Characterization of *mat A-2*, *mat A-3* and Δ*matA* Mating-Type Mutants of *Neurospora crassa*

Adlane V.-B. Ferreira,*[,1] Zhiqiang An,[†,2] Robert L. Metzenberg[†,3] and N. Louise Glass*

**Department of Botany and The Biotechnology Laboratory, The University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada and [†] The Department of Biomolecular Chemistry, University of Wisconsin, Madison, Wisconsin, 53706*

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ABSTRACT

The mating-type locus of *Neurospora crassa* regulates mating identity and entry into the sexual cycle. The *mat A* idiomorph encodes three genes, *mat A-1*, *mat A-2*, and *mat A-3*. Mutations in *mat A-1* result in strains that have lost mating identity and vegetative incompatibility with *mat a* strains. A strain containing mutations in both *mat A-2* and *mat A-3* is able to mate, but forms few ascospores. In this study, we describe the isolation and characterization of a mutant deleted for *mat* (Δ*matA*), as well as mutants in either *mat A-2* or *mat A-3*. The Δ*matA* strain is morphologically wild type during vegetative growth, but it is sterile and heterokaryon compatible with both *mat A* and *mat a* strains. The *mat A-2* and *mat A-3* mutants are also normal during vegetative growth, mate as a *mat A* strain, and produce abundant biparental asci in crosses with *mat a*, and are thus indistinguishable from a wild-type *mat A* strain. These data and the fact that the *mat A-2 mat A-3* double mutant makes few asci with ascospores indicate that MAT A-2 and MAT A-3 are redundant and may function in the same pathway. Analysis of the expression of two genes (*sdv-1* and *sdv-4*) in the various *mat* mutants suggests that the *mat A* polypeptides function in concert to regulate the expression of some sexual development genes.

MANY fungal mating-type genes encode products with characteristics of transcription factors (reviewed in Herskowitz 1989; Glass and Nelson 1994; Kothe 1996). Mating type in *Saccharomyces cerevisiae* is the best-characterized fungal mating system, and activation, repression, and protein-protein interactions have been demonstrated biochemically and genetically in that system. Mating-type genes in yeast regulate the fusion of haploid cells of the opposite mating type, as well as the entry of the diploid cell into meiosis (reviewed in Johnson 1995; Herskowitz 1989; Nasmyth and Shore 1987). In filamentous fungi, mating-type sequences also regulate the fusion of reproductive structures of two genetically distinct individuals, which results in the production of meiotic progeny.

The filamentous ascomycete fungus, *Neurospora crassa*, has two mating-types, *A* and *a*, which regulate entry into the sexual cycle (reviewed in Glass and Nelson 1994). The mating-types are determined by dissimilar DNA sequences at the mating-type locus (*mat*)[4] and have been termed idiomorphs (Metzenberg and Glass 1990). In *mat a* individuals, a single gene, *mat a-1*, is required for mating identity, postfertilization functions, and for vegetative incompatibility with *mat A* strains (Griffiths and Delange 1978; Chang and Staben 1994). MAT a-1 has an HMG domain and has been shown to bind to DNA by *in vitro* assays (Staben and Yanofsky 1990; Philley and Staben 1994). The *mat A* idiomorph contains three genes, *mat A-1*, *mat A-2*, and *mat A-3* (Ferreira *et al.* 1996). The *mat A-1* gene is required for *mat A* mating identity, postfertilization functions, and vegetative incompatibility with *mat a* strains (Glass *et al.* 1990; Saupe *et al.* 1996). MAT A-1 has similarity to the *S. cerevisiae* mating-type transcriptional activator MATα1 (Sprague *et al.* 1983). MAT A-2 shows ~20% identity with the mating-type polypeptide SMR1 of the pseudohomothallic ascomycete *Podospora anserina* (Ferreira *et al.* 1996). *SMR1* is one of the three genes (*FMR1*, *SMR1*, and *SMR2*) encoded by the *mat*− mating-type locus of *P. anserina* (Debuchy *et al.* 1993). MAT A-3 has an HMG domain and shows ~20% identity with both MAT a-1 and *P. anserina* SMR2 (Debuchy *et al.* 1993; Ferreira *et al.* 1996).

A mutant, A^{IIRIP}, which contains mutations in both *mat A-2* and *mat A-3*, normally mates as a *mat A* strain, but produces very few asci with ascospores (Glass and Lee 1992; Ferreira *et al.* 1996). The effect of individual

Corresponding author: N. L. Glass, Rm. 238 Wesbrook, The Biotechnology Laboratory, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada. E-mail: glass@unixg.ubc.ca

[1] *Current address:* Departamento de Microbiologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, 31270-910, Brazil.

[2] *Current address:* Millennium Pharmaceuticals, Inc., Cambridge, MA, 02139.

[3] Department of Biological Sciences, Stanford University, Stanford, CA, 94305-5020.

[4] The designation for the mating-type locus for *Neurospora crassa* has recently been changed from *mt* to *mat*, in accordance with the designation of other ascomycete mating type loci (Glass and Staben 1997).

mutations in *mat A-2* and *mat A-3* was not determined. Mutation of the *P. anserina SMR1* or *SMR2* genes also results in strains that mate qualitatively normally, but that produce a reduced number of progeny (Debuchy *et al.* 1993). Cytological analyses of reproductive structures from crosses between *P. anserina mat+ and SMR1* or *SMR2* mutants show haploid meioses and uniparental progeny (Zickler *et al.* 1995); uniparental spores are of the mutant *SMR1* or *SMR2* genotypes only.

The products of the mating-type locus of *N. crassa* and *P. anserina* are thought to act as master regulators of both mating and nuclear identity during sexual development. However, no direct target genes of any of the mating type products have been identified. Nelson and Metzenberg (1992) isolated genes (*sdv*) that are expressed early in sexual development. A functional *mat A-1* was required for the expression of at least seven of the *sdv* genes.

In this study, we describe the construction and phenotypes of a mating type deletion strain, as well as *mat A-2*- and *mat A-3*-specific mutants. We show that the mating-type genes are not essential for *N. crassa*, and that mutations in *mat A-2* or *mat A-3*, unlike their *P. anserina* counterparts, do not dramatically affect sexual development nor do they result in the production of uniparental asci. We also analyzed the expression of two *sdv* genes in the various *mat* mutants. Both genetic data and *sdv* gene analyses suggest that in *N. crassa*, the regulation of sexual development may involve the formation of complexes of the mating-type products that activate or repress genes in different pathways.

MATERIALS AND METHODS

***N. crassa* and *Escherichia coli* strains:** *N. crassa* strains used in this study and their relevant genotypes are listed in Table 1. *E. coli* XL1-blue (Stratagene, La Jolla, CA) was used for all DNA manipulations involving the construction of the mating-type deletion strain, and *E. coli* DH5α (BRL, Burlington, Ontario, Canada) was used for all other manipulations.

Construction of plasmids for making the Δ*matA* strain: The wild-type *Schizophyllum commune ADE5* gene encodes aminoimidazole riboside-5′-phosphate synthase (Alic *et al.* 1990) and is capable of complementing *ad-2* mutants of *N. crassa*, albeit weakly (our unpublished observations). *ADE5* was used as a selectable marker for gene replacement at the mating-type locus. The gene replacement plasmid (p2ADE-X) was constructed as follows: the 5.3-kbp *A* idiomorph and its left and right flanking regions (4 and 2 kbp, respectively) were cloned into pBR322 to give p2A4. The 5.7-kbp *Nde*I-*Nsi*I fragment (Figure 1), which contains *mat A*, was replaced with the 0.6-kbp multiple cloning site region from pGEM3Z(-) (Promega, Madison, WI) to give p2LAC3Z-4DA. The 3.8-kbp *Kpn*I-*Bam*HI fragment from pADE5-2g (Alic *et al.* 1990), which contains the *ADE5* gene of *S. commune*, was subcloned into the multiple cloning region of p2LAC3Z-4DA to give p2ADE-X.

Construction of plasmids for generating *mat A-2* and *mat A-3* repeat-induced point mutants: Plasmid pGq1310 (constructed by J. Grotelueschen, Department of Biomolecular Chemistry, University of Wisconsin) contains a 1.3-kb *Bcl*I/*Pvu*II *mat A* fragment (*mat A-2*) in a *qa-2*$^+$ vector. Plasmid pAL2 contains a 2.5-kb *Eco*RV *mat A* fragment (*mat A-3*) interrupted by a 1.3-kbp *Bam*HI fragment containing the hygromycin phosphotransferase gene (*hph*) inserted into the *Bam*HI site in the centromere distal flank region of the *mat A* locus (constructed by S. Saupe, Department of Botany and the Biotechnology Laboratory, University of British Columbia). Neither the pGq1310 nor the pAL2 plasmids contain the 5′ ends of *mat A-2* or *mat A-3* ORFs.

***N. crassa* transformation and repeat-induced point mutation analysis:** *N. crassa* spheroplasts were prepared from germinated conidia by the method of Akins and Lambowitz (1985), and they were transformed as described by Vollmer and Yanofsky (1986). Transformants were normally grown for 3–4 days at 30°. For screening of Δ*matA* strains, transformants were kept on plates in subdued light at 30° for 10 days and then moved to bright light at room temperature until all or nearly all transformants formed conidia. Mating-type tests were performed on the medium of Westergaard and Mitchell (1947).

Plasmids pGq1310 and pAL2 were introduced into RLM 52-22 (*qa-2; aro-9 A*) or NLG R2-39 (*thr-2 het-6PA A*) strains, respectively, for isolation of the *mat A-2* and *mat A-3* mutants. Transformants were randomly chosen (∼25 from each transformation) and grown under selective conditions. Homokaryons were isolated and crossed to wild-type *mat a* (FGSC 532) or a temperature-sensitive strain, *un-3 a* (NLG R1-09); *mat A* progeny were chosen for further screening as described in Glass and Lee (1992). Genomic DNA from the transformants and from first- and second-generation progeny was isolated (Raeder and Broda 1985) and digested with two isoschizomers, *Mbo*I (C-methylation insensitive) and *Sau3A*I (C-methylation sensitive). After gel electrophoresis, DNA was transferred to nylon membranes (Nytran; Schleicher & Schuell, Keene, NH). Membranes were probed with ^{32}P-labeled *mat A-2*, *mat A-3*, or *qa-2*–specific fragments, and were then exposed to X-ray films. DNA sequence analysis was performed using an automated sequencing procedure (Applied Biosystems, Inc., Mississauga, Ontario, Canada) at the Nucleic Acid-Protein Service Unit at Biotechnology Laboratory of the University of British Columbia (Vancouver, British Columbia, Canada).

Phenotypic characterization of mutants: Photographs of perithecial squashes from mutant and wild-type crosses were taken at ×10. The number of ascospores was quantified in the following manner: at 8 and 11 days after fertilization, 10 perithecia were picked from a crossing plate and transferred to an Eppendorf tube containing 25 μl of water. Perithecia were lightly squashed in the tube using a fitted plastic pestle. The number of ascospores present in a sample of the supernatant was counted using a hemacytometer. The ascospores present in five samples from each cross were counted. Statistical analyses were applied using analysis of variance and Student's *t*-test.

Heterokaryon tests: For heterokaryon tests, 25 μl of conidial suspensions containing approximately the same number of conidia (4×10^6/ml) from each auxotrophic strain were mixed and inoculated onto minimal medium (Vogel 1964) in race tubes (Davis and Deserres 1970). Linear growth of compatible and incompatible controls plus the experimental heterokaryons were measured over time. All heterokaryon tests were performed in triplicate.

RNA analyses: *N. crassa* strains for RNA analyses were grown as described by Nelson and Metzenberg (1992). Mycelial mats (grown for 3–4 days) were isolated by filtration and stored frozen at −70°. RNA isolation, reverse transcription (RT)-PCR, and Northern blots were performed as described (Ferreira *et al.* 1996), except that for Northern analyses, 3 μg of polyA$^+$ RNA was loaded per lane. Hybridization of membranes (Zeta-Probe; Bio-Rad, Richmond, CA) was carried out according to the manufacturer's suggestions. After a 24-h exposure to X-ray

TABLE 1

***Neurospora crassa* strains**

Strain	Genotype	Source
FGSC 2489	WT *A*	FGSC
RLM 52-22	*qa-2; aro-9 A*	RLM
NLG R4-50 (A^{IRIP})	*arg-2 mat A-1^{m92}*	Glass and Lee (1992)
NLG R2-39	*thr-2 het-6^{PA} A*	Smith *et al.* (1996)
NLG R5-38	*mat A-2^{ml}*	This study
NLG R5-40	*ad-3B mat A-2^{ml}*	This study
NLG R4-66	*mat A-3^{ml}*	This study
NLG R4-67	*pyr-4 mat A-3^{ml}*	This study
NLG R5-39	*thr-2 mat A-3^{m2}*	This study
NLG R4-16 (A^{IIRIP})	*mat A-2^{m3} mat A-3^{m3}*	Glass and Lee (1992)
FGSC 532	WT a	FGSC
FGSC 2088	*r al-1 a*	FGSC
NLG R1-51	*un-3 ad-3A nic-2 a*	NLG
FGSC 2226	WT *a*	FGSC
NLG R1-09	*un-3 a*	NLG
NLG R2-54	*qa-2; aro-9 a*	NLG
RLM 57-26	*pyr-4 arg-5; inl; pan-2 a*	RLM
RLM 57-30	*pyr-4; cyh-1 a*	RLM
FGSC 4565	*ad-3B cyh-1 mat a-1^{ml}*	Griffiths (1982)
RLM 41-10	*thi-4 ad-2; lys-1 A*	This study
RLM 57-11	*caf lys-1 A*	RLM
RLM 73-11	*ad-2; am132 inl inv mei2 A*	This study
RLM 73-12	*ad-2; am132 inl inv mei2 a*	This study
RLM 44-02 (FGSC 8292)	*thi-4; lys-1 ΔmatA*	This study
PS D25[a]	*his-5 tol A/a*	NLG

Strain names more commonly used are in parentheses. FGSC, Fungal Genetics Stock Center (Department of Microbiology, University of Kansas, Kansas City); L. Glass). NLG, L. Glass' laboratory; RLM, R. Metzenberg's laboratory; WT, wild type.

[a] Contains a partial duplication of linkage group 1; strain is derived from a cross between T(39311) *ser-3 A* (Perkins and Barry 1977) × *un-3; his-5 trp-4 tol a* (NLG R5–27).

film, membranes were exposed to a Phosphorscreen (Molecular Dynamics, Sunnyvale, CA). The screen was then scanned in a Phosphorimager scanner after 18–24 h of exposure and analyzed using Imagequant software (Molecular Dynamics). Radiation emitted from the blots was quantified for *sdv*-specific transcripts and for the constitutive gene controls. Controls included the *N. crassa crp-1* gene for the small ribosomal subunit protein, which produces two transcripts (Kreader and Heckman 1987), and/or the β-tubulin gene (*tub-1*) (Orbach *et al.* 1986). For subsequent analyses with other *sdv* genes, radioactivity on blots was allowed to decay or was stripped away by boiling the blot for 20 min in 0.1× SSC/0.5% SDS.

RESULTS

Deletion of the *A* mating type locus: The general strategy for removal of the mating type sequences was analogous to that used by Chang and Staben (1994) for replacement of one mating type by another. Their procedure took advantage of heterokaryon incompatibility between the resident *mat A* mating type and the introduced *mat a* mating-type gene to counterselect the very large majority of ectopic integrants that contained both mating types. Integrants in which the resident mating-type gene had been replaced were spared the incompatibility reaction and could grow. Because we wished to replace the *mat A* mating type genes with nonmating-type sequences (the *ADE5* gene of *S. commune*), we needed to introduce an additional step. Spheroplasts of RLM 41-10 (*thi-4 ad-2; lys-1 A*) were transformed to (partial) adenine independence with p2ADE-X, which contains the *S. commune ADE5* gene flanked by the flanking sequences of *N. crassa mat A* (see materials and methods). About 3600 transformants were selected on 18 plates containing lysine plus thiamine, and the ~200 colonies per plate were allowed to conidiate. The conidia from each plate were suspended in minimal medium (Vogel 1964), on which they were, of course, unable to grow. They could, however, be rescued by forming lysine- and thiamine-independent heterokaryons with a second strain, RLM 73-12 (*ad-2; am132 inl inv mei-2 a*). At least in principle, this nutritional helper strain of the *mat a* mating-type should allow survival and growth of only those transformants in which *mat A* had been deleted or otherwise inactivated. A comparable number of conidia of RLM 73-12 was therefore added to each of the 18 pools of conidia from the transformants. After 2 wk at 30°, one of the 18 tubes had formed a vigorous heterokaryon and had conidiated. Mating-type tests of this strain showed that it mated as

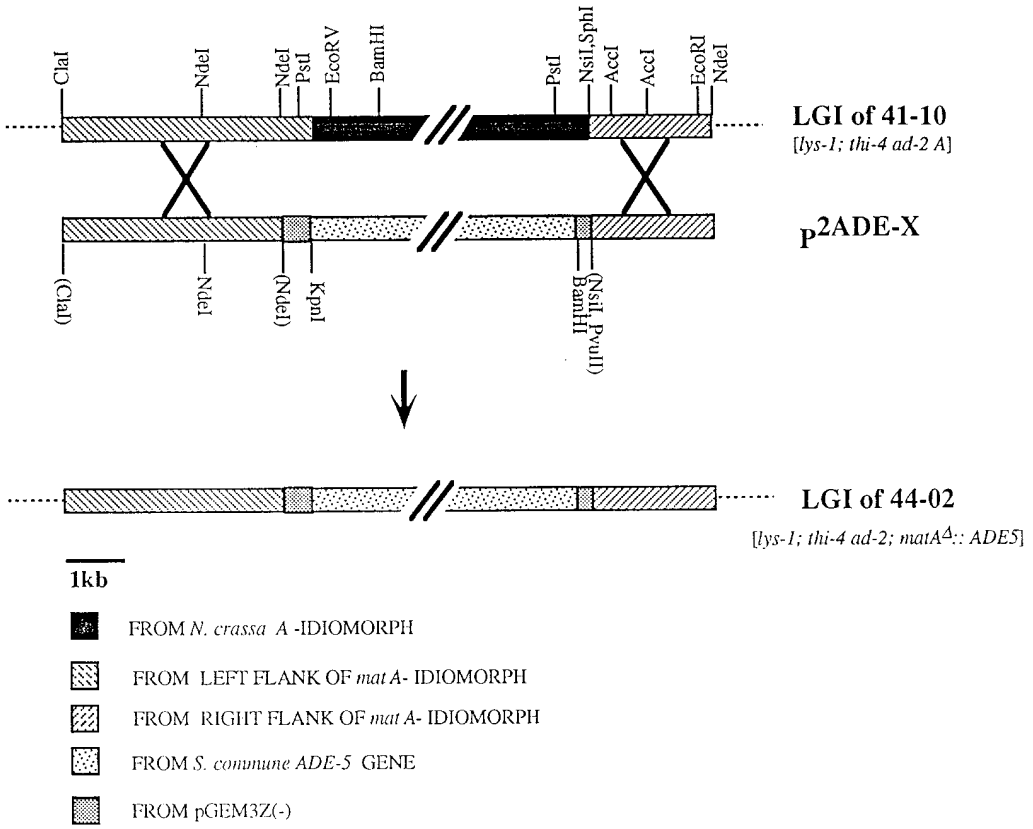

Figure 1.—Construction of mating-type deletion strain (Δ*matA*; RLM 44-02 deposited as FGSC 8292) by transformation of strain RLM 41-10 with plasmid p2ADE-X. LGI, *N. crassa* linkage group I.

a *mat a* strain, but not as a *mat A* strain, suggesting that it was a heterokaryon carrying the desired deletion. Conidia of this strain were streaked onto lysine plus thiamine plates, incubated at room temperature overnight, and 34 germinated conidia were picked to nonselective medium. Of the resulting cultures, 28 proved to require lysine and thiamine, and they were infertile with both mating types. Genomic DNA from four of these strains was tested on a Southern blot with several probes. As expected, this revealed that the *Nde*I-*Nsi*I fragment, which contains the *A* idiomorph, was absent, and that the *Kpn*I-*Bam*HI fragment, which contains the *ADE5* gene of *S. commune*, was present instead (data not shown). Figure 1 summarizes the double-crossover replacement event that is consistent with the data from blotting. One of these strains (Δ*matA*) was used in the remaining work and is deposited in the Fungal Genetics Stock Center as FGSC 8292. We refer to this strain as Δ*matA* to reflect the fact that it is a deletion of the *mat A* idiomorph; differences are present in the flanking regions of the *mat* locus between *mat a* and *mat A* strains (Randall and Metzenberg 1995).

The Δ*matA* strain was phenotypically identical to wild type during vegetative growth, and it produced female reproductive structures (protoperithecia) and conidia. The Δ*matA* strain, however, was sterile and did not mate with either *mat A* or *mat a* strains, but it formed a heterokaryon with either *mat A* or mat *a* strains, a phenotype identical to that described for *mat A-1* mutants (Griffiths 1982). The capacity of the Δ*matA* strain to mate and produce perithecia as either an *A* or *a* strain was restored by the introduction of ectopic copies of either *mat A* or *mat a* sequences, respectively, but ascospores were not produced (our unpublished results).

Isolation of *mat A-2* and *mat A-3* mutants: To determine the phenotype of *mat A-2*- and *mat A-3*-specific mutants, we chose to use a method termed repeat-induced point (RIP) mutation, which is a natural mutagenic process in *N. crassa* that involves duplicated sequences (Selker 1990). RIP occurs when transformants containing duplicated sequences introduced by the transformation process are taken through a cross; both introduced and resident copies suffer GC to AT transition mutations and typically show methylation of C residues in the duplicated segments (Selker and Garrett 1988; Cambareri *et al.* 1989). This strategy was adopted primarily because of the low frequency of gene replacement events at the mating-type locus (text above and unpublished results). The *mat A-2* plasmid (pGq1310) was introduced into RLM 52-22 (*qa-2; aro-9 A*) (see materials and methods), and two independent homokaryotic transformants (G11 and G12) were chosen. G11 and G12 were crossed to FGSC 532 (*OR a*), and more than 200 *mat A* progeny resulting from the two crosses

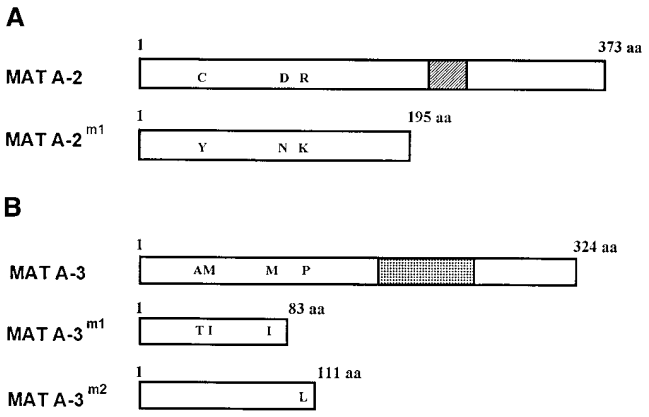

Figure 2.—MAT A-2 and MAT A-3 wild-type and mutant proteins. (A) Changes in MAT A-2^{m1} as compared to the MAT A-2 wild-type protein. A stop codon appears at position 196, and there are amino acid substitutions at positions 62 (cysteine to tyrosine), 93 (aspartic acid to asparagine), and 96 (arginine to lysine). Shaded area represents conserved region between MAT A-2 and SMR1. (B) Changes in MAT A-3^{m1} and MAT A-3^{m2} as compared to MAT A-3. MAT A-3^{m1} is 83 amino acids long, and it has a threonine at position 47 (instead of alanine) and isoleucine in place of methionine (positions 48 and 80). MAT A-3^{m2} has a stop codon at position 112 and a leucine replacing a proline in position 102 of the polypeptide. The HMG box is stippled.

were analyzed. These progeny displayed wild-type morphology during vegetative growth. In crosses with *mat a* strains, all the progeny produced perithecia and ascospores and were indistinguishable from wild-type crosses.

To get a preliminary indication of whether RIP had occurred in the *mat A* progeny of G11 and G12 transformants without giving an obvious phenotype, genomic DNA was analyzed for DNA methylation by digestion with the isoschizomers *Sau3A*I and *Mbo*I. When a *mat A-2* probe was hybridized to genomic DNA from random *mat A* progeny from the G11 and G12 crosses, methylation was observed in a few *mat A* progeny (data not shown).

Based on DNA methylation and RFLP analyses, a number of G11 and G12 *mat A* progeny were selected for DNA sequence analysis of the resident *mat A-2* sequences. One progeny of G11, NLG R5-38 (*mat A-2^{m1}*), had 27 bp changes in *mat A-2*, resulting in eight amino acid substitutions and two stop codons in the *mat A-2* ORF (Figure 2A). The first stop codon resulted in a truncated MAT A-2 of 195 amino acids. About 65% of the mutations occurred in 5′CpA 3′ dinucleotides, which is typical of RIP events (Grayburn and Selker 1989) (data not shown). All mutations were of the same polarity, resulting in C to T changes on the *mat A-2* noncoding strand.

To isolate *mat A-3*-specific mutants, we introduced pAL2, which contains *mat A-3*, into NLG R2-39 (*thr-2 het-6^{PA} A*) spheroplasts. Two homokaryotic *mat A-3* transformants (L22 and L25) were subjected to RIP by crossing to NLG R1-09 (*un-3 a*). As with the *mat A* progeny from the *mat A-2* RIP crosses (see above), 200 *mat A* progeny from the L22 and L25 crosses were examined, and none of them showed a mutant phenotype during either vegetative growth or sexual reproduction. A number of L22 and L25 first- and second-generation *mat A* progeny were subjected to RFLP, DNA methylation, and DNA sequence analyses (data not shown). DNA sequence analysis of *mat A-3* in one L22 progeny, NLG R4-66 (*mat A-3^{m1}*), showed 28 mutations in the first 600 bp that resulted in three amino acid substitutions and a stop codon at position 84 (Figure 2B). Fifty-seven percent of the changes occurred in 5′CpA3′ dinucleotides (data not shown). DNA sequence analysis of *mat A-3* in a second progeny of L22, NLG R5-39 (*mat A-3^{m2}*), revealed 16 mutations, two of which resulted in stop codons. The first stop codon would result in a truncated polypeptide of 111 amino acids (Figure 2B). Both the *mat A-3^{m1}* and the *mat A-3^{m2}* alleles have stop codons before the region encoding the HMG domain.

Mating-type specific cDNAs are absent in the *matA-2^{m1}*, *mat A-3^{m1}*, and A^{IIRIP} mutants: Because the *mat A-2^{m1}* and *mat A-3^{m1}* mutants still produce abundant ascospores (see below), the extremely low fertility seen in the A^{IIRIP} mutant (Glass and Lee 1992) was suspected of being caused by absence of both *mat A-2* and *mat A-3* function. The DNA sequencing of the *mat A-2* and *mat A-3* ORFs from A^{IIRIP} (NLG R4-16) showed mutations in *mat A-3* (*mat A-3^{m3}*) that lead to only three amino acid substitutions, while *mat A-2* was heavily mutated (*mat A-2^{m3}*) with a stop codon at amino acid 130 in MAT A-2 plus additional amino acid substitutions (Ferreira *et al.* 1996). We therefore sequenced the promoter region between *mat A-2^{m3}* and *mat A-3^{m3}*, and we found mutations in possible transcriptional regulatory regions of both *mat A-2* and *mat A-3* (Figure 3).

To determine if *mat A-2* and *mat A-3* transcripts were produced in the mating-type mutants, a series of RT-PCR experiments was performed. RNA from WT *A*, NLG R5-38 (*mat A-2^{m1}*), NLG R4-66 (*mat A-3^{m1}*), and NLG R4-16 (*mat A-2^{m3} mat A-3^{m3}*) strains grown in crossing medium was isolated and subjected to RT-PCR using mating-type-specific primers (Figure 4). Transcripts corresponding to *mat A-2* were absent in cDNA preparations from NLG R5-38 (*mat A-2^{m1}*) and transcripts corresponding to *mat A-3* were absent in cDNA preparations from NLG R4-66 (*mat A-3^{m1}*). Both *mat A-2* and *mat A-3* transcripts were absent from cDNA preparations from NLG R4-16 (*mat A-2^{m3} mat A-3^{m3}*). In contrast, *mat A-1* transcripts were present in all three mutants (Figure 4). These data indicated that the NLG R5-38 and NLG R4-66 strains were null for *mat A-2* or *mat A-3*, respectively, and that the mutant phenotype associated with the NLG R4-16 was caused by the absence of functional *mat A-2* and *mat A-3*.

Phenotypic analysis of *mat* mutants: MAT A-2 and MAT A-3 each show ~20% amino acid identity to the *P. anserina* SMR1 and SMR2 mating-type peptides, re-

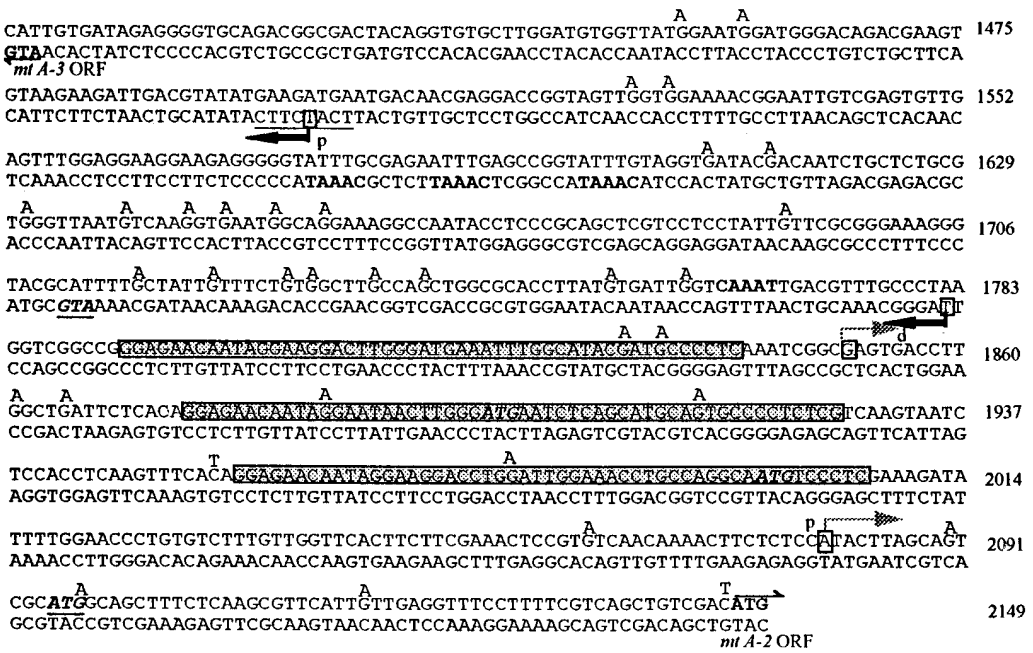

Figure 3.—DNA sequence changes in the promoter region of *mat A-2* and *mat A-3* in NLG R4-16 (*mat A-2^{m3} mat A-3^{m3}*). Nucleotide base changes are shown above promoter sequences. Most of the mutations were polarized G to A transitions in one strand; only two C to T mutations appear on the same strand. Transcriptional start sites and relevant motifs and repeats are shown as described in Ferreira *et al.* (1996). The DNA sequence between the proposed start codons of *mat A-2* and *mat A-3* is given. The arrows (in gray for *mat A-2* and black for *mat A-3*) indicate the positions of the proximal (p) and distal (d) transcriptional start sites. The putative CAAT box sequences are shown in bold type. The stippled boxes show the repeated sequences, which are possibly important for transcription of mat A-2 and mat A-3.

spectively (Ferreira *et al.* 1996). Crosses between *P. anserina SMR1* or *SMR2* mutants and wild type result in haploid meioses, a decrease in the number of meiotic progeny, and the occurrence of uniparental progeny of the *SMR1* or *SMR2* mutant genotype (Zickler *et al.* 1995). To determine if MAT A-2 and MAT A-3 function in a similar manner, crosses with the *mat A-2^{m1}*, *mat A-3^{m1}*, and *mat A-2^{m3} mat A-3^{m3}* mutants were monitored for the production of ascospores and for the occurrence of uniparental asci.

In crosses of the NLG R5-38 (*mat A-2^{m1}*) or the NLG R4-66 (*mat A-3^{m1}*) mutants and wild-type *a* strains (FGSC 532 and FGSC 2226), the number of ascospores produced at both 8 and 11 days after fertilization did not differ significantly from the number of progeny produced in wild-type crosses (data not shown). In contrast, crosses between wild-type *a* strains and NLG R4-16 (*mat A-2^{m3} mat A-3^{m3}*) produce only a few asci per perithecium. These few asci contain eight black ascospores (Glass and Lee 1992). To test for the production of uniparental asci, NLG R5-38, NLG R4-66, NLG R5-39, and NLG R4-16 strains were crossed to *R a* (FGSC 2088). The *R* (round-spore) mutation is ascus dominant (Mitchell 1966). Thus, in crosses with an *R* mutant, all eight ascospores (4*A* and 4*a*) in each ascus are round. If uniparental asci of the *mat A-2^{m1}*, *mat A-3^{m1}*, *mat A-3^{m2}*, or *mat A-2^{m3} mat A-3^{m3}* genotypes were produced in a cross with *R a*, all ascospores within an ascus would be spindle shaped. More than 50 perithecia from each cross were observed cytologically, and only round ascospores were seen. A perithecial squash from a cross of *mat A-3^{m2}* X *R a* is shown in Figure 5B. Figure 5C shows one of the few perithecia that contained asci and ascospores from *mat A-2^{m3} mat A-3^{m3}* X *R a.* Thus, mutations in *mat A-2* or *mat A-3*, or in both *mat A-2* and *mat A-3*, did not confer uniparental inheritance in *N. crassa*.

***mat A-2* and *mat A-3* are not involved in vegetative incompatibility:** The *mat A-1* gene confers both mating identity and mating-type-associated vegetative incompatibility. All of the known *mat A-1* mutants (with one exception) have lost their mating and heterokaryon incompatibility functions concomitantly (Griffiths 1982; Saupe *et al.* 1996). To determine if mutations in *mat A-2* or *mat A-3* affected mating-type-associated incompatibility, we assayed the growth of the *mat A-2^{m1}* and *mat A-3^{m1}* mutants as forced heterokaryons with an *a* strain. A heterokaryon between NLG R4-67 (*pyr-4 mat A-3^{m1}*) and NLG R2-54 (*qa-2; aro-9 a*) grew at a similar rate and had a phenotype similar to that of an incompatible heterokaryon between RLM 52-22 (*qa-2; aro-9 A*) and RLM 57-30 (*pyr-4; cyh-1 a*) (data not shown). A heterokaryon between NLG R5-40 (*ad-3B mat A-2^{m1}*) and NLG R2-54 (*qa-2; aro-9 a*) was inhibited and grew at a rate similar to an incompatible heterokaryon between RLM 52-22 (*qa-2; aro-9 A*) and RLM 57-30 (*pyr-4; cyh-1 a*) (data not shown). Thus, mutations in *mat A-2* and *mat A-3*

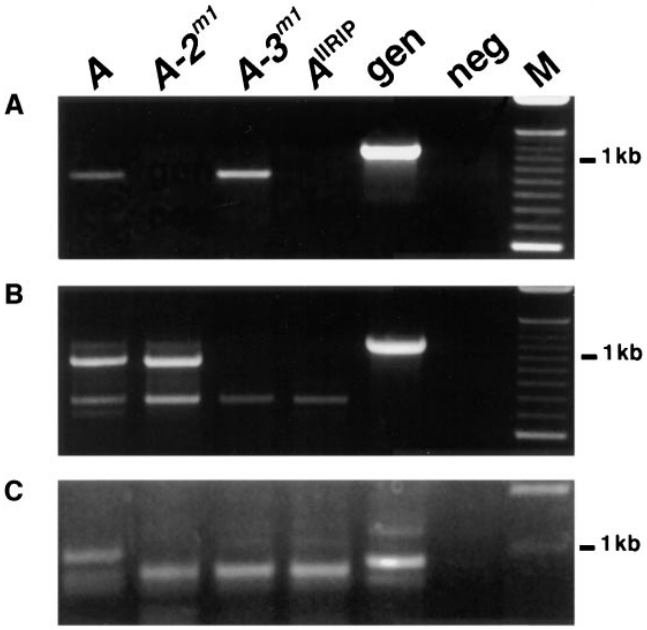

Figure 4.—Amplification of cDNA of mating-type genes in the FGSC 2489 (A), NLG R5-38 (A-2^{m1}), NLG R4-66 (A-3^{m1}), and NLG R4-16 (A^{IIRIP}) mutants by RT-PCR. Genomic DNA from FGSC 2489 (gen) was included in all experiments as a control for the amplification reaction and for size comparison with cDNA. PCR negative control (neg) is shown on the left of the DNA size marker (M). (A) Amplification products (~0.9 kb cDNA, 1.2 kb genomic) using primers specific for *mat A-2*. (B) Amplification products (~0.95 kb cDNA, 1.1 kb genomic) for *mat A-3*. A nonspecific 0.6-kb band in *mat A-3* cDNA amplifications was present. (C) Amplification of *mat A*-1 cDNA used as control for the reverse transcriptase reaction (~0.8 kb). Some cDNA preparations contained traces of genomic DNA that were also amplified by PCR.

do not appreciably affect heterokaryon incompatibility mediated by *mat A-1* and *mat a-1*.

Expression of *sexual development* (*sdv*) genes is altered in the mating type mutants: Several *sdv* genes isolated by subtractive hybridization require a functional *mat A-1* for expression and were therefore potential target genes for the mating type proteins (Nelson and Metzenberg 1992). We analyzed the expression of a number of *sdv* genes by Northern analyses in *mat A-1*^{m92} (*mat A-1*^{RIP}; Glass and Lee 1992), *mat a-1*^{m1} (Griffiths 1982), *mat A-2*^{m3} *mat A-3*^{m3} (Glass and Lee 1992), and the *mat A-2*^{m1}, *mat A-3*^{m1}, and Δ*matA* mutants. In initial RNA analyses, variable expression of some *sdv* genes was observed during time course experiments in various *mat* mutants although the requirement for *mat A-1* for expression was consistent (data not shown). The expression pattern of *sdv-1* and *sdv-4* in these strains, however, did not vary materially when analyzed from different RNA preparations during time course experiments, and these genes were therefore analyzed further.

Figure 6A shows analysis of *sdv-1* expression. Although the absence of functional MAT A-1 (*mat A-1*^{m92}) in strain NLG R4-50 resulted in a dramatic decrease in transcript

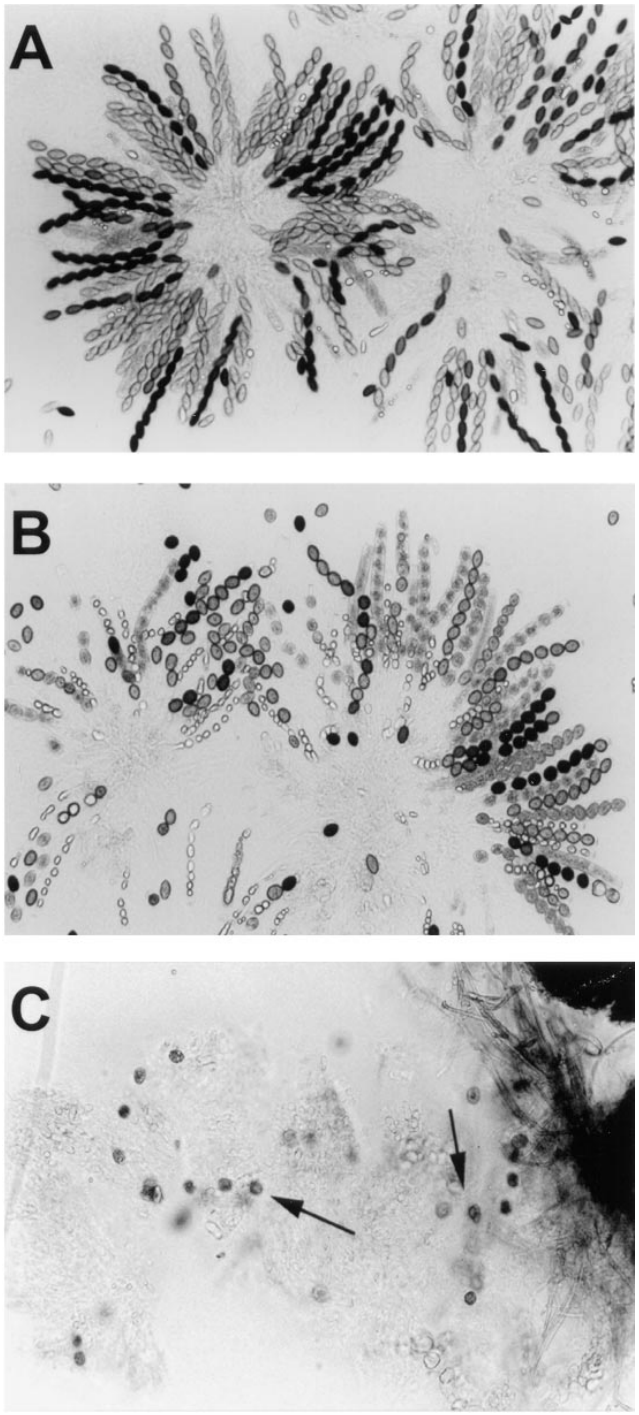

Figure 5.—Perithecial squashes of crosses of *mat A*, NLG R5-39 *(mat A-3*^{m2} *thr-2)*, and NLG R4-16 (*mat A-2*^{m3} *mat A-3*$^{m3)}$ to *R a*. (A) A rosette of asci with spindle-shaped ascospores from a wild-type cross. (B) A rosette 8 days after fertilization in a cross between *mat A-3*^{m2} and *R a* that shows production of round spores. (C) A cross between *mat A-2*^{m3} *mat A-3*^{m3} and *R a*. Only a small number of perithecia from this cross produce a few asci that bear eight ascospores. Arrows indicate round ascospores from asci.

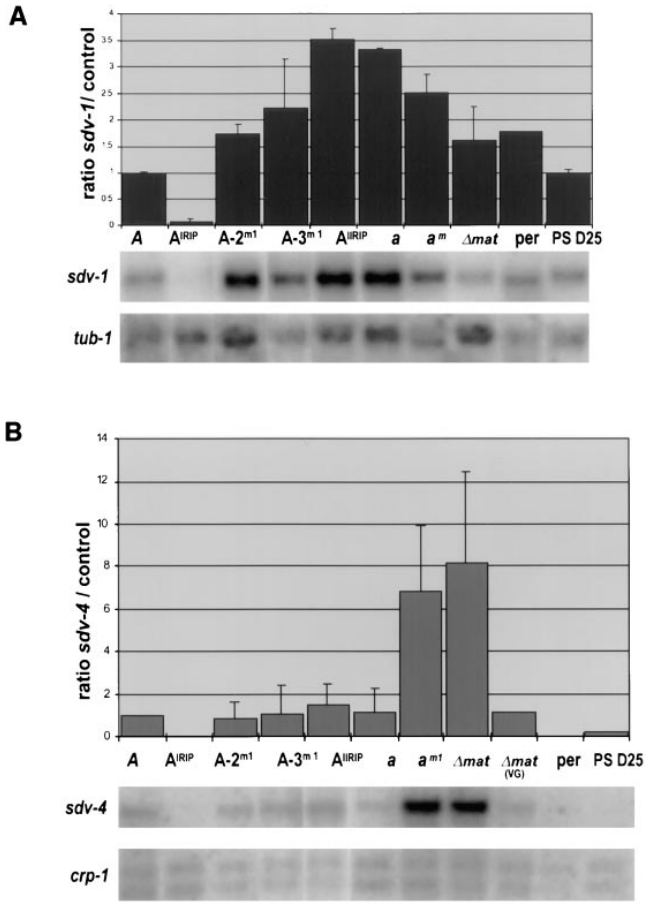

Figure 6.—Expression of *sdv-1* and *sdv-4* in *mat A*, *mat a*, and various mating-type mutants grown in crossing media (Westergaard and Mitchell 1947). Results are presented as the ratio of radioactivity emitted by hybridization to probes from the *sdv-1* or *sdv-4* genes compared to that emitted by hybridization to probes from the constitutive genes *tub-1* (Orbach *et al.* 1986) and/or *crp-1* (Kreader and Heckman 1987). All comparative analyses were done relative to the ratio obtained with RNA of wild-type *A* control (normalized to 1). Standard deviation bars that were calculated from at least three separate experiments show variability. (A) Expression levels of *sdv-1* in the various strains. (B) Expression levels of *sdv-4* in the various strains. A, FGSC 2489; A^{IRIP}, NLG R4-50; $A\text{-}2^{m1}$, NLG R5-38; $A\text{-}3^{m1}$, NLG R4-66; A^{IIRIP}, NLG R4-16; a, FGSC 532; a^{m1}, FGSC 4565; Δmat, RLM 44-02; D25, *mat A*/*mat a* partial duplication strain PS D25 (see Table 1); per, perithecial RNA from a wild-type cross; VG, culture grown in vegetative medium (Vogel 1964).

levels of *sdv-1*, transcripts for *sdv-1* were observed in the Δ*matA* strain, *i.e.*, in the absence of MAT A-1, MAT A-2, and MAT A-3. These observations indicated that either MAT A-2 or MAT A-3 or both repress the expression of *sdv-1* when *mat A-1* is inactivated by RIP. The transcript level of *sdv-1* in both NLG R5-38 (*mat* $A\text{-}2^{m1}$) and NLG R4-66 (*mat* $A\text{-}3^{m1}$) strains was higher than in a wild-type *mat A* strain (about twofold). In NLG R4-16 (*mat* $A\text{-}2^{m3}$ *mat* $A\text{-}3^{m3}$), the transcript level of *sdv-1* was more than threefold higher than in a *mat A* strain, consistent with our hypothesis that MAT A-2 and MAT A-3 modulate the expression of *sdv-1* in conjunction with MAT A-1. Because similar levels of *sdv-1* transcript were observed in FGSC 532 (*mat a*) and FGSC 4565 (*mat* $a\text{-}1^{m1}$), a functional MAT a-1 is not required for the expression of *sdv-1* in a *mat a* context. Transcript levels of *sdv-1* were typically about threefold higher in wild-type *a* strains than in *A* strains, indicating that factors in the *mat a* and *mat* $a\text{-}1^{m1}$ strains function to express *sdv-1* in the absence of *mat A-1*. Transcripts from *sdv-1* were also detected in RNA from perithecia and from a *mat A*/*mat a* duplication strain (PS D25) grown in crossing medium.

The analysis of *sdv-4* transcripts is shown in Figure 6B. Similar to the case with *sdv-1*, *sdv-4* transcripts were undetectable from RNA isolated from the NLG R4-50 (*mat* $A\text{-}1^{m92}$), indicating that a functional *mat A-1* is required for *sdv-4* expression. However, *sdv-4* transcripts were observed in RLM 44-02 (Δ*matA*) at a level approximately sevenfold higher than that observed in a wild-type *mat A* strain. Thus, as with *sdv-1*, either MAT A-2 or MAT A-3 (or both) must repress the expression of *sdv-4* in the absence of MAT A-1. Transcript levels of *sdv-4* were similar to those of a *mat A* strain in the NLG R5-39 (*mat* $A\text{-}2^{m1}$), NLG R4-66 (*mat* $A\text{-}3^{m1}$), and NLG R4-16 (*mat* $A\text{-}2^{m3}$ *mat* $A\text{-}3^{m3}$) strains, unlike what was observed in the Δ*matA* strain. Expression levels of *sdv-4* in FGSC 4565 (*mat* $a\text{-}1^{m1}$) were elevated as compared to a wild-type *mat a*, but they were similar to those of the RLM 44-02 (Δ*matA*), indicating the presence of factors in these mutants that increase *sdv-4* transcript levels in the absence of MAT a-1 or MAT A-1, MAT A-2, and MAT A-3. Interestingly, *sdv-4* was expressed in RLM 44-02 (Δ*matA*) grown in vegetative medium at levels similar to those found in a *mat A* strain (FGSC 2489) grown in crossing medium. In contrast, the expression of *sdv-4* is completely suppressed in a wild-type *mat A* strain grown in vegetative medium (Nelson and Metzenberg 1992; our unpublished results) and was indeed a prerequisite for its isolation.

DISCUSSION

The Δ*matA* strain described in this study was morphologically similar to the wild type in vegetative characteristics, but it did not mate as either a *mat A* or *mat a* strain, even though female and male reproductive structures were formed. In this respect, the *N. crassa* Δ*matA* strain resembles mating type deletion mutants from other ascomycete species, such as *P. anserina* and *Cochliobolus heterostrophus*, *i.e.*, Δ*mat* strains are morphologically indistinguishable from the wild type during vegetative growth, but they are incapable of mating with strains of either mating type (Coppin *et al.* 1993; Wirsel *et al.* 1996). Fertility is restored to the Δ*mat* mutants of *P. anserina* and *C. heterostrophus* by the introduction of ectopic copies of mating type sequences. In *N. crassa*, Δ*matA* transformants containing either the *a* or *A* idio-

morph in an ectopic position will mate as a female or a male, but they fail to produce ascospores (our unpublished results). The reason for the failure of ectopic copies to fully complement a mating type deletion in *N. crassa* is unknown. Apparently mating-type regulation of sporulation in *N. crassa* differs from that in *P. anserina* and *C. heterostrophus*.

As with the *N. crassa* Δ*matA* mutant, the *mat A-2*^{m1}, *mat A-3*^{m1}, or *mat A-2*^{m3} *mat A-3*^{m3} mutants do not have an altered vegetative growth phenotype. All of the *mat A* mutants produce a large number of conidia and form normal protoperithecia when grown in crossing medium (nitrogen-limiting conditions). Unlike the Δ*matA* mutant, however, the *mat A-2*, *mat A-3*, and *mat A-2 mat A-3* mutants mate as a *mat A* strain and are vegetatively incompatible with *mat a* strains; these functions are attributable to *mat A-1* (Glass *et al.* 1990; Staben and Yanofsky 1990). The fact that the *mat A-2*^{m1}, *mat A-3*^{m1}, and *mat A-2*^{m3} *mat A-3*^{m3} mutants retain *mat A-1* function, that all three contain a transcript for *mat A-1*, and that RIP of *mat A-1* during the sexual cycle does not affect ascosporogenesis (Glass and Lee 1992) indicates that *mat A-1* is functional in all three mutants. The *mat A-2*^{m1} and *mat A-3*^{m1} mutants were shown by DNA sequence analysis to contain stop codons in the *mat A-2* and *mat A-3* ORFs, respectively, and transcripts of the mutated genes were undetectable in RT-PCR experiments. The absence of the transcripts could be caused by either lack of transcription or mRNA instability. In the *S. cerevisiae* phosphoglycerol kinase gene, a nonsense codon present within the first two thirds of the coding region led to mRNA instability and degradation, a phenomenon termed nonsense-codon-mediated mRNA degradation (Peltz *et al.* 1994). The promoter region of *mat A-2* and *mat A-3* in the *mat A-2*^{m3} *mat A-3*^{m3} mutant showed mutations in putative transcriptional regulatory regions of both genes, and transcripts for *mat A-2* and *mat A-3* were absent in the *mat A-2*^{m3} mat A-3^{m3} mutant.

In contrast to the case with *mat A-2* and *mat A-3* single mutants, the *mat A-2*^{m3} *mat A-3*^{m3} mutant displays reduced fertility in crosses with *mat a* strains. Similar to *mat A-2* or *mat A-3* mutants, however, the few asci that are produced in crosses between *mat A-2*^{m3} *mat A-3*^{m3} and *mat a* segregate mating-type 1:1 (Glass and Lee 1992) and form biparental asci. Our results are in contrast to those observed with *P. anserina* mutants in *SMR1* (*mat A-2* homolog) and *SMR2* (*mat A-3* homolog). Crosses between a *mat*+ strain and an *SMR1* or *SMR2* mutant result in the production of uniparental progeny of only *SMR1* or *SMR2* mutant genotype, respectively (Zickler *et al.* 1995). Interestingly, crosses between *SMR1* mutants and an *FPR1* mutant (*mat a-1* homolog) result in a restoration of predominantly biparental asci, indicating that *SMR1* is not the only factor involved in compartmentation of opposite mating-type nuclei in the crozier (Zickler *et al.* 1995). It is also possible that *mat A-2* and *mat A-3* are involved in the recognition of opposite mating-type nuclei during sexual development. Because the *mat A-2*^{m1}, *mat A-3*^{m1}, and *mat A-2*^{m3} *mat A-3*^{m3} mutants form only biparental asci in crosses to *mat a*, however, factors other than MAT A-2 and MAT A-3 must be sufficient for compartmentation of opposite mating-type nuclei in the crozier. The fact that the few progeny of crosses with the *mat A-2*^{m3} *mat A-3*^{m3} mutant segregate normally for mating-type indicates that it is not an occasional spore that can survive from one ascus, but rather, it is an occasional successful compartmentation of opposite mating-type nuclei into a crozier (Glass and Lee 1992). An alternative hypothesis to the one above is that MAT A-2 and MAT A-3 are involved in orchestrating the multiple mitotic divisions of opposite mating-type nuclei in the ascogenous hyphae before the migration of opposite mating-type nuclei into the crozier (for review of asci development, see Raju 1992). In the *mat A-2*^{m3} *mat A-3*^{m3} mutant, this process—and not compartmentation of opposite mating-type nuclei into the crozier—may be impeded.

Our results suggest that *mat A-1* and *mat a-1* are the critical factors for both mating and sexual development in *N. crassa*; *mat A-2* and *mat A-3* increase the efficiency of the process but are not essential for the production of ascospores. Mating-type genes homologous to *mat A-1* and *mat a-1* have been found in many filamentous ascomycetes (Arie *et al.* 1997). *mat A-2* and *mat A-3* homologs, however, are absent or nonfunctional in other ascomycetes such as *C. heterostrophus* and related species, as well as in *Neurospora terricola* (Turgeon *et al.* 1993, 1995; T. Vellani and L. Glass, unpublished results), suggesting that other means of increasing ascospore production have evolved in these fungi.

Direct target genes of the *N. crassa*, *P. anserina*, or *C. heterostrophus* mating-type polypeptides have not been reported. Nelson and Metzenberg (1992) isolated a number of putative target genes of *mat A-1* by subtractive hybridization. Mutational analysis of two of the *sdv* genes (*asd-1* and *asd-2*) resulted in recessive mutants that displayed blocks during sexual development. Mutational analysis of the remaining *sdv* genes by RIP did not result in mutants with a detectable vegetative or sexual phenotype (Nelson and Metzenberg 1992). Additional analyses showed that the expression patterns of some of the *sdv* genes are erratic (Nelson *et al.* 1997b; this study). The two genes analyzed in this study, *sdv-1* and *sdv-4*, however, are promising candidates as target genes for regulation by the mating-type polypeptides. Analyzing genes that are preferentially expressed during sexual development may also identify additional mating-type target genes. As part of the *N. crassa* genome project, several clones specific for conidial, mycelial, and sexual stages have been identified (Nelson *et al.* 1997a). In addition, several genes from *N. crassa* that appear to be transcriptional regulators of vegetative and sexual development have been isolated recently (Aramayo *et al.* 1996; Ballario *et al.* 1996; Yamashiro *et al.* 1996;

Linden and Macino 1997). Expression studies using strains with mutations at these genes may also be of help in identifying additional regulators of *sdv* gene expression.

In the analysis of *sdv* gene expression in the *N. crassa mat* mutants, it was shown that both *sdv-1* and *sdv-4* required MAT A-1 for expression, but both *sdv-1* and *sdv-4* transcripts were observed in the Δ*matA* strain, which lacks MAT A-1, MAT A-2, and MAT A-3. These data indicate that MAT A-2 and/or MAT A-3 (or the products they regulate) repress the expression of *sdv-1* and *sdv-4* unless MAT A-1 is also present. A simplistic model consistent with our data is that MAT A-1, MAT A-2, and MAT A-3 form a complex that regulates the expression of *sdv-1* and *sdv-4* in a *mat A* context under crossing conditions. In *S. cerevisiae*, direct protein-protein interactions of the mating-type polypeptides MATα2 and MAT**a**1 have been described (Dranginis 1990); the **a1**/α2 heterodimer signals that karyogamy has occurred and is involved in the regulation of entry into meiosis (Goutte and Johnson 1988). The mating type polypeptides of the *P. anserina mat*− idiomorph, FMR1 and SMR2, showed an interaction in assays using the yeast two-hybrid system (Zickler *et al.* 1995; R. Debuchy, personal communication). In *N. crassa*, interactions may also occur between polypeptides from the same mating type, *i.e.*, MAT A-1, MAT A-2, and MAT A-3, and may be important in maintaining opposite mating-type nuclear identity before karyogamy.

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