (10 mm Tris, pH 7.0, 1 mm NaCl) containing proteinase inhibitors (Sigma) and flash frozen in liquid nitrogen for >1 hr (Wilson *et al.* 1999). An equal amount of boiling 2× SDS gel-loading buffer was added to the frozen samples and the samples were boiled immediately for 5 min. Proteins were separated by 10% acrylamide SDS–PAGE and transferred to Immobilon-P membranes as above. Membrane was blocked and inoculated with anti-Mtd1 antibody (1:1000 dilution) and peroxidase-conjugated

goat-anti-rabbit secondary antibody (1:10,000 dilution, Bio-Rad) sequentially.

RNA preparation and Northern blotting: For RNA isolation, 10^8–10^9 cells were collected and resuspended in RNA lysis buffer (20 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0, 5% SDS, and 50 μg/ml proteinase K). The cell mixture was incubated at RT without stirring for 4–24 hr. Sodium acetate (3 M, pH 5.2) was added to the cell mixture to a final concentration of 0.3 M and vortexed. RNA was extracted by an equal volume of phenol/chloroform (1:1) and precipitated by an equal volume of isopropanol. Precipitated RNA was washed and dissolved in DEPC water. RNA was further purified by precipitation using an equal volume of lithium chloride overnight at 4°, followed sequential precipitation using 2.5 vol of ethanol. RNA from ethanol precipitation was vacuum dried and resuspended in DEPC water. RNA concentration was determined by spectrophotometry at 260 nm. For Northern blotting, RNA was loaded to 1% agarose formaldehyde gels and the gels were run at 35 V overnight at RT. After electrophoresis, RNA was transferred to nylon membranes (Hybond-XL, Amersham, Piscataway, NJ) by dry blotting overnight and crosslinked at 1200 μJ × 100 (UV Stratalinker 1800, Stratagene, La Jolla, CA). For hybridization, cDNA probes were randomly radiolabeled. Hybridization and washes were done following Church and Gilbert (1984).

cDNA synthesis and RT–PCR: Poly(A) RNA was isolated from 5 μg of total RNA using Dynabeads oligo$(dT)_{25}$ (Invitrogen, San Diego), according to the manufacturer's protocol. Beads with bound mRNA were inoculated with RQ1 RNase-free DNase (Promega, Madison, WI) in a 10-μl reaction at 37° for 30 min. SuperScript II reverse transcriptase (Invitrogen) was used for cDNA synthesis using random primers with reaction conditions of 25° for 10 min, 42° for 1 hr, 50° for 30 min, and 65° for 15 min. After these reactions, RNA was digested by the addition of RNase H (Invitrogen) at 37° for 30 min. One microliter from the reaction was used in a 20-μl PCR using Taq polyermase (Promega). PCR cycle numbers were determined experimentally to ensure that the products were within a linear range. In the study of *MID* expression, the intensity of individual RT–PCR *MID* and *CRY1* signals was measured by Quantity One software (Bio-Rad). The relative amount of the *MID* was standardized by the intensity of corresponding *CRY1* and further standardized by the relative amount of *MID* in vegetative cells.

Primers used in this study are the following: *MID* (5′-ATG GCCTGTTTCTTAGCC-3′; 5′-CTACATGTGTTTCTTGACG-3′); *MTD1* (5′-GCTACCGGAGGCTCCTAC-3′; 5′-GACACGTTG CAAGACAGA-3′); *CRY1* (5′-TTCGGCGTTGCTCACATCTT-3′; 5′-TCGATGCGGCCAATCTTCAT-3′); *GSM1* (5′-CAGTGGA CACGGCGACTG-3′; 5′-CCGAAGAAACTCAGAGTACG-3′); *SAD1* (5′-TTCAGAGCGCTGGATCTG-3′; 5′-GCCATGCTGG TGTACCTG-3′); *NSG6* (5′-TGAGCGGCAGTTTGCTGA-3′; 5′-ACCATGGCGCCCATCAAT-3′); and *NSG17* (5′-TGCAGGC CATGCAAATGA-3′; 5′-ACAACCGCGTGCGAAACT-3′).

Ribonuclease protection assay: RNA probes for ribonuclease protein assay (RPA) were synthesized by *in vitro* transcription using linearized plasmids containing full-length *MID* or partial *MAT3* cDNA sequences as templates. T7 or T3 RNA polymerase (Ambion) and radiolabeled UTP were used. The transcribed probes were gel purified using 5% acrylamide/8 M urea gel. RPA was performed using an RPAIII kit (Ambion) according to the manufacturer's instructions.

RESULTS

The RWP-RK protein family in Chlamydomonas: Two years after Mid was identified (Ferris and Goodenough 1997), a protein named NIN (*n*odule *in*ception) was identified in lotus (*Lotus japonicus*) (Schauser *et al.* 1999). Sequence comparison between NIN and Mid revealed a conserved RWP-RK motif, which contains an invariant RWP×RK sequence (Schauser *et al.* 1999). More NIN-like proteins were lately identified in pea (Borisov *et al.* 2003), rice, and Arabidopsis (Schauser *et al.* 2005). In total, 14 proteins in Arabidopsis and 16 in rice contain this conserved motif.

TABLE 1

RWP-RK proteins in Chlamydomonas

RWP-RK proteins	Localization in JGI Genome Project
NIT2	Chlre3/scaffold_9:322348-327145
RWP1	Chlre3/scaffold_34:980727-982909
RWP2	Chlre3/scaffold_3:54841-64925
RWP3	Chlre3/scaffold_43:243606-250716
RWP4	Chlre3/scaffold_27:360353-364613
RWP5	Chlre3/scaffold_26:825065-832955
RWP6	Chlre3/scaffold_26:1532616-1536494
RWP7	Chlre3/scaffold_14:53086-56143
RWP8	Chlre3/scaffold_15:44952-49225
RWP9	Chlre3/scaffold_12:1929625-1934390
RWP10	Chlre3/scaffold_17:985300-986840
RWP11	Chlre3/scaffold_27:345625-354103
RWP12	Chlre3/scaffold_72:313007-321422
RWP13	Chlre3/scaffold_22:137799-143173

Fourteen RWP-RK proteins in addition to Mid were identified in the current Chlamydomonas genome using the conserved domain (aa 96–147) of Mid (materials and methods). One protein is the gene product of the nitrate assimilation regulatory gene *NIT2* (Schnell and Lefebvre 1993; Galvan and Fernandez 2001); the rest are unknown proteins, named RWP1–13 (Table 1). Sequence comparisons (Figure 1A and 1B) and phylogenetic analysis (Figure 1C) reveal that Mid is phylogenetically closer to Nit2 in Chlamydomonas and to NIN and NIN-like proteins in lotus, pea, rice, and Arabidopsis (group 1) than to other RWP proteins in Chlamydomonas (group 2). Some amino acids other than RWP×PK are conserved in all proteins (Figure 1A, inverted triangles), including the lysine that is mutated in the *mid-1* mutant (Figure 1A, circle) (Ferris and Goodenough 1997). Other sites are conserved among group 1 but not group 2 proteins (Figure 1A, squares). Given that group 1 proteins are all involved in processes induced by nitrogen limitation, these sites may play a role in the nitrogen response.

Patterns of *MID* expression: During Chlamydomonas mating, cell adhesion triggers elevation of intracellular cyclic AMP (cAMP), which in turn activates a series of mating responses; addition of exogenous, membrane-permeant dibutyryl cyclic AMP (db-cAMP) is able to mimic these responses (Pasquale and Goodenough 1987; Goodenough 1989). To ask whether *MID* expression is affected by cAMP, db-cAMP was added to wild-type *plus* and *minus* gametes. Western blotting using anti-Mid

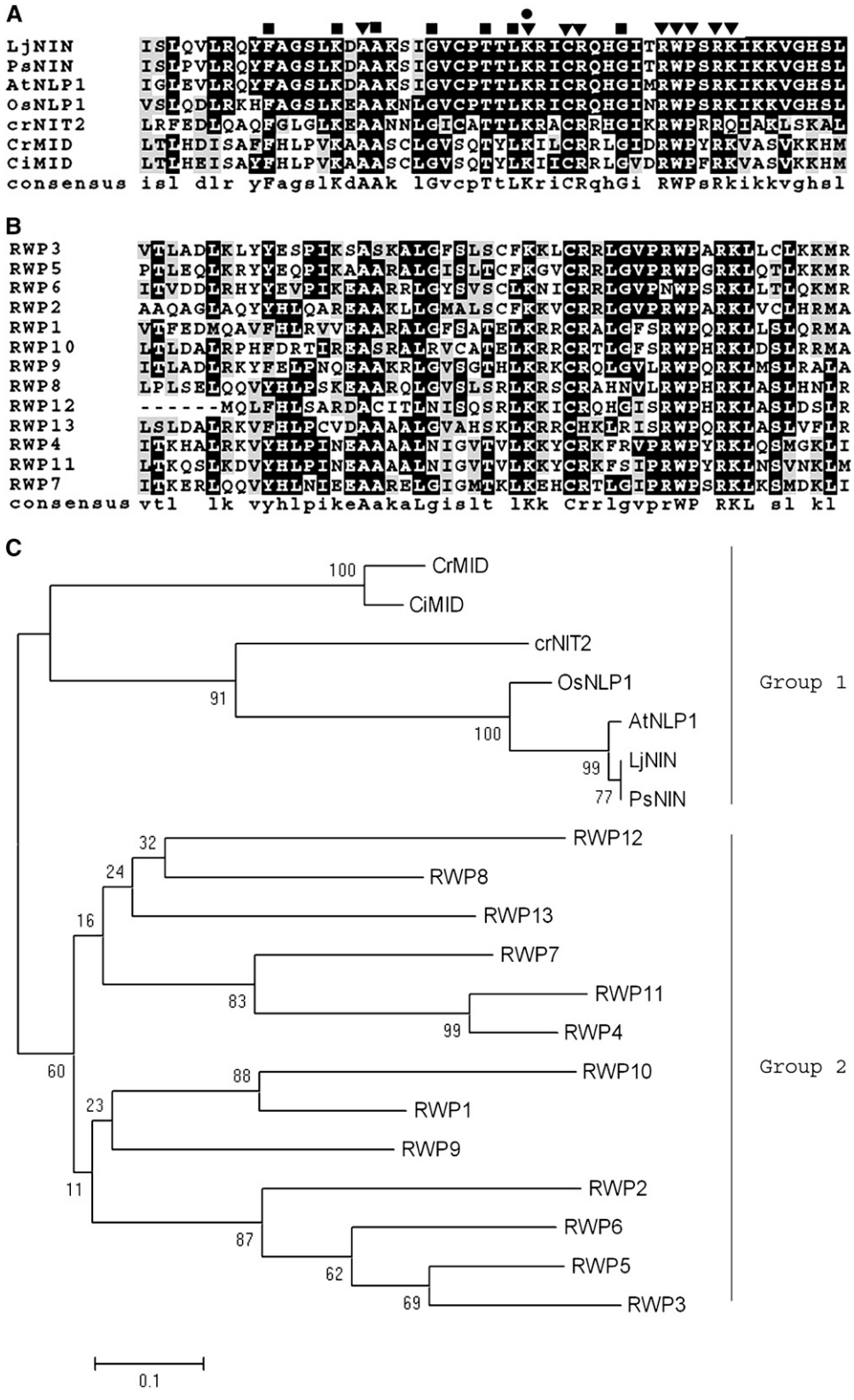

FIGURE 1.—RWP-RK proteins. (A) Alignment of RWP-RK domains from *C. reinhardtii* Mid (CrMid, AAC49753), *C. incerta* Mid (CiMid, AAB60944), *C. reinhardtii* Nit2 (CrNit2, ABC42493), and several Nin-like plant proteins: Lj, *L. japonicus* (CAB61243); Ps, *Pisum sativum* (CAD37949); At, *Arabidopsis thaliana* (F84548); Os, *Oryza sativa* (AAM22710.1). ▼, conserved amino acids within all listed proteins (except in CrNit2, in which lysine in RWP-RK is replaced by glutamine); ●, mutation of this amino acid from lysine to isoleucine in *mid-1* mutant leads to *pseudo-plus* gametes; ■, conserved amino acids within all proteins listed in A but not in B. (B) Alignment of Chlamydomonas RWP proteins. (C) Neighbor-joining tree showing the relationship of all listed RWP-RK proteins. Numbers at nodes represent bootstrap percentages of 1000 repeated runs. Proteins in group 1 all respond to nitrogen limitation in different organisms; the function of proteins in group 2 is currently unknown.

antibody showed that addition of db-cAMP in *minus* cells made no difference in Mid protein level (Figure 2A). Western blotting also confirmed that Mid protein is present in *minus* but not *plus* gametes, with the observed molecular weight (~17 kDa) close to that calculated (16,390 Da) (FERRIS and GOODENOUGH 1997).

When *MID* was first identified, Northern blotting showed a very weak and hence ambiguous *MID* signal in *minus* vegetative cells and a strong signal in mature gametes (FERRIS and GOODENOUGH 1997). To ask whether or not *MID* is indeed expressed in vegetative cells, and to understand when and under what conditions expression

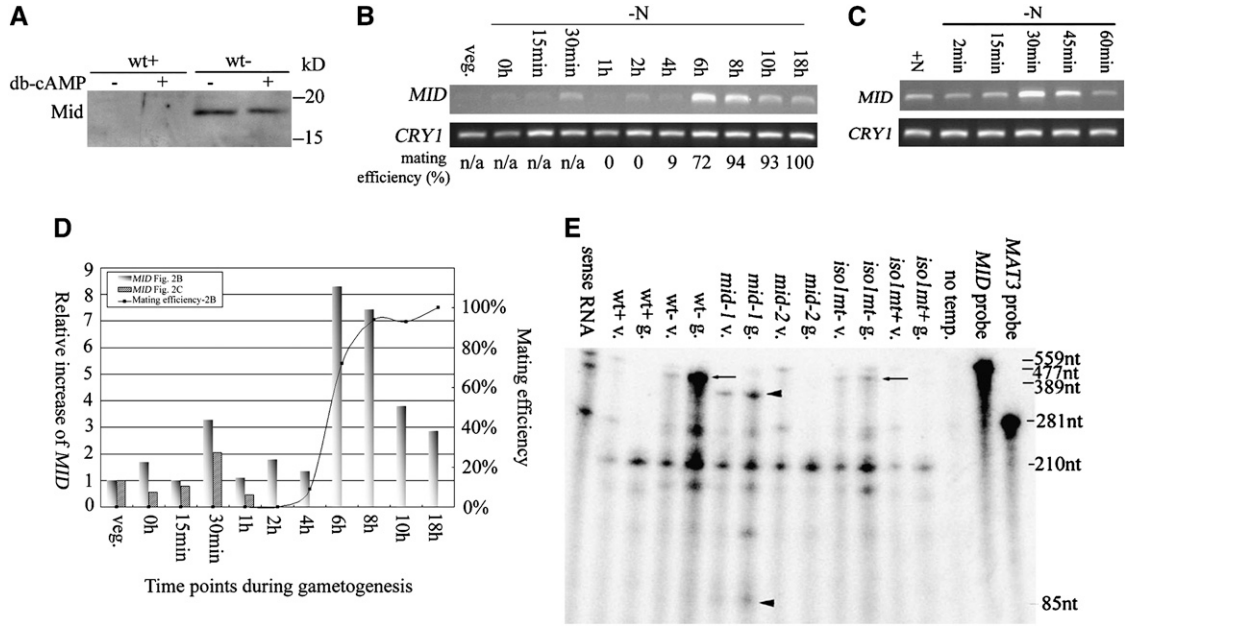

FIGURE 2.—Patterns of *MID* expression. (A) Western blot of Mid in wild-type *plus* (*wt*+) and *minus* (wt−) gametes with or without the addition of db-cAMP. (B) RT–PCR of *MID* during gametogenesis. Synchronous wild-type *minus* cells were transferred to nitrogen-free (−N) media and samples were collected at various time points as indicated. RT–PCR products of poly(A) selected RNA were detected by ethidium bromide staining. *CRY1*, encoding ribosomal protein S14, is used as an internal control. Mating efficiencies of cells when samples were collected are standardized using mating efficiencies of wild-type tester cells. (C) RT–PCR of *MID* during early gametogenesis. Wild-type *minus* cells from 3-day-old TAP plates were transferred to nitrogen-free media and collected at various time points as indicated. Mating efficiencies of cells were not determined since vegetative cells do not differentiate into gametes within 1 hr. (D) Relative increases of *MID* during gametogenesis. The expression levels of *MID* in B and C were obtained by quantitation of the *MID* RT–PCR signals with the internal loading control, *CRY1*, and standardized by the relative amount of *MID* in vegetative cells. The relative increases of *MID* were plotted against time points when samples were removed during gametogenesis. The mating efficiency of individual samples from B is also plotted. (E) Expression of *MID* in vegetative cells. Total RNA isolated from wild-type and various mutant cells was hybridized with both a *MID* antisense RNA probe and a *MAT3* antisense RNA probe and subjected to the ribonuclease protection assay. Arrows and arrowheads indicate the protected fragment of *MID*. *mid-1*, *MID* mutant with point mutations; *mid-2*, *MID* deletion mutant; *iso1 mt*$^-$, an isoagglutination mutant; and *iso1 mt*$^+$, mutant that carries the same mutation as in *iso1 mt*$^-$ but has a normal *plus* phenotype. The *MID* probe, 559 nucleotides (nt); the protected *MID* messages in wild-type *minus* and *iso1 mt*$^-$ cells, 477 nt (arrows); and the protected *MID* fragments in *mid-1*, 389 and 85 nt, respectively (arrowheads). *MAT3*, encoding a retinoblastoma homolog (UMEN and GOODENOUGH 2001), was used as an internal loading control. The *MAT3* probe, 281 nt; and the protected *MAT3* message, 210 nt. The third-to-last lane, no template, serves as a negative control with no RNA template.

is upregulated, vegetative cells were transferred into nitrogen-free medium and the level of *MID* was studied at different time points during gametogenesis using RT–PCR. Cells were prepared for these studies in two different ways.

In the first, wild-type *minus* cells were synchronized by light/dark cycles. Cells remain in a prolonged G_1 during the light phase, undergo alternative rounds of S and mitosis in the dark, and re-enter G_1 when reilluminated (UMEN and GOODENOUGH 2001). Cells were collected at this G_1 and immediately transferred into nitrogen-free medium (set as time 0) (ABE *et al.* 2004). As shown in Figure 2B, some cells started to become gametic at 4 hr, capable of agglutinating and fusing with wild-type *plus* testers with a relative mating efficiency of 9%, and the mating ability of the culture increased dramatically to 72% within the next 2 hr. Sensitive RT–PCR was able to detect *MID* transcript in vegetative cells at what will be referred to as basal levels. Its expression increased approximately threefold (level 1) at 30 min (Figure 2D, shadowed bars), returned to basal levels at 1 hr, and was then strongly upregulated to approximately eightfold (level 2) at 6 hr (Figure 2D) in concert with the augmentation of mating ability (Figure 2D, curve).

A second approach was taken to study in detail the upregulation of *MID* within the first 30 min. It has been shown that cells growing on TAP plates are uniformly vegetative after 3 days in culture (MARTIN and GOODENOUGH 1975). Such 3-day vegetative cells were washed into N-free medium, collected at different time points, and subjected to RT–PCR. Consistent with the results from Figure 2B, basal levels of *MID* message were detected in the vegetative cells; levels increased at 30 min to approximately twofold those of the basal levels, and abated at 60 min (Figure 2, C and D, diagonal bars).

An RPA was employed to detect the *MID* transcripts in various strains in both vegetative and gametic cells. As shown in Figure 2E, and consistent with results

summarized in Figure 2D, a low level of *MID* mRNA was found in wild-type *minus* vegetative cells, with strong expression (approximately fourfold) in mature wild-type *minus* gametes.

Basal levels of *MID* transcripts were also observed in *mid-1* vegetative cells, with a slightly upregulated message (approximately twofold) in mature *mid-1* gametic cells. Ferris and Goodenough (1997) found that the *mid-1* mutant carries two very close nucleotide changes within the coding region of *MID*: one leads to a synonymous change while the other leads to the change of a single amino acid, conserved within all the RWP-RK proteins reported here, from lysine to isoleucine. These nucleotide changes caused mismatches between the *in vitro*-transcribed antisense *MID* probe and the endogenous *MID* message in *mid-1*; therefore two shorter *MID* fragments were observed. Observation of the *MID* messages at the basal levels in vegetative cells suggests that the mutations within the coding region do not affect its initial transcription. However, upregulation of the *MID* message from its basal levels in *mid-1* gametes (approximately twofold) is not as robust as that in wild-type *minus* gametes (approximately fourfold), suggesting that the mutations may affect some feature of its stability.

Also shown in Figure 2E are results with the *iso1 mt−* strain. This mutant, described in Campbell *et al.* (1995), displays an isoagglutinating gametic phenotype because cells in a clonal population differentiate as either *minus* or *pseudo-plus*. Since *MID* is not expressed in the *pseudo-plus* cells, the mixed cell population fails to display evidence of *MID* upregulation from basal levels.

Expression levels of Mid correlate with mating efficiency: Experiments were next performed to evaluate the relationship between Mid protein levels and gametogenesis. Although anti-Mid antibody is able to detect Mid (Figure 2A), it also recognizes many other bands in immunoblots even after affinity purification and/or preabsorption with *plus* gametic proteins. Therefore, nine copies of an epitope tag, FLAG, were introduced to the C terminus of Mid right before the stop codon. Under the regulation of its own promoter, either *MID* or *MID-F* was cotransformed into the *MID*-deletion mutant *mid-2* with pSI103, which provides paromomycin resistance. In previous studies with the missense *mid-1* mutant (Ferris and Goodenough 1997), *MID* transformation was found to yield only partial rescue: some cells differentiated as *minus* while the rest continued to differentiate as *pseudo-plus*, the result being clonal isoagglutination. The same outcome was obtained with *mid-2* transformants: 1 of 88 paromomycin-resistant colonies from the *MID* transformation displayed an isoagglutination phenotype, and 1 of 183 paromomycin-resistant colonies from the *MID-F* transformation displayed isoagglutination. Such partial rescue presumably reflects the sensitivity of *minus* differentiation to *MID* expression levels (Figure 2, B–D) and the failure to achieve full expression in exogenous integration sites.

An ~25-kDa protein (size change due to nine copies of FLAG) was recognized by anti-FLAG antibody in *MID-F* transformants at both vegetative and gametic stages (Figure 3A, *mid-2::MID-F* v. and g.) with the level of Mid-F strongly upregulated in fully differentiated gametes. This protein was absent in wild-type *minus*, *mid-2*, or *mid-2* gametes transformed with non-epitope-tagged *MID* (Figure 3A).

This *mid-2::MID-F* transformant was used to analyze the relationship between the level of Mid expression and mating ability. The strain proved to undergo gametogenesis slowly in synchronous culture and to mate with low efficiency even after long periods (>20 hr) in nitrogen-free medium due to its mixed *minus/pseudo-plus* phenotype. In addition, individual subclones isolated from the same strain displayed different mating abilities at a given time point after nitrogen removal. Six individual subclones were grown synchronously and cell samples were taken at different time points after nitrogen deprivation. As detected by immunoblots, Mid-F protein increased gradually even though mating capacity was not observed until >20 hr later. Figure 3B showed the expression pattern of Mid-F in one of the subclones. At 28 hr after nitrogen removal, when cells were considered to have reached maximum mating efficiency, the relative amounts of Mid-F in each subclone were quantitated against an internal transketolase control and further standardized by the relative amounts of Mid-F in vegetative samples, yielding an estimate of the relative increase of Mid in each subclone. These values were plotted against the mating efficiencies of individual subclones at 28 hr after nitrogen deprivation (Figure 3C). Although the values reflect population levels and give no indication of the Mid levels attained in individual cells, the experiment demonstrates that *MID* protein levels indeed correlate with the ability to differentiate as *minus* gametes.

Expression of *MTD1* during gametogenesis: We next turned to the *MTD1* gene. Previous studies using Northern blotting showed that expression of *MTD1* was restricted to *minus* gametes (Ferris *et al.* 2002). To follow expression during synchronous gametogenesis, we used the same RNA samples as in Figure 2B but employed Northern blotting. *MTD1* expression was activated 4 hr after cells were transferred to nitrogen-free medium, coincident with the onset of mating ability (Figure 4A). It was also coincident with the onset of the expression of two other *minus* gamete-specific genes, *SAD1* and *GSM1* (Figure 4A), but whereas *MTD1* expression reaches a sustained plateau level at 4 hr, *SAD1* and *GSM1* expression do not reach their maximum until 2 hr later, and expression abates with time.

Recent studies on synchronous gametogenesis in *plus C. reinhardtii* strains (Abe *et al.* 2004, 2005) identified 18 novel nitrogen-starved gametogenesis (*NSG*) genes that were assigned to three temporal classes: early, middle, and late. Among them, *NSG17* was fully induced within

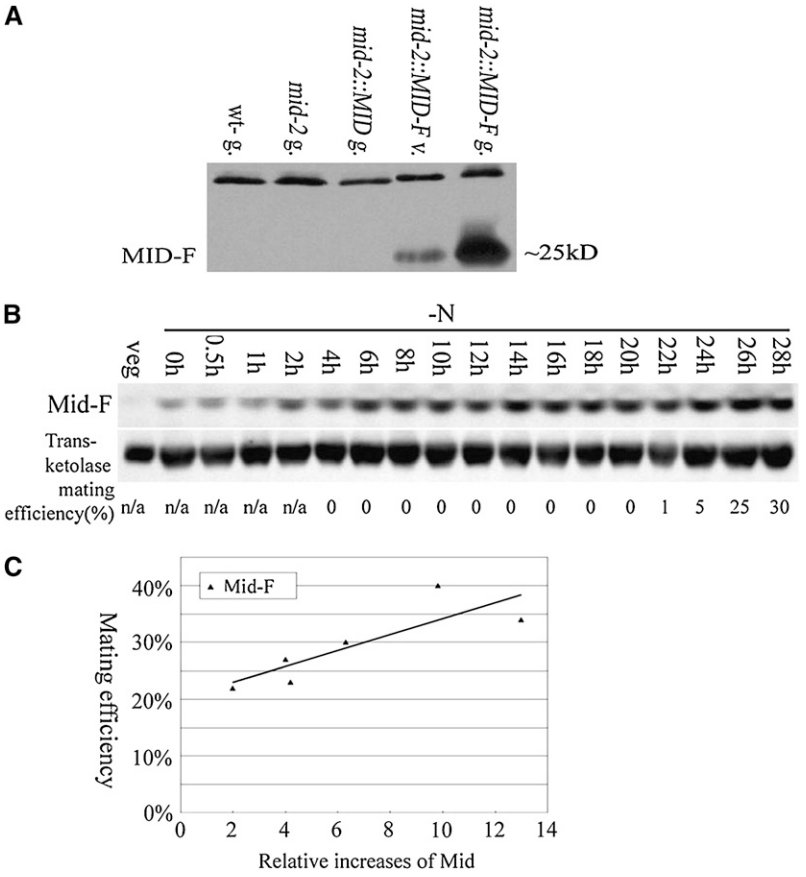

FIGURE 3.—Mid protein levels related to *minus* mating efficiency. (A) An anti-FLAG antibody was used to detected FLAG-tagged Mid in *mid-2::MID-F* cells. Gametes from wild-type *minus*, *mid-2*, and *mid-2::MID* (a *mid-2* transformant carrying MID without FLAG tag) were used as negative controls. The top ~35-kDa band in all lanes was due to cross-hybridization between the anti-FLAG antibody and an unknown Chlamydomonas protein and served as a loading control. (B) *mid-2::MID-F* expression in synchronous culture. The anti-FLAG antibody was used to detect Mid-FLAG; an anti-transketolase antibody was used to detect transketolase as an internal control. Mating efficiencies of cells when samples were collected were standardized using mating efficiencies of wild-type tester cells. (C) Relationship between the amount of Mid-F and the mating efficiency of mature *mid-2::MID-F* cells. Six individual *mid-2::MID-F* subclones were grown synchronously and the mating abilities of individual subclones were tested at 28 hr after nitrogen removal. The increases of Mid-F levels at 28 hr were obtained by quantitation of the Mid-F protein signal with the transketolase signals and standardized by the relative amount of Mid in corresponding vegetative cells. The relative increases of Mid-F were plotted against the mating efficiency of individual *mid-2::MID-F* subclones.

1 hr after cells were transferred to nitrogen-free medium and therefore classified as an early gene, while *NSG6* was induced between 3 and 4 hr and placed in the middle class (ABE *et al.* 2004). To determine in which class *MTD1* belongs, expression patterns of *MTD1*, *NSG17*, and *NSG6* were evaluated using RT–PCR (Figure 4B). Consistent with our Northern blotting results (Figure 4A), activation of *MTD1* expression starts at 4 hr, placing it in the middle class. The *NSG6* pattern agreed with the published data for *plus* cells (ABE *et al.* 2004): very weak expression up to 1 hr (barely detectable by Northern blotting); a relatively weak upregulation (~10-fold) at 2 hr; and an ~30-fold upregulation compared to vegetative cells at 4 hr and after. By contrast, *NSG17* expression differed from the data published for *plus* cells, showing a pattern of relative weak upregulation (~10-fold) between 15 min and 2 hr and strong upregulation (~40-fold) at 4 hr and after. It is not yet known whether this reflects a *plus/minus* difference or results from different experimental conditions in the two laboratories.

RNAi demonstrates an essential role for *MTD1* in *minus* gametogenesis: *MTD1* function was evaluated using RNAi (SINESHCHEKOV *et al.* 2002; KOBLENZ *et al.* 2003). A hairpin RNAi plasmid containing inverted pairs of the third exon of *MTD1*, with the third intron serving as the middle loop, was driven by the constitutive HSP70A/rbcS2 promoter (Figure 5A) (SCHRODA *et al.* 2000; KOBLENZ *et al.* 2003). This RNAi construct was cotransformed into wild-type *mt*$^-$ cells with pSI103. From two individual transformation experiments, 195 and 168 paromomycin-resistant colonies were isolated. Crude DNA was extracted from each colony and subjected to a PCR screen targeting the HSP70A/rbcS2 promoter. Four individual clones were identified: #1 and #4 from the first transformation and #210 and #216 from the second transformation. Among them, #4 and #216 showed no agglutination or fusion with either wild-type *plus* or wild-type *minus* gametes. Addition of db-cAMP did not change the nonmating phenotype of these transformants, indicating that, like *MID*, *MTD1* expression is cAMP independent. Prolonged incubation with *plus* gametes overnight did not lead to any zygote formation. Gametes of a third strain (#210) showed weak agglutination and fusion with *plus* gametes and ~1/3 of number of wild-type zygotes with overnight incubation, while a fourth (#1) mated normally, and produced normal zygote levels. As shown in Figure 5B, levels of *MTD1* message in the various strains were strongly correlated with mating ability: strong *MTD1* transcripts were found in both wild-type *minus* and *MTD1* RNAi #1 gametes; reduced level of *MTD1* in #210; and no *MTD1* message found in #4 and #216, which had no mating ability.

Strain #4 was selected for further study. RT–PCR was performed to detect any weak level of *MTD1* missed by

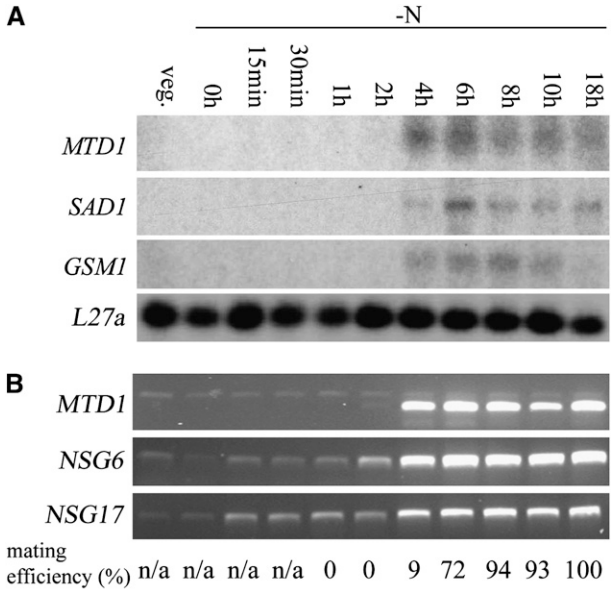

Figure 4.—Transcription of *MTD1* during gametogenesis. (A) Total RNA samples were prepared as in Figure 2B. Ten micrograms of total RNA were loaded in each lane. A membrane was probed with *MTD1*, stripped, and reprobed with *SAD1*, *GSM1*, and *L27a*, sequentially. *SAD1* encodes *minus* agglutinin; *GSM1* encodes a homeodomain protein expressed in *minus* gametes. *L27a*, encoding a 60S ribosomal protein, serves as a loading control. (B) RT–PCR results of *MTD1* and two nitrogen-starved gametogenesis (*NSG*) genes, *NSG6* and *NSG17*. mRNA samples were prepared as in Figure 2B. The top band in the *MTD1* lanes derives from contaminating genomic DNA. Mating efficiencies of cells when samples were collected are standardized using mating efficiencies of wild-type tester cells.

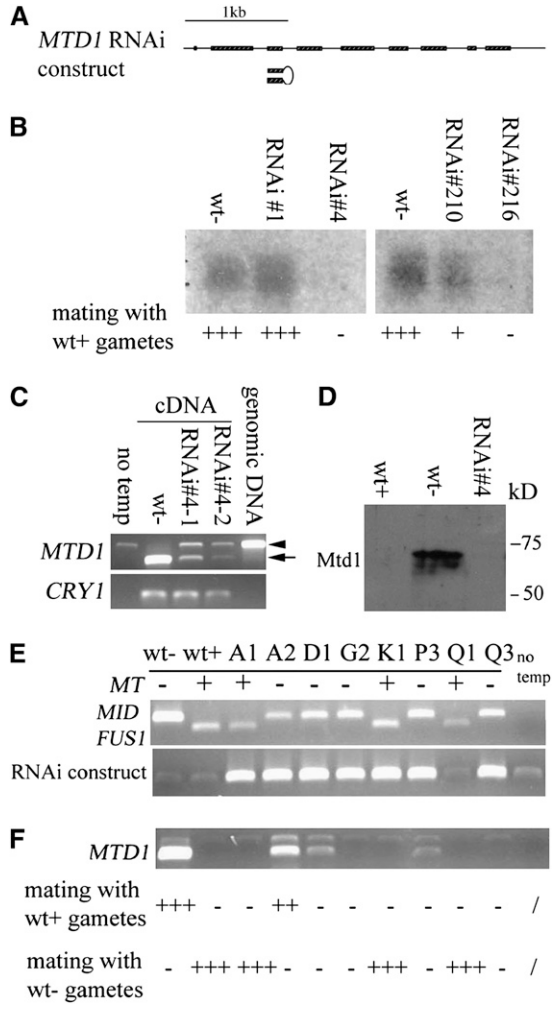

Figure 5.—RNAi of *MTD1* in *minus* cells. (A) Structure of the *MTD1* gene and RNAi construct. Boxes, exons; lines, 5′-UTR, introns, and 3′-UTR. RNAi hairpin structure is presented by inverted pairs of exon 3 with intron 3 serving as the loop. The construct was driven by a constitutive HSP70A/rbcS2 promoter. (B) Northern blotting of *MTD1* levels in different RNAi lines. The mating abilities of individual lines with wild-type *plus* gametes are indicated: +++, strong mating efficiency (80–100%); +, weak mating efficiency (20–50%); −, no mating. (C) RT–PCR of *MTD1* in strain 4. Poly(A) RNA was isolated from two different cultures (#4-1 and #4-2). The arrow indicates the amplified cDNA fragment. The arrow head represents a weak contaminating genomic DNA fragment amplified in PCR. *CRY1* is used as an internal control. The "no temp" control served as a negative control for PCR with no addition of DNA template. "Genomic DNA" served as a positive control for PCR by using genomic DNA as template. (D) Western blotting of Mtd1 in wild type and in the Mtd knockdown strain 4. (E) PCR of genomic DNA from progeny obtained from a cross between wt+ and strain 4. (Top) PCR of *MID* and *FUS1* to determine mating types of individual progeny. (Bottom) PCR to detect the existence of RNAi construct. "No temp," no DNA template control. (F) RT–PCR to detect the expression level of *MTD1* in these progeny. The mating efficiency of individual progeny with either wild-type *plus* or wild-type *minus* gametes is indicated: +++, strong mating efficiency (80–100%); ++, moderate mating efficiency (50–80%); +, weak mating efficiency (20–50%); −, no mating.

Northern blotting. RNA was isolated from two different cultures of strain #4 at different times. Both samples show strongly reduced levels of *MTD1* (Figure 5C, arrow)—~1/10 of that in wild-type *minus* gametes—while the control message, *CRY1*, displayed a similar expression level in all three samples (Figure 5C). [An additional band is observed in the RNAi samples (Figure 5C, arrowhead) with the same size as genomic DNA amplified by the same set of primers. Since this band is also present in the negative PCR control (no template), it suggests that some genomic DNA contaminated the PCR reaction. This contaminating genomic DNA is not observed in the wild-type *minus* sample, presumably either because it is masked by the intensive signal from the amplified cDNA copy of *MTD1* or because the abundant cDNA template overwhelmed the low-abundance genomic DNA contamination for binding and amplification by PCR primers.]

Consistent with the RNA results, immunoblotting using anti-Mtd1 antibody showed an ~73-kDa protein in wild-type *minus* gametes but not in *plus* or strain #4 gametes (Figure 5D). The size of the detected protein was somewhat larger than the calculated molecular weight of 64.7 kDa, presumably due to glycosylation on putative N-glycosylation sites (Ferris *et al.* 2002).

Two months after isolation, the original RNAi lines had gradually recovered, with increasing levels of *MTD1* correlating with increased mating ability. Such RNAi instability has been observed in mice (Bartlett and Davis 2006) and in Chlamydomonas (Koblenz *et al.* 2003). Strain #4 was therefore backcrossed to wild-type *plus*, and progeny were screened by PCR to amplify the HSP70A/rbcs2 promoter in the RNAi construct. The mating types of these progeny were determined by amplification of both *MID* and *FUS1* from crude genomic DNA extract: all the *plus* progeny contained the *plus*-specific *FUS1* gene only, while all the *minus* progeny carried *MID* only. Analyses of several progeny are shown in Figure 5, E and F: five *minus* progeny strains carrying the construct (A2, D1, G2, P3, Q3) displayed various levels of mating ability: one (A2) agglutinated and fused with wild-type *plus* but not with wild-type *minus* gametes, while the rest displayed little or no agglutination and fusion ability with either *plus* or *minus* testers (Figure 5F, bottom). RT–PCR revealed that transcription of *MTD1* was greatly reduced or absent in those four progeny but only slightly reduced in A2 (Figure 5F). Two *plus* progeny carrying the construct (A1, K1) agglutinated and fused normally with wild-type *minus* gametes but not with wild-type *plus* gametes (Figure 5F, bottom), indicating that the presence of the construct *per se* is not toxic to gametic differentiation. The G2, P3, and K1 strains were used in subsequent studies.

***MID* deletion and *MTD1* knockdown affect *minus* gamete-specific gene expression:** The inability of *MTD1* knockdowns to agglutinate as *minus* indicates a block in Sad1 agglutinin synthesis. This mimics the phenotype of *mid* mutants with one important difference: in *mid* mutants, *SAG1* expression occurs instead, and the gametes agglutinate as *plus*, whereas in the *MTD1* knockdowns, no agglutinin of either type is made. It therefore became important to understand the relationship between *MID* and *MTD1*.

RT–PCR was performed to detect *MID*, *MTD1*, *SAD1*, and *GSM1* expression in wild type, in *mid* mutants, and in *MTD1* knockdowns. As shown in Figure 6A, expression of *MTD1* is greatly reduced, but not eliminated, in *mid-2* cells, suggesting that Mid strongly influences *MTD1* expression but that a weak Mid-independent *MTD-1* expression pathway exists as well. Expression of *MID* was also greatly reduced in *MTD1* knockdowns, suggesting that Mtd1 is a determinant of strong (level 2) *MID* expression. Expression of *SAD1* and *GSM1* was inhibited in both *mid-2* and *MTD1* knockdowns, indicating that their expression is dependent on both Mid and Mtd1. Given that *MID* has two activation stages during gametogenesis (Figure 2, B–D), these observations generate the proposal (see DISCUSSION) that the very early activation of *MID* by nitrogen starvation may act as a positive regulator of *MTD1* and that *MTD1* expression in turn leads to a second increase of *MID* to its threshold level, which is necessary to activate *SAD1* and *GSM1*.

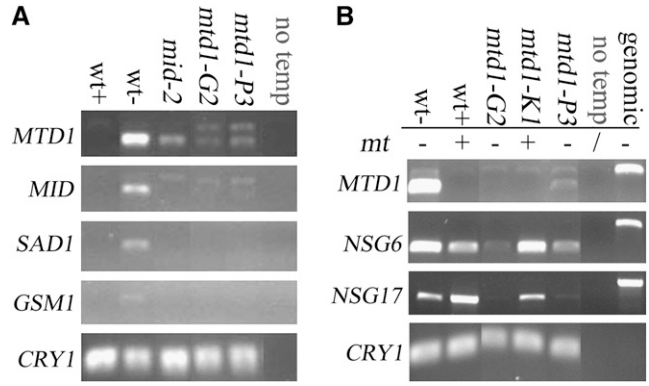

Figure 6.—Effects of *MID* mutation and *MTD1* knockdown on expression of some gamete-specific genes. (A) RT–PCR of various *minus*-specific genes in wild type, *mid-2*, and *MTD1* RNAi *minus* progeny G2 and P3. *CRY1* is used as an internal control. "No temp," negative control in PCR with no addition of DNA template. (B) RT–PCR to detect expression levels of *NSG6* and *NSG17* in different *MTD1* RNAi progeny. "No temp," negative control in PCR with no addition of DNA template. "Genomic," positive control in PCR using genomic DNA as templates.

We also tested the effect of *MTD1* knockdown on the expression of *NSG6* and *NSG17*. RT–PCR (Figure 6B) revealed that *NSG6* and *NGS17* were affected in *minus* knockdown strains G2 and P3, but not in the *plus* (K1) strain carrying the RNAi construct, as expected. Thus expression of these two *NSG* genes during *minus* gametogenesis is also influenced by Mtd1 levels.

DISCUSSION

Responses to ammonium depletion in Chlamydomonas: Adverse environmental conditions such as nitrogen deprivation commonly trigger gametogenesis in algae (Sager and Granick 1954; Harris 1989) and in fungi such as *Schizosaccharomyces pombe* (Mochizuki and Yamamoto 1992; Breeding *et al.* 1998; Davey 1998) and *Candida lusitaniae* (Young *et al.* 2000). In fission yeast, nitrogen starvation stimulates activation of a heterotrimeric G protein followed by activation of adenylate cyclase, with cAMP activating the signal transduction pathway leading to sexual development (Davey 1998). By contrast, in *C. reinhardtii*, nitrogen starvation activates the expression of gamete-specific genes, while sexual agglutination activates a cAMP-dependent signal transduction pathway that triggers subsequent events in the mating reaction (Pasquale and Goodenough 1987; Goodenough 1989; Saito *et al.* 1993).

Ammonium depletion has been shown to trigger three changes in vegetative *C. reinhardtii* cells:

1. A rapid (within 1 hr) transcriptional activation of genes involved in nitrate utilization that are repressed by ammonium [*NIA1*-encoding nitrate reductase (Loppes *et al.* 1999), *NRT2;1*-encoding nitrate transporters (Quesada *et al.* 1994), and *NIT2*, a positive regulator

of nitrate-assimilation genes (Schnell and Lefebvre 1993)].

2. An onset of massive protein and nucleic acid catabolism (Beck and Haring 1996) and ribosome degradation (Martin *et al.* 1976), accompanied by an early (within 1–3 hr) expression of genes encoding proteasome subunits (Abe *et al.* 2004), amino acid oxidase (Vallon *et al.* 1993), urate oxidase (Merchan *et al.* 2001), and other proteins presumably involved in this catabolic response (Abe *et al.* 2004). The outcome of this is that cells enter a stable G_0 stage in which they can survive for weeks without an exogenous nitrogen source. Following the terminology of Abe *et al.* (2005), we refer to this as the N-adaptation program.
3. A later (3–4 hr) expression of genes encoding proteins necessary for mating and early zygote development, hereafter called the gamete program (Abe *et al.* 2004). These changes are fully reversible: gametes provided ammonium will redifferentiate into cycling vegetative cells within 18 hr (Sager and Granick 1954; Goodenough 1991).

The fact that many of the genes involved in the N-adaptation program are expressed prior to most of the genes involved in the gamete program suggests that the former are responsive to transcription factors that are directly activated by ammonium depletion and that the latter are responsive to transcription factors that are expressed or activated as a consequence of some feature(s) of the N-adaptation program. Such a two-stage model helps to clarify an otherwise puzzling facet of the process: once catabolism is underway, intracellular ammonium levels presumably rise, dampening the N-starvation signal, but gametogenesis is able to proceed because it is regulated by a system acting downstream of N-starvation.

Distinctive features of the *MID* gene and Mid protein: The *MID* gene, resident in the MT^- locus and required for expression of *minus*-specific mating- and zygote-related genes, is shown here to have a unique expression pattern:

1. Unlike most gamete-specific genes, including *MTD1*, *MID* is expressed at low (basal) levels in *minus* vegetative cells.
2. *MID* undergoes a small transient rise in expression (to level 1) within 30 min of ammonium depletion, in concert with the ammonium-repressed and N-adaptation-program genes.
3. Several hours later, *MID* undergoes a second, sustained increase in expression (to level 2) in concert with the onset of mating-related *minus* gene expression. The upregulation to level 2, which we propose serves as a threshold level, is required to drive the expression of *minus*-specific genes necessary for agglutination and fusion, possibly because their *cis*-regulatory elements have relatively low Mid affinity.

These observations indicate that *MID* expression is under complex regulation: basal expression in vegetative cells is possibly constitutive; level 1 expression appears to be a direct response to ammonium depletion; and level 2 expression is presumably dependent on features instantiated by a pathway downstream of the initial ammonium-withdrawal response.

A second feature of the Mid protein is also documented in this report: Of the 15 genes (13 of unknown function) in the *C. reinhardtii* genome that encode proteins with an RWP-RK motif, the Mid sequence alone carries a contiguous additional set of seven conserved amino acids found in the N-sensitive Nit2 transcription factor of *C. reinhardtii* (Schnell and Lefebvre 1993; Galvan and Fernandez 2001) and in transcription factors involved in nitrogen-deprivation-induced nodulation events in lotus (Schauser *et al.* 1999) and pea (Borisov *et al.* 2003). Hence, *MID* possesses two properties of a protein influenced by nitrogen limitation: upregulated expression in response to ammonium depletion and a protein motif that possibly adopts distinctive configurations in response to ammonium levels. The fact that vegetative cells express basal levels of Mid is consistent with the possibility that Mid may play some role in the ability of *minus* vegetative cells to sense the occurrence of ammonium depletion.

Our study also clarifies a third feature of the *MID* system. Previous reports showed that whereas deletion (*mid-2*) or loss of function (*mid-1*) of Mid disallows *minus*-specific gene expression, *plus*-specific gene expression is not affected, and the cells differentiate as *pseudo-plus* gametes, lacking only those *plus* functions that are encoded in the absent MT^+ locus (Ferris *et al.* 1996; Ferris and Goodenough 1997). This observation has been open to two interpretations: (1) Mid acts both as a transcriptional activator of *minus* genes and as a transcriptional repressor of *plus* genes or (2) the *plus* program is the "default" program expressed when the *minus* program fails without positing any direct repressor activity for Mid. We show here that in an *MTD1*-knockdown background, wherein functional Mid protein is expressed at low (but not high) levels, *plus* genes fail to be expressed, in contrast to their full expression when Mid is absent or nonfunctional. This observation strongly suggests that low levels of Mid are adequate for preventing the expression of *plus* genes.

The role of Mtd1 in *minus* gametogenesis: Sex-determination systems are typically complex, an example being the elaborate interplay between *SRY* and *Sox-9* in mammalian testis determination (Koopman 1999; Kanai *et al.* 2005). This study indicates that sex determination in Chlamydomonas entails a similarly complex pattern of gene regulation.

Our working model is shown in Figure 7. Upregulation of *MID* to level 1 immediately follows nitrogen depletion and leads to activation of *MTD1* expression, which in turn leads to the second stage of *MID*

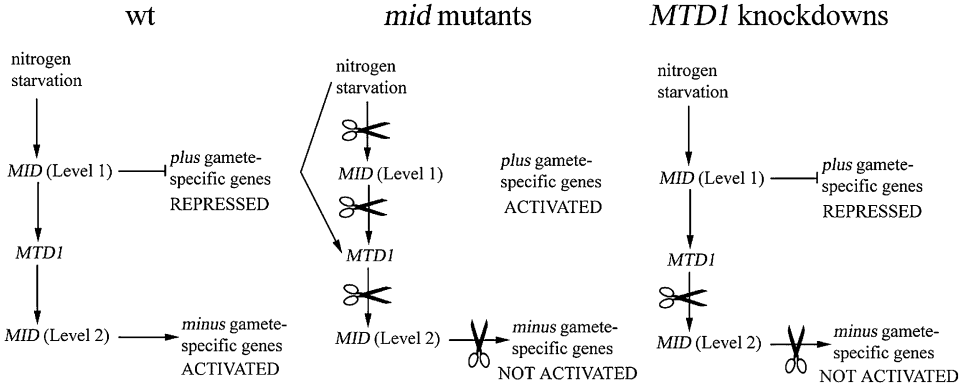

FIGURE 7.—Proposed model of gametogenesis in *minus* cells. In wild-type (wt) cells, *plus* gamete-specific genes are repressed and *minus* gamete-specific genes are activated by *MID*. In *mid* mutants, loss of function of *MID* fails to repress *plus* gamete-specific genes and activate *minus* genes; therefore cells differentiate into *pseudo-plus* cells. Nitrogen starvation is able to activate *MTD1* through a *MID*-independent pathway. In *MTD1* knockdowns, low levels (level 1) of *MID* are able to repress *plus* gamete-specific genes. However, *MID* cannot reach its threshold level (level 2) to activate *minus* gamete-specific genes due to the defect in *MTD1*. Therefore, cells fail to display any gametic phenotypes.

activation, level 2. Level 1 is sufficient for preventing expression of *plus* gamete-specific genes while level 2 represents the threshold necessary to activate *minus* gamete-specific genes. When *MID* undergoes loss-of-function mutations, expression of *plus* gamete-specific genes is not prevented, nor does activation of *minus*-specific genes occur. As a result, cells differentiate as *pseudo-plus* gametes. Expression of *MTD1* is strongly reduced but not eliminated in the *mid* mutants, indicating that nitrogen deprivation controls the expression of *MTD1* via both *MID*-dependent and -independent pathways. On the other hand, when *MTD1* is knocked down, cells fail to differentiate as either *pseudo-plus* or *minus* gametes: *plus* gamete-specific genes are not expressed due to the level 1 presence of *MID*, and *minus* gamete-specific genes are not induced since level 2 cannot be reached. That is, a threshold level of *MTD1* is apparently required for the critical second stage of *MID* activation in *minus* cells.

This model does not account for two published observations: An mt^+ strain carrying the *MID* gene transposed to an autosome differentiates as *minus*, as do mt^+ cells transformed with the *MID* gene, even though neither possesses a copy of the *MTD1* gene (FERRIS and GOODENOUGH 1997). To reconcile these observations with the results reported here, we are led to propose that *plus* gametes express a system, the "*MTD1*-equivalent system," that is functionally equivalent to the "*MTD1* system" but achieves this outcome without requiring the Mtd1 protein itself. When *MID* is introduced into a *plus* background, the *MTD1*-equivalent system enables sufficiently high *MID* expression to allow transformants to undergo *minus* differentiation, albeit success is usually incomplete (see RESULTS and FERRIS and GOODENOUGH 1997), meaning that the *MTD1*-equivalent system is not repressible by Mid. Importantly, at least one essential gene in the posited *plus MTD*-equivalent system must be resident in the MT^+ locus. If the system were fully encoded elsewhere in the genome and Mid repressible, then the mt^+ cells carrying a *MID* gene would fail to differentiate. If it were fully encoded elsewhere in the genome and not Mid repressible, then *MTD1* knockdowns would presumably be complemented by this second system and would not have a mating-null phenotype.

That *plus* cells possess an *MTD*-equivalent system is also indicated by the phenotypes of two mutant strains, the conditional *dif2* and the nonconditional *dif3*, both studied in a *plus* background (ABE *et al.* 2005). In the *dif2* mutant, expression of all known N-adaptation genes and of one *plus*-specific gamete-program gene (*FUS1*) is blocked at restrictive temperature and the cells are unable to mate; gene expression and mating ability occur normally with temperature downshift. In *dif3*, three N-adaptation genes (*NSG3*, *NSG6*, and *NSG7*) and *FUS1* are not expressed, and gametogenesis also fails. When *dif2* is crossed into an mt^- background, the nondifferentiation phenotype is also observed (SAITO and MATSUDA 1991); therefore, *dif2* does not correspond to the posited gene linked to mt^+. The *dif3* mutant is sterile and cannot be tested for mt^+ linkage.

The MT^+ locus contains no ORF with any similarity to *MTD1*, and indeed *MTD1* lacks homology with any known gene. Hence the posited *plus MTD*-equivalent system must be specified in a fashion different from the *minus* system. The one gene currently known to be unique to the MT^+ locus—expressed exclusively in gametes, not repressible by Mid (FERRIS *et al.* 2002), and not yet assigned a function—is *MTA1*, which encodes a small protein with a predicted coiled-coil domain and repeated motifs predicted to form a leucine histidine zipper (FERRIS *et al.* 2002). This is a totally different species from Mtd1, which is predicted to be a transmembrane protein (FERRIS *et al.* 2002). Experiments to test the phenotype of *MTA1* knockdowns are clearly of high priority. Alternatively, the posited MT^+-linked gene may await identification.

Of high priority as well is the ascertainment of the cellular location of Mtd1 since, despite considerable effort, our antibodies have failed to generate definitive immunolocalization. Given its predicted three-spanner

transmembrane configuration with N-glycosylation sites, however, one can reasonably speculate that Mtd1 functions to monitor or respond to features of the external environment, the obvious feature being nitrogen status.

This study provides an answer to a puzzle pertaining to the sex-determination system of *C. reinhardtii*. When it was assumed that *MID* was the sole determinant of mating type, it was not obvious why *C. reinhardtii* possesses complex *MT* loci under recombinational repression. Would it not be sufficient that cells carrying the *MID* gene differentiate as *minus*, and cells not carrying *MID* differentiate as *plus*? Our finding that *MID* and *MTD1* are mutually dependent on one another for bringing about *minus* gametogenesis, and that at least one component of the posited complementary system in *plus* is encoded in the MT^+ locus, indicates that it may be essential that *MID* and *MTD1* remain in genetic linkage. If so, the puzzle shifts to the question of how such a system evolved in the first place.

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