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Mating in *Cryptococcus neoformans*

Tracey Dee Elisabeth Moore

ABSTRACT

Cryptococcus neoformans is a basidiomycetous human fungal pathogen. Mating type in *Cryptococcus neoformans* is controlled by one locus with two alleles, *MAT**a*** and *MAT*α. Little is known on a molecular level about the progression of *C. neoformans* through its sexual cycle. Nonetheless, an understanding of mating is important since the α-mating type is more virulent than **a**-mating type. To begin a molecular and genetic study of mating in *C. neoformans*, a set of auxotrophic and drug resistant strains in a common genetic background was constructed. The *MAT*α locus was isolated in order to identify the genetic differences between mating types and their contribution to virulence.

The *MAT*α locus was found to be a 35-45 kb region present only in *MAT*α strains. A 2.1 kb *XbaI/HindIII* fragment was identified that directs filament formation in starved *MAT**a*** cells, but not in *MAT*α cells. This fragment contains a gene, designated *MF*α, encoding a pheromone precursor with similarity to the lipoprotein mating factors found in *Saccharomyces cerevisiae, Ustilago maydis* and *Schizosaccharomyces pombe*. *MAT**a*** cells transformed with the *MF*α gene can transcribe, modify, and secrete the *MAT*α pheromone, suggesting a common mechanism for these processes in both cell types. These results also suggest that the production of pheromone is under a type of nutritional control common to both cell types.

To identify genes in *C. neoformans* involved in mating, twenty-seven nonmating and non-filament forming (Nmf) mutants were isolated. Four

mutants have undergone progeny analysis. One mutant has a defect in the *MAT**a*** locus (*nmf1*), another has a *MAT**a*** specific defect (*nmf2*). Two others produce a non-specific Nmf phenotype (*nmf3*, Nmf64). The *nmf1* mutant forms filaments following transformation with the *MAT*α pheromone gene. These results are consistent with the *nmf1* strain containing a defect in the *MAT**a*** pheromone gene.

These studies have provided information about cell-cell signaling and the function and control of mating type genes in *C. neoformans*. Both the cloned mating type locus and the Nmf mutants can be expected to provide more information about mating and possibly virulence in *C. neoformans* in the future.

Jeffrey C. Edman M.D.

Chairman, Thesis Committee

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CHAPTER ONE: INTRODUCTION

A. *C. NEOFORMANS*-BACKGROUND

Disease. *Cryptococcus neoformans* is a human fungal pathogen which causes a serious and often fatal meningitis in AIDS and other immunocompromised patients (Rippon 1988). Since the first clinical isolates of *C. neoformans* were found in patients with cancer, the fungus was assumed to be cancer causing, and therefore was given the species name neoformans (Rippon 1988). The primary infection usually occurs in the lungs following inhalation of the organism, most likely the spore form. The pulmonary infection is usually sub-clinical and almost always resolves spontaneously. Cryptococcal infections which arise as complications of other debilitating conditions may become systemic, most often traveling to the central nervous system and presenting as a meningeal infection. *C. neoformans* is unique among pathogenic fungi in that it produces a thick polysaccharide capsule. Cryptococcal meningitis can be diagnosed by the presence of an encapsulated yeast in the cerebrospinal fluid (CSF), which is detected by placing a CSF sample in India ink in which the capsule displaces the ink particles to give a distinctive halo.

Treatment. The antifungal agents used to treat cryptococcal infections belong to three classes: polyene antibiotics (amphotericin B), pyrimidine analogs (5-FC), and ergosterol biosynthesis inhibitors (fluconazole) (Sugar, Stern et al. 1990, Bossche, Willemsens et al. 1987). Amphotericin B disrupts cell membranes by interacting with the sterol portion of the fungal cell membrane and causing changes in its permeability and fluidity. These perturbations affect the function of the membrane itself and the enzymes

found within. Amphotericin B has a higher affinity for ergosterol than cholesterol. Ergosterol is the primary sterol found in fungal cell membranes. Amphotericin B will also bind to the cholesterol component of animal cells resulting in the many toxic side effects observed with this agent. Prior to the discovery of amphotericin B, cryptococcal meningitis had a 80 to 90 % fatality rate (Sande and Mandell 1980). The prodrug 5-FC is taken up by cells and converted to 5-FU by cytosine deaminase, an enzyme that occurs only in fungi and bacteria. The lack of a mammalian cytosine deaminase accounts for the low host toxicity of this agent. However, there is a high frequency of resistance to 5-FU. Amphotericin B and 5-FU are often used together to minimize both the toxicity of amphotericin B and the resistance to 5-FU. The azole antifungals interfere with ergosterol biosynthesis by inhibiting the cytochrome P-450-dependent 14α-demethylation of lanosterol. The lack of ergosterol and/or the accumulation of 14α-methylsterols causes the fungal membranes to be leaky. The altered sterol composition of the membrane also affects the enzyme chitin synthase, resulting in the uncoordinated synthesis of chitin. The azoles are effective and well-tolerated by patients in most cases (Sugar, Stern et al. 1990).

Life cycle. *C. neoformans* is a heterothallic basidiomycetous yeast with two mating types, *MAT**a*** and *MATα* (Kwon-Chung 1976, Kwon-Chung 1975). *C. neoformans* has a well-defined life cycle in which mating occurs only between opposite mating types (Figure 1-1). The haploid *MAT**a*** or *MATα* yeast cells divide vegetatively by budding and are induced to mate by starvation. Haploid yeast cells of opposite mating types fuse during mating and differentiate into the dikaryotic hyphal phase. Basidia form at the hyphal tips, where nuclear fusion, meiosis, and spore formation occur.

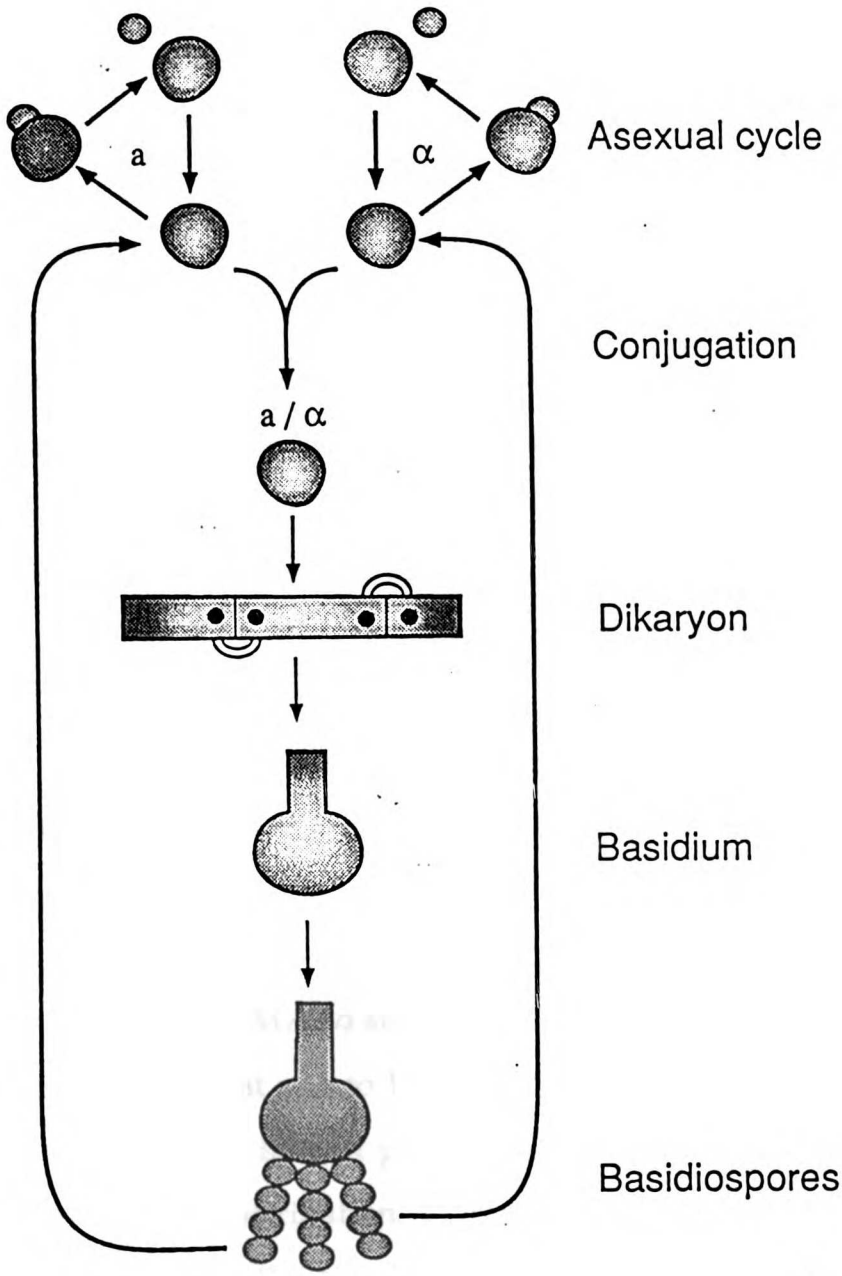

Figure 1-1. *Cryptococcus neoformans* can divide vegetatively by budding, or when starved, cells of opposite mating types mate and form dikaryotic hyphae. The basidia form at the hyphal tips inside of which meiosis and spore formation occur (Whelan 1987).

Mating type. Mating type in *C. neoformans* is determined by a single locus (MAT), which has two alleles, *MAT**a*** and *MAT*α (Kwon-Chung 1976). Basidiomycetes with a single mating type locus are uncommon but not unprecedented. For example, *Ustilago violacae* (anther smut) is also bipolar having only two mating types, **a1** and **a2** (Day and Garber,1988). However, two mating type loci are more common. *U. maydis* (corn smut) has two mating type loci: the **a** locus with two alleles, and the **b** locus with 25-30 alleles (Banuett 1992). *Tremella* (jelly fungi) species have a similar system to *U. maydis* (Wong and Wells 1985). The higher basidiomycetes which form complex fruiting bodies to house their basidia, such as *Schizophyllum commune* and *Coprinus cinereus*, often have two or more mating type loci (Casselton 1978).

With a single locus-two allele system, one would expect the mating types to occur in equal frequency. In the laboratory, microdissection analysis of progeny isolated from a cross fall equally into *MAT**a*** and *MAT*α classes. In nature, however, the distribution of mating types in the strains isolated is strongly biased in favor of *MAT*α strains (Whelan 1987). Strains isolated from soil and pigeons occur at a 40 to 1 (α/a) ratio and clinical isolates have a 30 to 1 bias (α/a). Another human pathogen, *Histoplasma capsulatum*, also has a biased mating type distribution (Maresca and Kobayashi 1989). What forms the biologic basis for the bias in either *C. neoformans* or *Histoplasma capsulatum* is unknown. No basic differences between mating types in viability such as growth rate or ability to sporulate have been reported. The association of increased virulence with an congenic *C. neoformans MAT*α strain has recently been established, although the molecular basis for this

increase in virulence among *MAT*α strains is still unknown (Kwong-Chung, Edman et al. 1992).

Habitat. *C. neoformans* can be isolated from pigeon nests, roosts, and droppings, but rarely infect the pigeon (Whelan 1987). The organism does survive passage through the pigeon, however, whose average body temperature is 42° C. At this temperature, *C. neoformans* can survive but not grow. The actual habitat for *C. neoformans* may be the grains or grasses that the birds feed upon. Four serotypes have been isolated: A, B, C, and D (Whelan 1987)). Serotypes A and D have a worldwide distribution, are commonly found in pigeon habitats worldwide, and were given the variety name *C. neoformans neoformans*. The B and C serotypes are found associated with eucalyptus trees, have a tropical and subtropical distribution, and were given the variety name *C. neoformans gattii*.

Virulence. Genetic analysis of *C. neoformans* has identified four factors which are associated with virulence: ability to grow at 37° C, a polysaccharide capsule, production of melanin, and α–mating type. Thermotolerance is an obvious prerequisite for the infection of mammalian hosts, and the capsule and melanin appear to offer some protection from host defense mechanisms (Flegel 1981, Kwon-Chung and Rhodes 1986, Jacobson and Emery 1991). No morphological or biochemical differences which could account for the difference in virulence have yet been identified between *MAT*a and *MAT*α congenic strains. Therefore, the *MAT* loci must be isolated and analyzed to understand the mating process in *C. neoformans* and the role of α-mating type in virulence.

This study. The study of mating in *C. neoformans var. neoformans* by both genetic and molecular biological methods will be discussed in the

following chapters. In the remaining portion of Chapter 1, what is known about mating in fungi will be discussed. Chapter 2 describes a variety of auxotrophic and drug resistant strains which were isolated, characterized, and constructed in a common genetic background. The isolation of the *MAT*α locus from *C. neoformans*, and the isolation and identification of a pheromone precursor gene in the locus are covered in Chapter 3. Chapter 4 is concerned with the production, isolation, and characterization of sterile (nonmating and nonfilamentous) mutants. A summary and projections are provided in Chapter 5.

B. FUNGAL MATING

Ascomycetes

Saccharomyces cerevisiae-life cycle

Saccharomyces cerevisiae is a single-celled yeast that grows vegetatively by budding (Figure 1-2). Individual cells are *MAT**a***, *MAT*α, or *MAT*α/***a*** cell type (Herskowitz 1988, Fincham, Day et al. 1979). Mating type specific pheromones are used by cells of each mating type to signal their presence to cells of the opposite mating type. The diffusible pheromone or mating factor produced by a *MAT**a*** cell is a dodeca-peptide, **a**-factor (Brake, Brenner et al. 1985, Figure 1-3). The peptide is post-translationally modified and secreted from the *MAT**a*** cell using a cell-type specific peptide transport system (Kuchler, Sterne et al. 1989; Wilson and Herskowitz 1984, Wilson and Herskowitz 1987, Raymond, Gros et al. 1992, Michaelis, Chen et al. 1992). A *MAT*α cell has a receptor encoded by the *STE3* gene that specifically binds **a**-factor specifically (Hagen, McCaffrey et al. 1986). Likewise, *MAT*α cells secrete

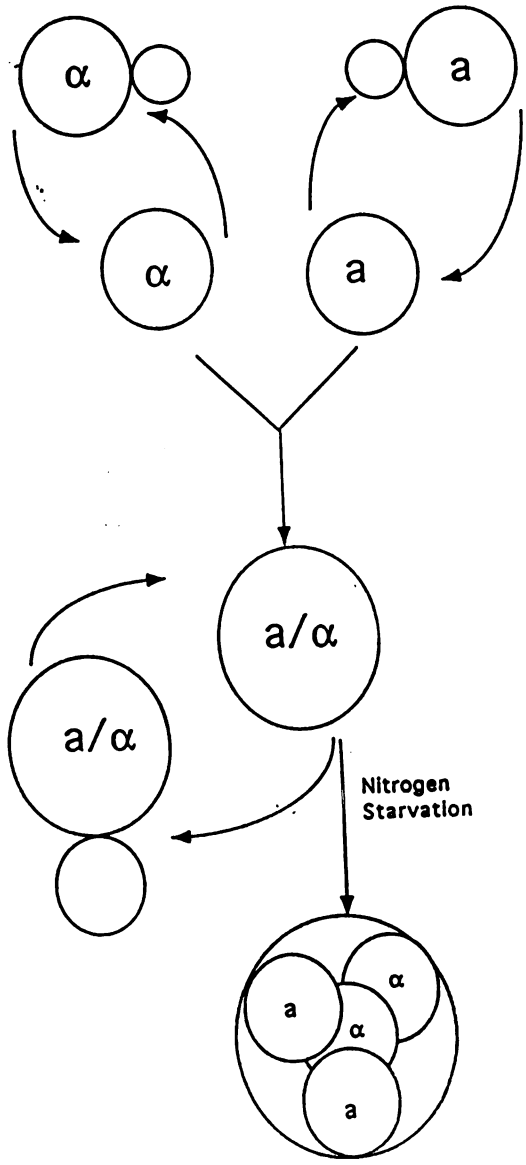

Figure 1-2. The budding yeast *Saccharomyces cerevisiae* has two mating types, which will mate when exposed to cells of the opposite mating type. The resulting diploid a/α cell will continue to divide by budding, until starvation for nitrogen triggers the cells to undergo meiosis and sporulation (Herskowitz 1988).

(a.)

```
Cn-MFα..MDAFTAIFTTFTSAATSSSEAPR--------N-QEAHPGGMTL-CVIA
Sc-MFa1.MQPSTA--T-AAPKEKTSSEKKD--------N-YIIKGVFWDPACVIA
Sc-MFa2.MQPITTASTQATQKDK-SSEKKD--------N-YIIKGLFWDPACVIA
Sp-MFm1.MDSMANSVSSSSVVNAGNKPAQTLNKTVK--N-YTPKVPYM---CVIA
Sp-MFm2.MDSIATNTHSSSIVNAYNNNPTDVVKTGNIKN-YTPKVPYM---CVIA
Um-MFa1.MLSIFAQ-TTQTSASEPQQPTAPQGR-----N-DNGSPIGYSS-CVVA
Um-MFa2.MLSIFETWAAAAPVTVAETQQAS--------N-NENRGQPGYY-CLIA
```

(b.)

```
Sc-MFa1...YIIKGVFWDPAC
Sc-MFa2...YIIKGLFWDPAC
Sp-MFm1...YTPKVPYM---C
Sp-MFm2...YTPKVPYM---C
Rt-A10....YPQISWTRNG C
Tm-A10....QHDPSAPGNGYC
Tm-a13....GGGGRGDPSGVC
Tb-A13-1.DGGRDPGASSYGC
Tb-A13-2.DGSRDPGASSYGC
```

Figure 1-3. Fungal pheromone gene (a) and peptide (b) sequences. Amino acid alignment of: both copies of the MFa and MFm precursors from *S. cerevisiae* and *S. pombe* respectively, and pheromone precursors from both *U. maydis* mating type alleles, a1 and a2 are shown with the *C. neoformans* MFα pheromone precursor. In (b) mature sized but unmodified peptide sequences are compared.

a pheromone, α-factor, a 13 amino acid peptide which is processed and secreted using the standard secretory pathway (Kurjan and Herskowitz 1982, Julius, Brake et al. 1984). The *MATa* cells also contain a receptor, the product of the *STE2* gene, which is specific for α-factor (Blumer, Reneke et al. 1988). Pheromones binding to their respective receptors on the membrane of the opposite mating type cell induces a cascade of events, such as cell division arrest, agglutination, induction of specific transcripts and cell fusion, all of which ultimately lead to cell and nuclear fusion (Figure 1-4, Ref. Marsh, Neiman et al. 1991, Kurjan 1992). Cell cycle arrest in the G1 phase ensures that only a single copy of the *MATa* or *MATα* genome is present at the time of cell fusion. Agglutination is precipitated by binding of the pheromone to its receptor through the production of cell surface-bound glycoproteins and agglutinins that help to bring the cells together to mate. Mating requires the breakdown and reformation of cell membranes and cell walls to form the zygote from the two parent cells. In *S. cerevisiae,* nuclear fusion occurs immediately following cell fusion to form a diploid cell, the form which is most commonly found in nature. Diploid cells neither produce nor respond to pheromones and are incapable of mating. Both diploid and haploid cells are capable of unlimited vegetative growth. Starvation triggers the diploid cell to stop mitotic growth and undergo meiosis and sporulation. Sporulation results in the formation of an ascus which contains four haploid ascospores, two of each mating type.

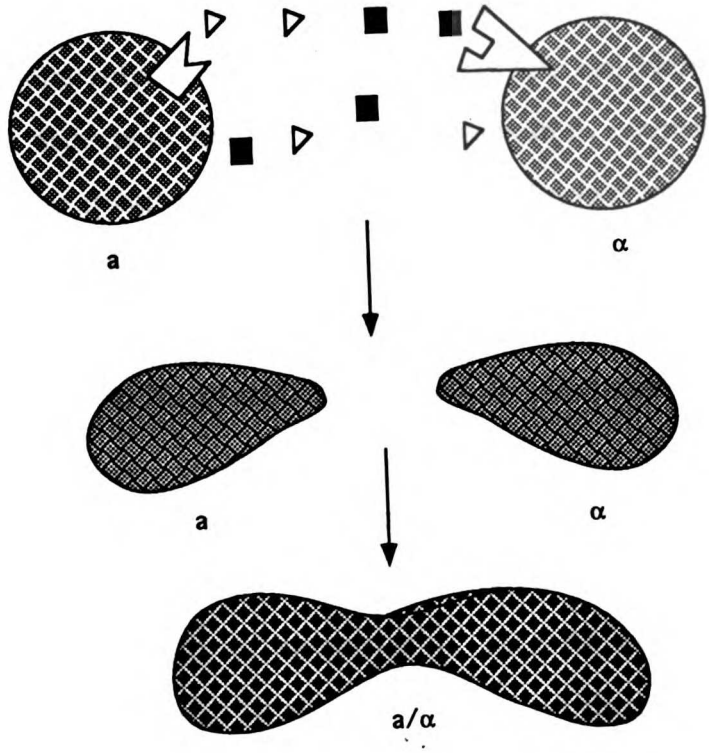

Figure 1-4. Pheromone response in *S. cerevisiae*. Haploid cells of both mating types secrete peptide pheromone and express a receptor for the opposite mating type pheromone on their surface. In response to pheromone binding, the normally oval cells become pear-shaped and form a tip extension in the direction of the opposite mating type cell. The cells fuse at their extension tips to form the diploid a/α cell (Sprague 1991).

S. cerevisiae-MAT loci

The organization and function of the mating-type locus in *S. cerevisiae* has been extensively characterized (for a review see Herskowitz 1989, Figure 1-5). Certain aspects of mating type processes in *S. cerevisiae* will be discussed here in relative detail because much more is known about mating type in *S. cerevisiae* than for other fungi. The specific topics discussed here were chosen to provide a basis for comparison and contrast with the other fungal species: *S. pombe, N. crassa, U. maydis, S. commune, C. cinereus,* and *C. neoformans*.

Mating and cell type in *S. cerevisiae* are controlled by a single locus, the mating type locus (MAT) which has two alleles, *MAT*α and *MAT*$\boldsymbol{a}$. The mating type locus of *S. cerevisiae* is located near the centromere on chromosome III. The *MATa* allele is 642 bp and the *MAT*α allele is 747 bp of unique DNA sequence (Nasmyth and Tatchell 1980). In addition to the expressed allele present at the *MAT* locus near the centromere, there are archival copies of both *MATa* and *MAT*α information near the left and right telomeres of chromosome III, at loci designated *HML* and *HMR* respectively. These loci serve as storage sites of mating type information that usually can be activated only by transposition to the *MAT* locus. This is accomplished by a "cassette" mechanism following a double-strand break produced by the HO endonuclease, the repair of which results in a switch of mating type information expressed at the *MAT* locus (Tatchell, Nasmyth et al. 1981). A cell which is competent to switch mating types is termed homothallic. Cells unable to switch mating type are called heterothallic.

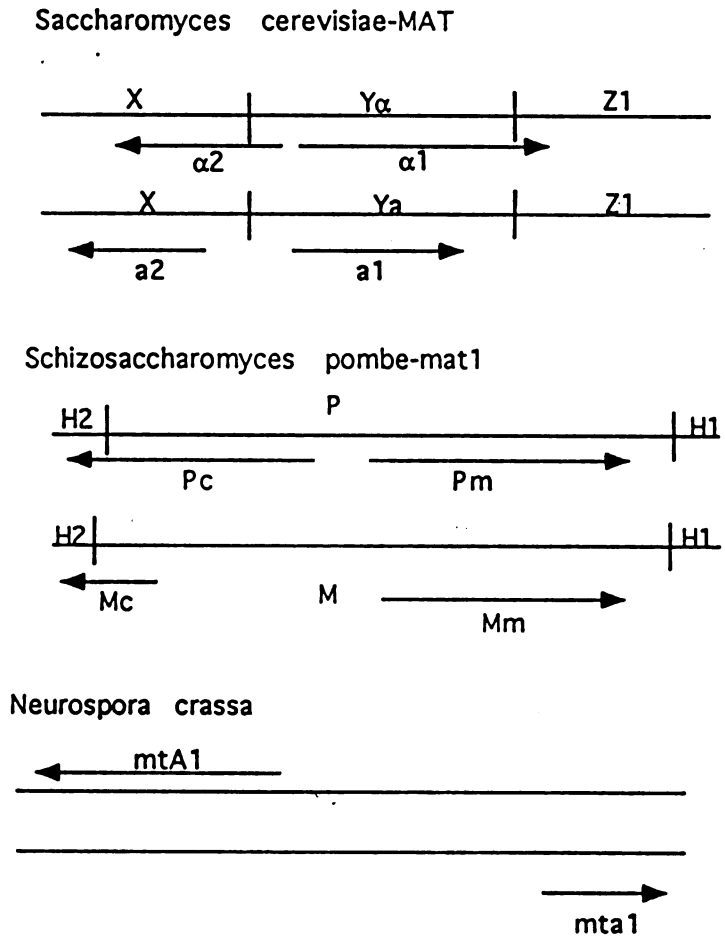

Figure 1-5. The basic structural characteristics of the mating type loci of three ascomycetes: *S. cerevisiae, S. pombe,* and *N. crassa.* Transcripts are identified by arrows in the direction of transcription. Areas of homology between opposite mating type loci are shown with identical alphanumeric symbols. The regions without similarity are Ya and Yα in *S.cerevisiae,* P and M in *S. pombe,* and **A** and **a** in *N. crassa* (Glass and Lorimer 1991).

The three distinct cell types; *MATa*, *MATα*, and *MATa/α*, are dependent on the presence of the *MATa*, *MATα*, or both *MATa* and *MATα* allele(s) respectively (Nasmyth and Tatchell 1980, Tatchell, Nasmyth et al. 1981, Hersksowitz 1989). Both alleles code for regulatory proteins which control expression of cell-type specific gene sets: the *MATa*-specific genes, the *MATα*-specific genes, the haploid-specific genes, and the sporulation-specific genes. The *MATa* allele codes for the **a**1 protein, while the *MATα* allele codes for two divergently transcribed genes corresponding to proteins α1 and α2 (Astell, Ahlstrom-Jonasson et al. 1981). These proteins are responsible for three regulatory activities: α1, α2, and the **a**1/α2 heterodimer (Figures 1-5, 1-6). The α1 protein is a positive regulator of transcription of the α-specific genes (Ammerer, Sprague et al. 1985, Sprague and Herskowitz 1981). The α2 protein is a negative regulator of the **a**-specific genes, while **a**1/α2 forms a novel regulatory protein that turns off the synthesis of the α1 protein, and the haploid-specific genes, including *RME1* (repressor of meiosis) (Dranginis 1990). Meiosis is does not occur in haploid cells and is repressed by the *RME1* product until the cell becomes diploid. These three regulatory proteins determine the cell type characteristics of the cell in which they are expressed: a *MATa* cell will mate with a *MATα* cell, a *MATα* cell will mate with a *MATa* cell, and neither can undergo meiosis. The diploid **a**/α cell will not mate, but it can undergo meiosis and sporulation.

Two other regulatory proteins interact with the mating type polypeptides to form regulatory activities, the MCM1 and Ste12 proteins. The MCM1 protein is present in all three cell types, while the Ste12 protein is present in both haploid cell types, but only at a low level in the **a**/α diploid (Fields and Herskowitz 1985, Smith and Johnson 1992). In *MATa* cells, where

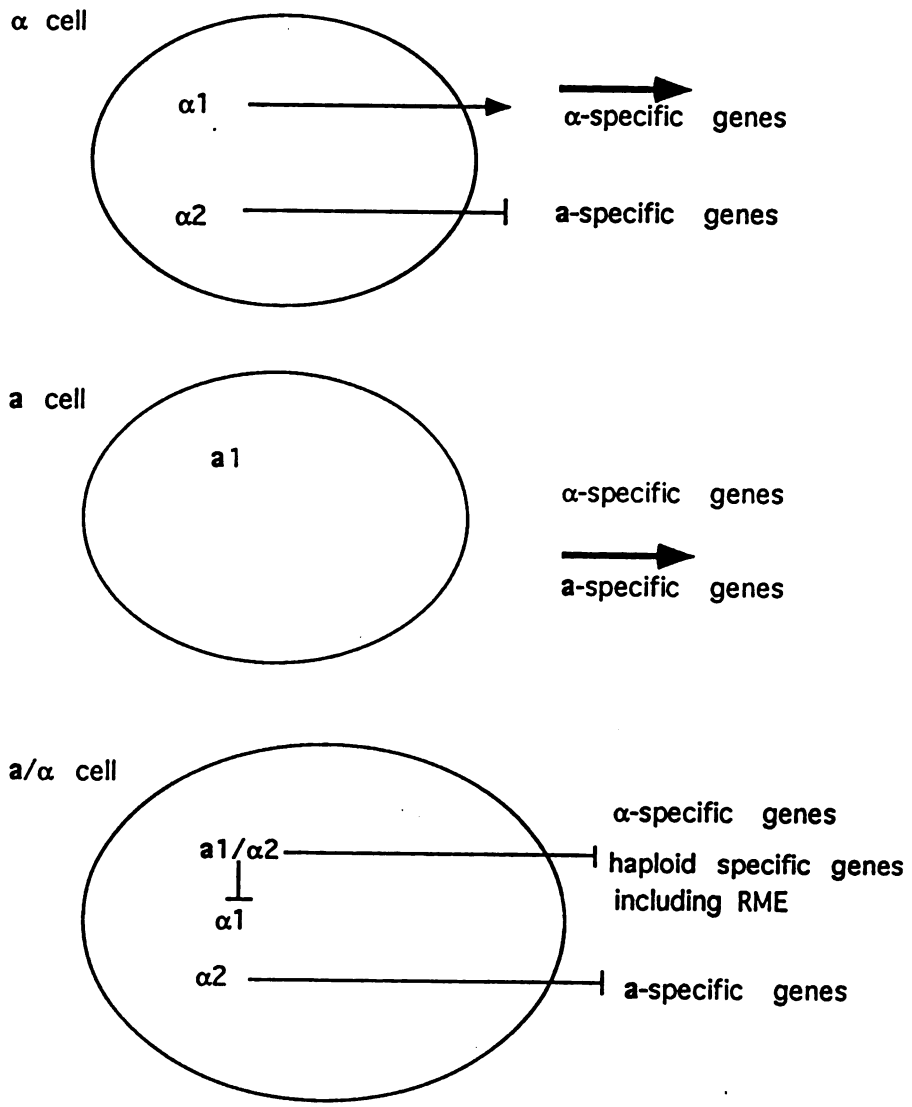

Figure 1-6. A simplified diagram of cell type control in *S. cerevisiae*. The *MATα* cell expresses the α-specific genes because it contains a transcriptional activator of those genes, the α1 protein. The α cell does not express the a-specific genes due to the specific repression of those genes by the α2 protein. In contrast, the *MATa* cell does express the a-specific genes since it does not contain the α2 protein, while the α-specific genes are not expressed due to lack of the activator protein, α1. In the diploid a/α cell the a1 and α2 proteins form a heterodimeric repressor of α1 and haploid specific gene transcription (Herskowitz 1988).

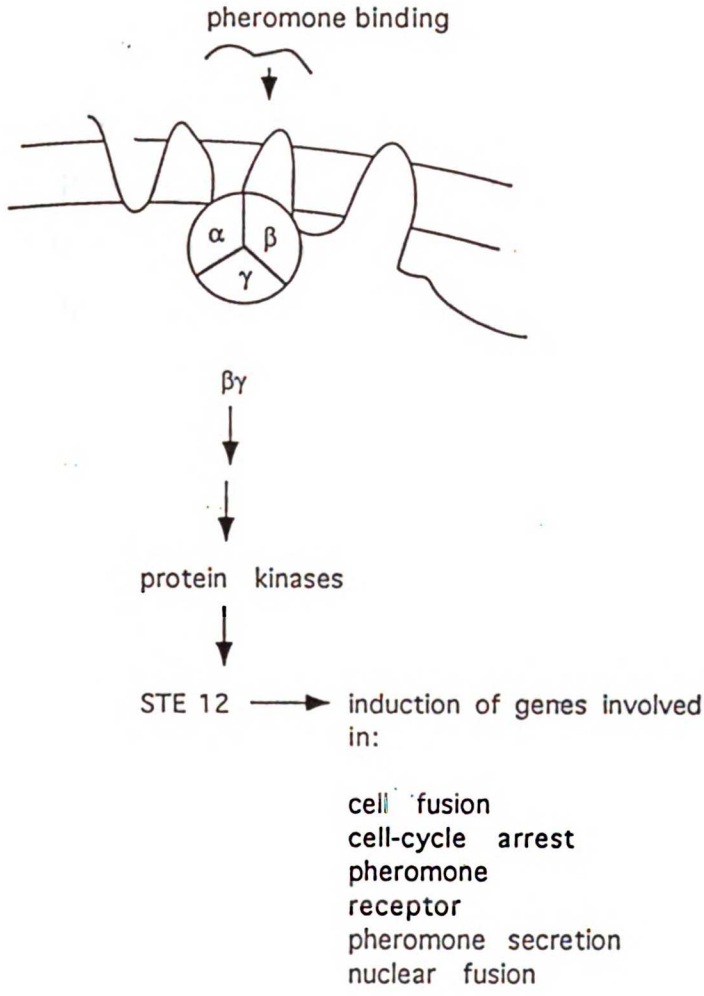

Figure 1-7. The peptide pheromone ligand binds to the extracellular portion of the receptor, which has seven transmembrane domains. The receptor is associated with a trimeric G-protein. Binding of pheromone causes the dissociation of the βγ subunit from the α subunit. The βγ subunit interacts with an as yet undetermined effector. It is known that several protein kinases inhabit the pathway and that the ultimate response of the pathway is the activation of the nuclear transcription factor, Ste12. Ste12 activates the transcription of many genes involved in mating, and nuclear and cell fusion (Marsh, Neiman et al. 1991).

a-cell specific genes are expressed independently of any mating type locus contribution, MCM1 binds to sites upstream of **a**-specific genes and stimulates their transcription. In *MAT*α cells, MCM1 still binds to a sequence upstream of the *MAT**a***-specific genes. However, *MAT*α cells also contain the α2 protein which interacts with MCM1 and DNA upstream of the **a**-specific genes to prevent their transcription. Both the MCM1 and α1 proteins bind to sites upstream of α-specific genes. These proteins bind to DNA and interact to activate transcription of this gene set. The STE12 and MCM1 proteins are thought to interact and the resulting complex is essential for a normal basal level of expression of *MAT**a*** and *MAT*α specific genes. The induced levels of transcription of *MAT**a*** and *MAT*α specific genes, and other genes involved with mating, are strongly dependent upon the STE12 protein which is itself induced upon binding of pheromones to their receptors and may act without MCM1 in this case.

Both pheromone receptors are coupled to a common signal transduction pathway which has been extensively characterized (Marsh, Neiman et al. 1991; Figure 1-7). The receptors themselves are integral membrane proteins which are members a family of receptors with seven transmembrane domains. A characteristic feature of this family of receptors is that they are coupled to heterotrimeric G proteins. When pheromone binds to the receptor, there is a change in Gα, such that it changes from the GDP-bound to the GTP-bound state. This change leads to the release of the $\beta\gamma$ subunit which activates downstream components of the signaling pathway. The activity of the STE12 protein is ultimately induced as a result of the pathway, which leads to differentiation of the cell by increased transcription

of several genes. These genes include those encoding proteins involved in nuclear fusion, pheromone secretion, pheromone recovery, cell fusion, and cell cycle arrest. The genes encoding the pheromones and receptors are also induced. The signaling pathway is similar in both *MAT**a*** and *MAT*α cells with the exception of the receptors.

***Schizosaccharomyces pombe*-life cycle**

Schizosaccharomyces pombe is a single celled yeast which divides vegetatively by medial fission (Refs; Egel, Nielsen et al. 1990, Fincham, Day et al. 1979, Figure 1-8). Homothallism is very common in *Schizosaccharomyces* species. However, both homothallic and heterothallic strains are found. Cells grow by tip extension and divide to form two equal-sized daughter cells. There are two types of haploid cells, the P (or h+), and the M (or h-) mating types. In *S. cerevisiae,* conjugation occurs readily in rich medium while sporulation of the diploid is induced by starvation (Kelly, Burke et al. 1988). In *S. pombe,* conjugation and sporulation are both induced by starvation (Figure 1-8). When starvation of the cells occurs (vegetative growth and mitotic division of the yeast cells are no longer sustained by the medium), mating, meiosis, sporulation, and ascospore formation occur (Egel 1989, Egel, Nielsen et al. 1990). Mixing of cells of opposite mating type, under starvation conditions induces the secretion of pheromones which causes the cells to elongate toward each other (Davey 1992). The cells become arrested in the G1 phase of the cell cycle and agglutinate. Karyogamy and meiosis occur immediately following cell fusion. This results in the formation of a four-spored ascus, which contains two spores of each mating type. The spores do not germinate until they encounter favorable growth conditions. Unlike *S.*

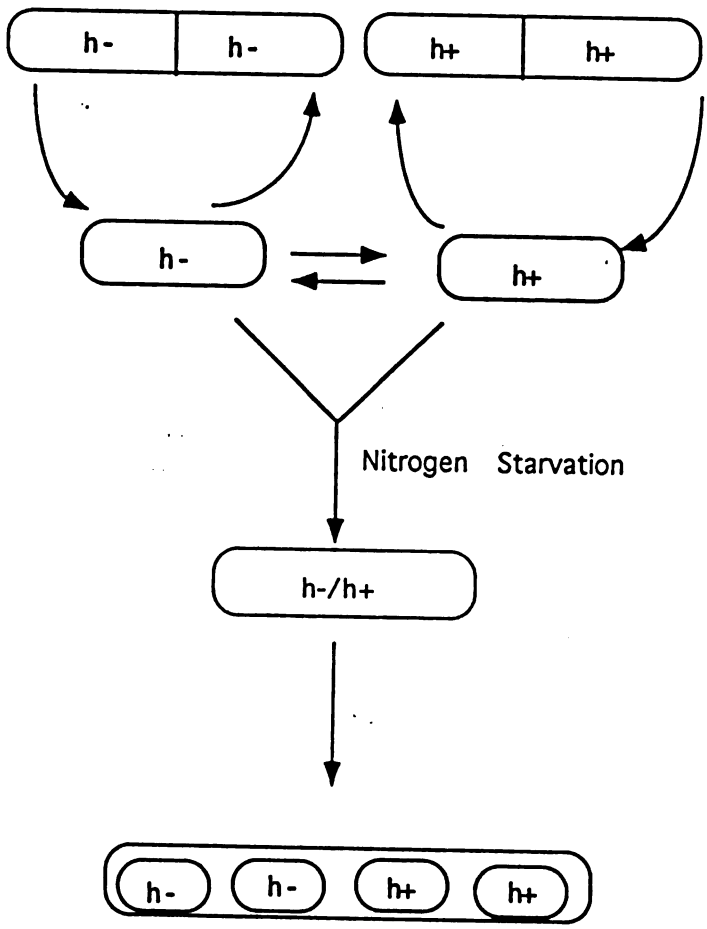

Figure 1-8. In *S. pombe* the haploid cells of both mating types (P or h+, and M or h-) divide by medial fission. The cell lengthens and then divides into two cells. The cells do not mate unless starved for nitrogen. The cells immediately undergo meiosis and sporulation following mating (Egel 1989).

cerevisiae, the diploid stage in *S. pombe* is transient. *S. pombe* normally reproduces vegetatively only as a haploid, although the diploid stage can be propagated in the laboratory by manipulating growth conditions.

***Schizosaccharomyces pombe*-MAT loci**

The mating type locus in *S. pombe* is organized in a similar way to that of *S. cerevisiae* (Refs; Kelly, Burke et al. 1988, Egel, Nielsen et al. 1990; Figure 1-5). This similarity involves the presence of active and archival mating type loci on one chromosome. The *mat1* locus is present of the right arm of chromosome II, and the mating type of a given cell is determined by the mating type sequence that is located at *mat1* (*mat1-P* or *mat1-M*). In common with the *MAT* locus in *S. cerevisiae, mat1 is* the only site at which expression of either mating type allele can occur. The P allele is 1104 bp; the M allele is 1128 bp, and as in *S. cerevisiae* the two mating-type alleles of *S. pombe* are dissimilar in sequence. There are two archival loci present on chromosome II, *mat2* and *mat3*, that each contain a copy of the M and P allele respectively. These archival mating type alleles enable *S. pombe* to switch mating type via transfer of archival *mat* alleles to the active *mat1* locus (Nielsen and Egel 1989, Beach 1983). A 15 kb segment of DNA separates *mat2* from *mat1* and another 15 kb separates *mat2* from *mat3*. Surrounding the *mat2, mat3,* and *mat1* loci are short regions of homology. The archival copies of mating type information at *mat2* and *mat3* are present as direct repeats and are not expressed in haploid or diploid cells. The active cassette at *mat1*, either P or M, gives rise to two transcripts by divergent transcription: Pc and Pi, or Mc and Mi. A constitutive transcript, Pc or Mc, is detectable in the haploid cell

cultures under vegetative growth conditions. Mutations in these genes results in strains unable to mate or conjugate with either P or M cells. It has been postulated that Pc and Mc both encode positive regulators that are responsible for conferring both mating-type and conjugation functions in haploid cells. This is similar to the situation in *S. cerevisiae MATα* cells, which require the α1 protein to activate transcription of the α-specific gene set, and in contrast to *MAT**a*** cells, which require no *MAT* encoded gene to express the **a**-specific gene set. Some of the presumed target genes of Pc and Mc include genes that control the production of pheromones and their receptors. While the Pc and Mc transcripts are detectable under vegetative conditions, an increase in the level of message is observed when cells are shifted from rich to nitrogen-limited medium. The Pc and Mc genes are actively transcribed in both haploid and diploid cells where they are required for mating type, conjugation, meiosis, and sporulation. An additional transcript, Mi or Pi, is induced by starvation and is required for meiosis and sporulation. Although the molecular mechanisms have not been defined, it is known that the products of all four mating type genes are required for meiosis and sporulation. The Pm protein has limited homology with the homeodomain of *MATα2*, suggesting that it may have similar DNA-binding properties (Figure 1-16). Meiosis in *S. pombe* is regulated by the *mei3+* product, which is expressed only in *matP/matM* diploids and binds to a protein kinase encoded by the *pat1+* (*ran1+*) gene, inhibiting its enzymatic activity (McLeod, Stein et al. 1987; McLeod and Beach 1988). The *pat1+* protein kinase is a negative regulator of meiosis through its inhibition of the *mei2* gene, and its inactivation therefore commits the cell to meiosis. Since products from both M and P *mat* loci are required to activate genes required

for meiosis, meiosis consequently limited to functionally diploid cells. Stimulation of the pheromone response pathway is required even after conjugation for meiosis to be successful.

Several components of the *S. pombe* cell signaling pathway have been isolated and identified. The two genes encoding M-factor have been cloned and show similarity to both **a**-factor genes of *S. cerevisiae* and to *mfa1* and *mfa2*, the pheromones from the **a** alleles of *U. maydis* (Refs. Davey 1992, Bolker, Urban et al. 1992; Figure 1-3). The peptide M-factor itself was also isolated and shown to be a nanopeptide which is carboxy-methylated at the carboxy-terminal cysteine residue and S-alkylated, probably with a farnesyl residue. Another gene, *GPA1*, which encodes a protein homologous to the α subunits of mammalian guanine nucleotide-binding proteins has been isolated and is involved in mating and sporulation pathways (Obara, Makafuku et al. 1991). A mutant in *GPA1* is viable and therefore the protein is not essential for vegetative growth, but the mutant strain is sterile and cannot sporulate, which shows it is involved in that developmental pathway.

***Neurospora crassa*-life cycle**

Neurospora crassa is a filamentous ascomycete that grows vegetatively as a single individual with several genetically identical nuclei per cell. Fusion occurs readily between hyphae within an individual colony (Refs. Fincham, Day et al. 1979, Glass and Lorimer 1991; Figure 1-9). Productive vegetative fusion occurs between genetically nonidentical colonies. Hetero-alleleism at any one of the many heterokaryon-incompatibility or *het* loci prevents productive vegetative fusion. Nitrogen starvation will induce either *MATA* or *MAT**a***-mating-type cultures to form spherical aggregates of hyphae that become the female reproductive structures (protoperithecia). Specialized

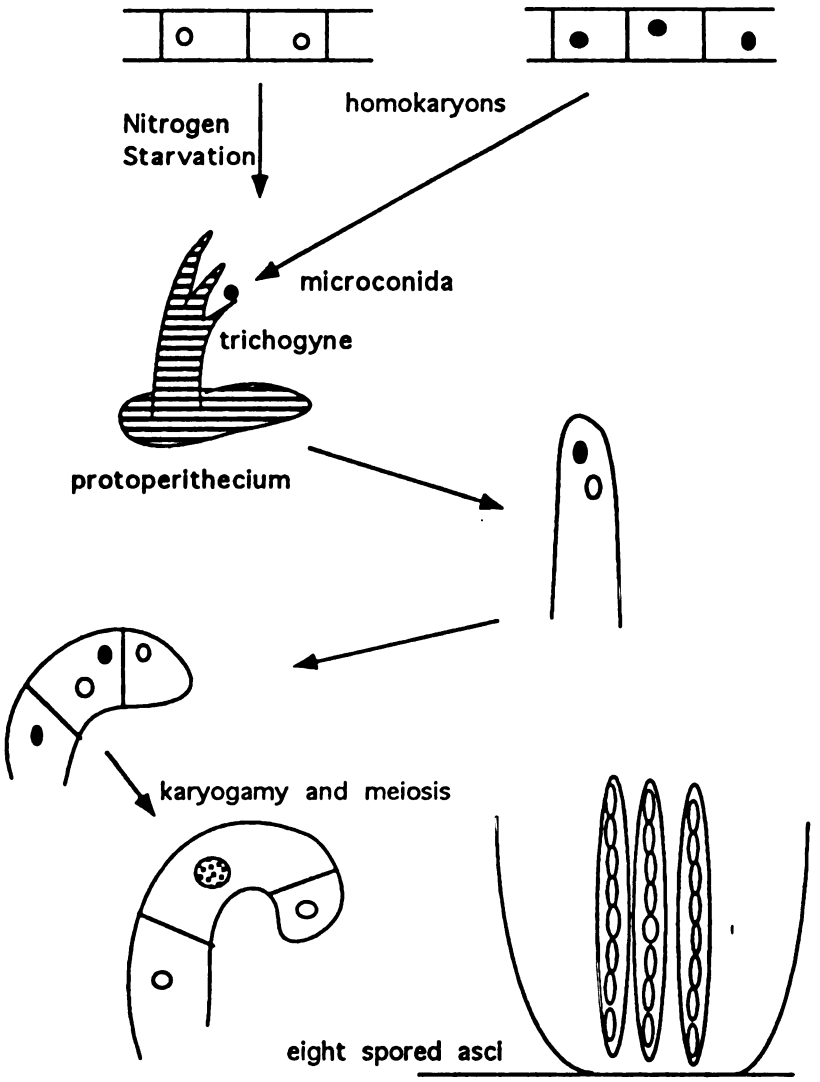

Figure 1-9. The two mating types of *N. crassa,* A and a, grow vegetatively as filamentous hyphae as long as the media sustains their growth. Following nitrogen starvation, either mating type will form the female reproductive structures, the protoperithecia and the trychogyne. Male cells from an opposite mating type strain can then fuse with the trychogyne The paired nuclei undergo 8-10 synchronous divisions before fusing. After karyogamy, meiosis occurs followed by a single mitotic division to form 8 spores in the ascus, four of each type (Fincham, Day et al. 1979).

hyphae (trichogynes) which are slender and receptive extend from these protoperithecia. Mating occurs between these trichogynes and a male fertilizing cell of the opposite mating type. This male cell may be a conidium, a microconidium, or a vegetative hypha of the opposite mating type. In both *S. cerevisiae* and *S. pombe,* meiosis is under nutritional control, although at different steps of the life cycle. In *N. crassa,* nitrogen starvation leads to female differentiation, which will occur in either mating type. It is female differentiation which enables mating to occur. Mating type specific pheromones seem to mediate the ability of trichogynes to attract target cells of opposite mating type. Trichogynes fuse only with male cells of opposite mating type. After fusion of the cells, the male nucleus is transported through the trichogyne and into the protoperithecium. Inside the protoperithecium, the associated nuclei divide synchronously to form a small mass of dikaryotic fertile (ascogenous) hyphae. After 8 to 10 divisions, karyogamy occurs between the associated nuclei in the penultimate cell of each ascogenous hypha. Unlike *S. cerevisiae* and *S. pombe,* cell fusion and nuclear fusion are separated in *N. crassa* temporally and spatially. Like *S. pombe,* however, sporulation occurs directly after nuclear fusion. Nuclear fusion in *N. crassa* is followed immediately by two meiotic divisions and a single mitotic division to form characteristic eight-spored asci, hundreds of which are formed in each perithecium.

***Neurospora crassa*-MAT locus**

Mating type in *N. crassa* is controlled by one locus with two alleles (Glass, Vollmer et al. 1988, Fincham, Day et al. 1979). The *MATA* or the *MATa*

allele is present as a single copy per haploid genome (Figure 1-5). This organism is therefore structurally heterothallic and incapable of mating type switching. The mating type locus is located on the left arm of chromosome I and encompasses a 3235 bp region in the case of the *MATa* allele (Staben and Yanofsky 1990) and a 5301 bp region in the case of the *MATA* allele (Glass, Grotelueschen et al. 1990). One transcript from each allele (**A** and **a**), has been detected under vegetative and mating growth conditions. Mutants which harbor mutations within the transcripts, mt A-1 and mt a-1, are unable to conduct any of the steps involved in the sexual phase of the life cycle. The lack of these mating type proteins is not lethal: vegetative growth is unaffected and the cells still respond to nitrogen starvation by forming protoperithecia. The trichogynes in these mutants strains, however, fail to respond to cells of the opposite mating type in the area. Likewise, mutant conidia fail to attract trichogynes of the opposite mating type. The mt A-1 and mt a-1 products have been postulated to encode positive regulators of sex-specific gene set which confer cell type, for example, genes for pheromones and their respective receptors. The mt A-1 and mt a-1 products in *N. crassa* may provide functions analogous to those of Mc and Pc in *S. pombe* and the α1 protein in *S. cerevisiae* in establishing mating cell type in the haploid phase. How the mt A-1 and mt a-1 products control cell type has not been elucidated at the molecular level. The mt A-1 product is required for meiosis as well as for mating and has some similarity to the αl protein (Figure 1-16). The mt a-1 product, which is predicted to have similarity to the Mc polypeptide of *S. pombe,* is also required after cell fusion. The *N. crassa* mating type locus, along with the unlinked *tol* locus, is also necessary for heterokaryon incompatibility functions.

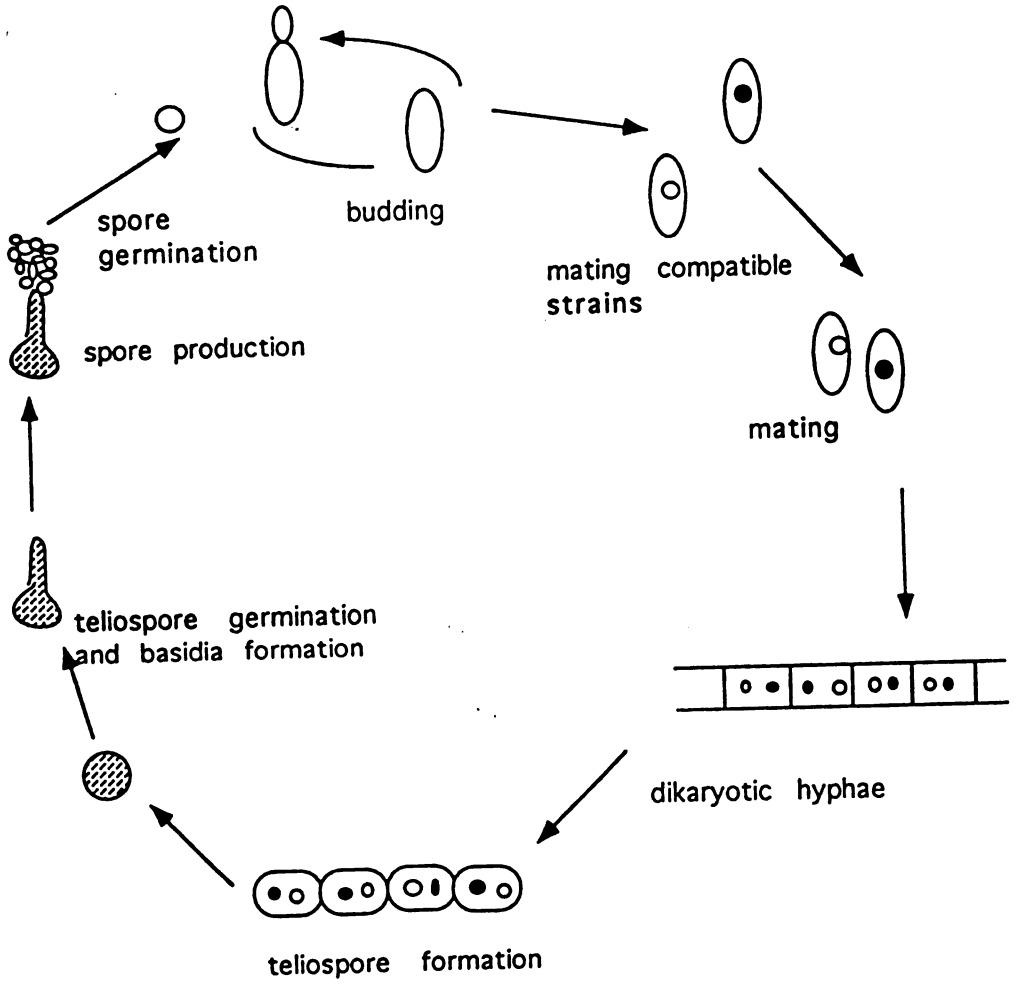

Figure 1-10. The haploid yeast-like cells of *U. maydis* can divide vegetatively by budding or mate to form dikaryotic hyphae. The sexual cycle is transversed only if the cells are passed thru the plant host. Compatible strains mate and penetrate the plant tissue where the fungus is filamentous in structure and induce tumors in the plant if compatible at the **b** locus. The dikaryotic hyphae can "round up" inside the plant to form teliospores. The teliospores can germinate and form simple basidia inside which meiosis and spore formation occurs. Spores bud from the basidia and germinate to regenerate the haploid yeast-like phase (Banuett 1992, Fincham, Day et al. 1979).

BASIDIOMYCETES

Ustilago maydis-life cycle

Ustilago maydis is a basidiomycetous fungus that causes corn smut disease. Dimorphism and many of the steps in the life cycle are under the genetic control of two mating type loci, **a** and **b** (Refs. Fincham, Day et al. 1979, Banuett 1992; Figures 1-10, 1-11). The ability of *U.maydis* to traverse its life cycle and to cause disease in corn plants are inseparable. The mating reaction and all stages of subsequent development are controlled by the two mating type loci **a** and **b**. For a successful mating reaction, partners must carry different alleles at both the **a** and **b** locus (Schultz, Banuett et al. 1990, Banuett and Herskowitz 1989, Banuett 1991). The sexual cycle is initiated by the fusion of two haploid cells. This leads to the formation of a filamentous dikaryotic mycelium that is able to infect corn plants and induce tumors. The life cycle is completed by the production of a large number of diploid teliospores in the corn plant. Upon germination, meiosis occurs, and haploid sporidia are produced. Even though meiosis occurs outside the plant, competence to undergo meiosis is acquired by passage through the plant. Meiosis occurs, followed by germination of the teliospore and the formation of a short filament (promycelium). The diploid nucleus migrates into the promycelium for the meiotic divisions. Mature tumors crack open and release spores that can be spread by the wind and carried to another susceptible host plant. The spores germinate on a plant and begin the infection/sexual process (Banuett 1992).

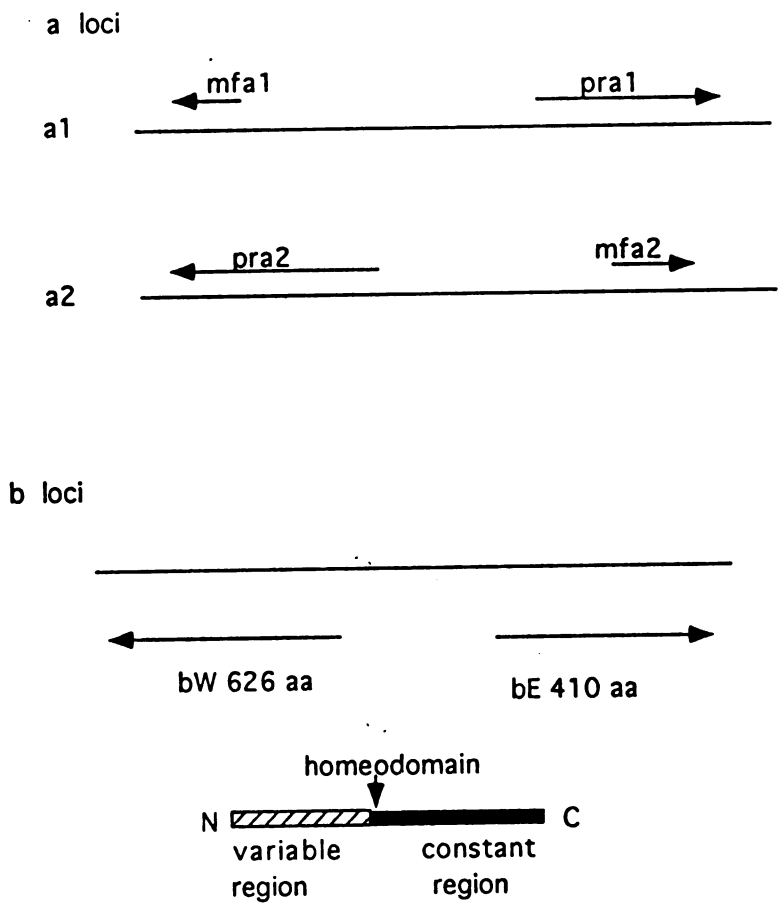

Figure 1-11. *U. maydis* has two mating type loci. The **a** locus has two alleles, **a1** and **a2**. The **a** locus is responsible for mating and maintenance of the filamentous hyphal stage. The **b** locus has more than 30 different alleles and is responsible for pathogenicity and maintenance of the hyphal stage. Each **a** locus contains a pheromone precursor gene and the receptor for the opposite mating type pheromone. All the **b** alleles analyzed to date encode two genes containing homeodomains (Banuett 1992).

***Ustilago maydis*-MAT loci**

The two unlinked loci, **a** and **b**, control mating type, dimorphism, and pathogenicity (Refs. Banuett 1992; Figure 1-11). A successful mating will occur when the parent strains differ at both loci. The developmental pathways regulated by **a** and **b** are not known with certainty. The **a** locus has only two alleles, **a1** and **a2**, which have been associated with cell fusion in classical genetic studies (Raper 1966). The **a** alleles are comprised of two heterologous regions of DNA; **a1** is 4.5 kb, and **a2** is 8.0 kb in length (Bolker, Urban et al. 1992). Each **a** allele contains a pheromone gene and a gene which encodes the receptor for the opposite mating type pheromone. The putative pheromone genes contain small open reading frames (ORF) of approximately 40 amino acids, with a CAAX (Cysteine-Aliphatic-Aliphatic-Any amino acid) prenylation motif at the carboxy terminus. The small size of the ORF and presence of a CAAX motif are characteristics shared by other pheromone precursor genes, such as those found in *S. cerevisiae* **a**-factor, *S. pombe* M-factor (Refs. Clarke 1992; Figure 1-3), and which were discovered in the pheromone genes in *Rhodosporidium toruloides*. Peptide pheromones isolated from *R. toruloides*, *Tremella brasiliensies* and *Tremella mesenterica* are farnesylated. The amino acid sequences of the pheromones themselves are dissimilar, as would be expected since they are used to identify specific mating partners of the opposite mating type but of the same species. No peptide pheromone has been isolated from *U. maydis* to date, and are no visible morphological changes are associated with exposure to the opposite mating type cells. The genes which are thought to encode receptors are similar to the *S. cerevisiae* **a**-factor receptor, and have seven predicted

transmembrane domains (Bolker, Urban et al. 1992). The **a** locus, which is thought to be involved in cell fusion, has been shown to be required for maintenance of filamentous growth after cell fusion (Banuett and Herskowitz, 1989). The **b** locus has 25 known alleles and is a complex locus consisting of two divergently transcribed genes, designated bE and bW (Schultz, Banuett et al. 1990, Gillisen, Bergemann et al. 1992). Each **b** allele contains two separate genes. For example, the b1 allele contains both the bE1 and bW1 genes. The **b** locus is required for filamentous growth and tumor production. The bW genes encode polypeptides of 626 amino acids and the bE genes encode polypeptides of 410 amino acids. Each **b** gene has a variable and a constant region. The **b** genes analyzed so far, (b1, b2, b3 and b4), all contain homeodomains which are similar to homeodomains found in *MAT*α2 and *MAT**a**1* from *S. cerevisiae* (Kues and Casselton 1992, Egel 1992, Gillissen, Bergemann et al. 1992, Herskowitz 1992). The current model for the function of the **b** genes in the control of filamentous growth and tumor production is that heteromultimers are the active regulatory species (Kues and Casselton 1992, Herskowitz 1992). In other words, the bE and bW polypeptides from different alleles are proposed to multimerize and form positive regulatory species which is required for filament formation and pathogenicity. The locus is functionally redundant in that only one pair of different genes is needed for the **b** developmental pathways, such as bE1 and bW2. In this way it is postulated that it is possible for all 25 **b** mating specificities to be compatible with each other, to distinguish self from non-self, and to complete the sexual cycle. The heterodimer is thought to be a novel regulatory activity in dikaryotic cells, similar to the a1/α2 heterodimer formed in *S. cerevisiae*.

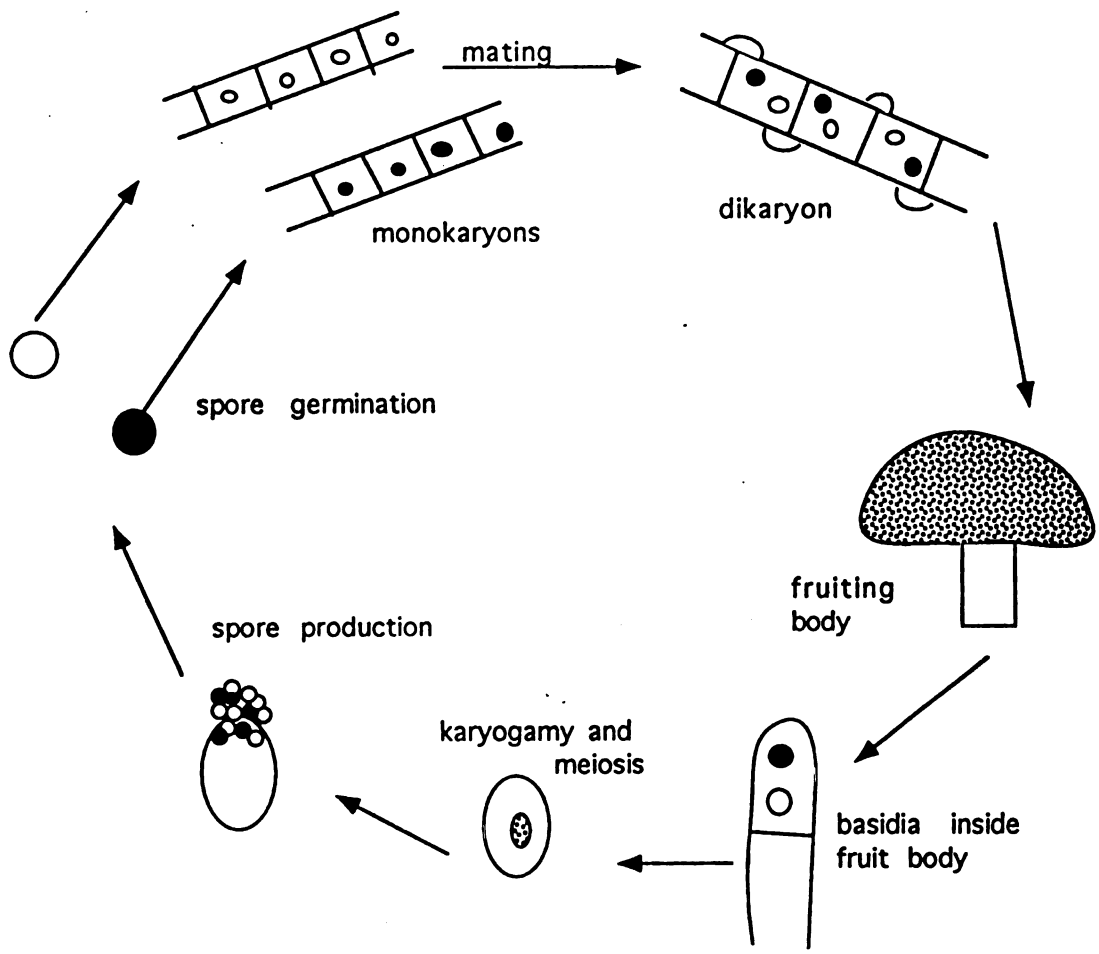

Figure 1-12. Both *S. commune* and *C. cinereus* follow a typical higher basidiomycete life cycle. Monokaryons which are compatible can mate and form a dikaryon with clamp connections. Under appropriate conditions (nutrition, light), a fruiting body is formed which contains many basidia. Karyogamy and meiosis occur in the basidia, and spores are produced. These spores germinate to regenerate the monokaryotic phase of the lifecycle (Fincham, Day et al. 1979, Novotny, Stankis et al. 1991).

***Schizophyllum commune*-life cycle**

Schizophyllum commune is a filamentous basidiomycete. Haploid spores are produced by basidial cells located within the fruiting bodies (Refs. Fincham, Day et al. 1979, Novotny, Stankis et al. 1991, Casselton 1978; Figure 1-12). Spores germinate and grow into a monokaryon or heterokaryon which is a mycelium of uninucleate cells. All monokaryons are capable of fusing, regardless of mating type, but in fusions between cells of the same mating type there is no sexual development and the fused cells die. If mates have different alleles at both the **A** and **B** mating-type loci, the fungi will progress through the full cycle of sexual development. These strains are fully mating compatible. If the nuclei have different alleles at either the **A** locus or the **B** locus, sexual development is incomplete. Following fusion, these cells neither die as in fully incompatible matings or show complete sexual development as is the case in compatible matings. Study of these hemicompatible matings have revealed two distinct pathways of sexual development, one controlled by the **A** mating type locus and one controlled by the **B** locus. In the fully compatible mating, the products of the **A** and **B** alleles trigger a cascade of events that transform the two monokaryotic mycelia into a single fertile mycelium called the dikaryon. The two mates undergo a reciprocal exchange of nuclei. The newly-exchanged nuclei migrate throughout the mycelium of the mate (Casselton 1978). The nuclei divide as they travel, and the monokaryotic septa degrades to allow the nuclei to pass. A different kind of septum characteristic of dikaryotic cells replaces the degraded monokaryotic septa. The two monokaryotic mycelia which have mated develop by this method into a single dikaryotic mycelium. Like the *S. cerevisiae* diploid cell, each cell in the dikaryotic mycelium can be propagated

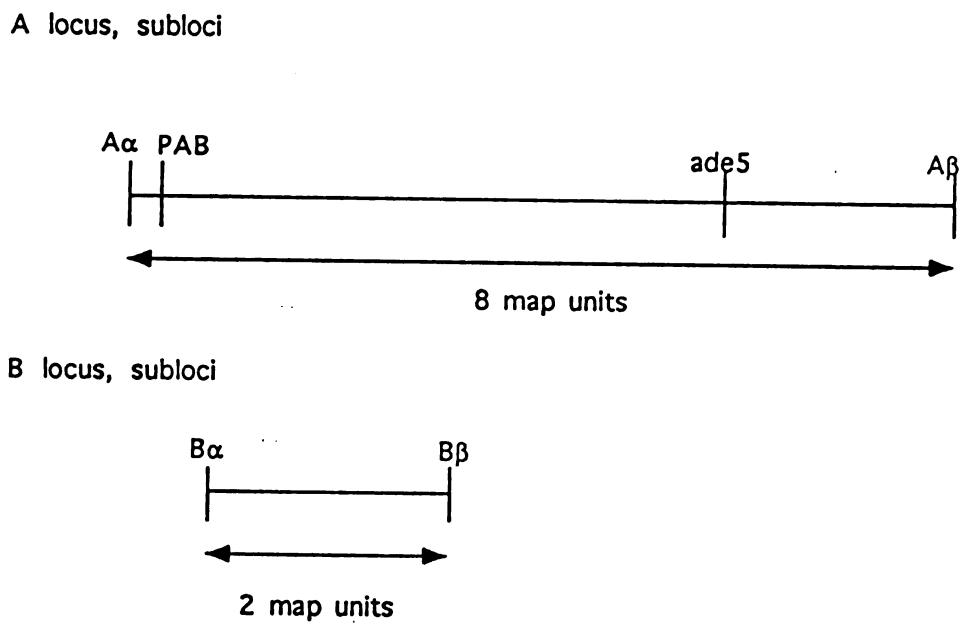

Figure 1-13. *S.commune* has two mating type loci each consisting of two linked subloci. The **A**α and **A**β subloci are separated by a distance of 8 map units while the **B**α and **B**β subloci are separated by 2 map units (Raper 1966).

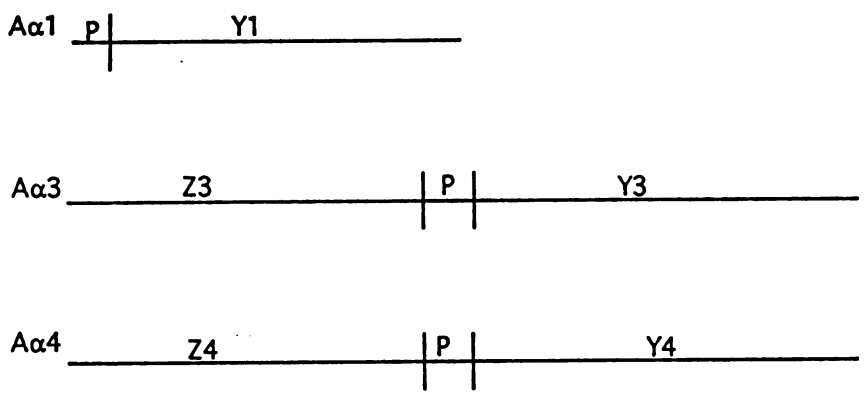

Figure 1-14. Analysis of three *S. commune* Aα alleles revealed the existance of multiple genes in 2 cases, Aα3 and Aα4. Aα1 has a single coding region Y1, with homology to the Y3 and Y4 regions of the Aα3 and Aα4 alleles respectively. In addition the Aα3 and Aα4 alleles contain a second coding region (Z3 and Z4), with homology (Stankis, Specht et al. 1992).

indefinitely. Similar to *S. cerevisiae* appropriate environmental conditions will trigger sporulation. These conditions trigger the dikaryotic mycelium to give rise to fruiting bodies. Nuclear fusion and meiosis occur in the basidial cells of the fruiting body, followed by sporulation.

***Schizophyllum commune*-MAT loci**

Schizophyllum commune has four mating-type loci, ***Aα, Aβ, Bα,*** and ***Bβ,*** which are located in linked pairs on chromosomes I and II (Refs. Novotny, Stankis et al. 1991, Raper 1966; Figures 1-13, 1-14). The **A** loci are separated by 6 map units and the **B** loci are separated by 2 map units. Many alleles have been found for each locus, ***Aα*** (9 alleles), ***Aβ*** (32 alleles), ***Bα*** (9 alleles), and ***Bβ*** (9 alleles). Full sexual development occurs after the fusion of two monokaryons only if the two mates have different alleles at the **A** locus and at the **B** locus. Nuclear pairing, cellular septation, hook cell formation, conjugate nuclear division, and hook cell septation are controlled by the **A** loci. The **B** loci control nuclear migration and hook cell fusion to the apical cell. The mating type loci appear to be functionally redundant because allelic differences of the mates at either ***Aα*** or ***Aβ,*** and ***Bα*** or ***Bβ*** will fully activate the **A** and **B** pathways respectively with equal effectiveness. Several ***Aα*** alleles (***Aα1, Aα3,*** and ***Aα4***) have been analyzed. The ***Aα3*** and ***Aα4*** alleles each contain two deduced ORFs, Y and Z (Giasson, Specht et al. 1989, Stankis, Specht et al. 1992). The Aα1 allele contained only one ORF, Y. All the ORFs analyzed have been found to contain homeodomains, and the *S. commune* Z and Y proteins have homology to the *U. maydis* bE and bW proteins and to the *C. cinereus* Aβ1-1 protein (Refs. Specht, Stankis et al. 1992; Figure 1-16).

The model for the $A\alpha$ locus function is similar to that presented for *U. maydis* and supported by transformation experiments (Specht, Stankis et al. 1992). The Z and Y genes are able to interact with each other if they are from different alleles to form novel regulatory activities in the dikaryon. To get complete sexual development in *S. commune* only one Z polypeptide and one Y polypeptide are required provided that they come from different $A\alpha$ alleles, for example, Y1 and Z3, or Z3 and Y2.

***Coprinus cinereus*-life cycle**

The mating type system of the filamentous basidiomycete *C. cinereus* closely resembles *S. commune* (Refs. Casselton 1978, Fincham, Day et al. 1979, Pukkila and Casselton 1991, Raper 1966; Figure 1-12: see above for discussion). There are two pairs of multiallelic mating type loci. *C. cinereus* has a typical higher basidiomycete life cycle with two functionally distinct mycelial stages. A spore germinates to give a uninucleate-celled mycelium, the monokaryon. For sexual reproduction, two compatible monokaryons must mate to give the dikaryon. The dikaryon cells each contain one nucleus from each parent. When the dikaryon is exposed to the proper temperature and light conditions, it differentiates into the fruiting bodies. Hyphal fusion between compatible monokaryons is sufficient for mating and initiates the developmental pathway. Like *S. commune,* any monokaryotic hyphae can fuse and, if nonidentical at $A\alpha$ or $A\beta$ and $B\alpha$ or $B\beta$, will form dikaryotic hyphae. Hyphal fusion triggers a cascade of events, regulated in part by **A** and in part by **B**. Mating of fully-compatible strains converts the two monokaryons into a single dikaryon. As in other higher basidiomycetes, nuclear migration and conjugate nuclear division occur to produce and maintain the dikaryoic state. Matings between *C. cinereus* strains with one common factor are incompatible sexually but can generate sterile heterokaryons if suitable forcing markers are present. The dikaryotic hyphae can differentiate to form fruiting bodies. In the specialized basidia that develop on the gill surfaces, the dikaryotic nuclei eventually fuse. After karyogamy, meiosis occurs to produce nuclei which enter the basidiospores.

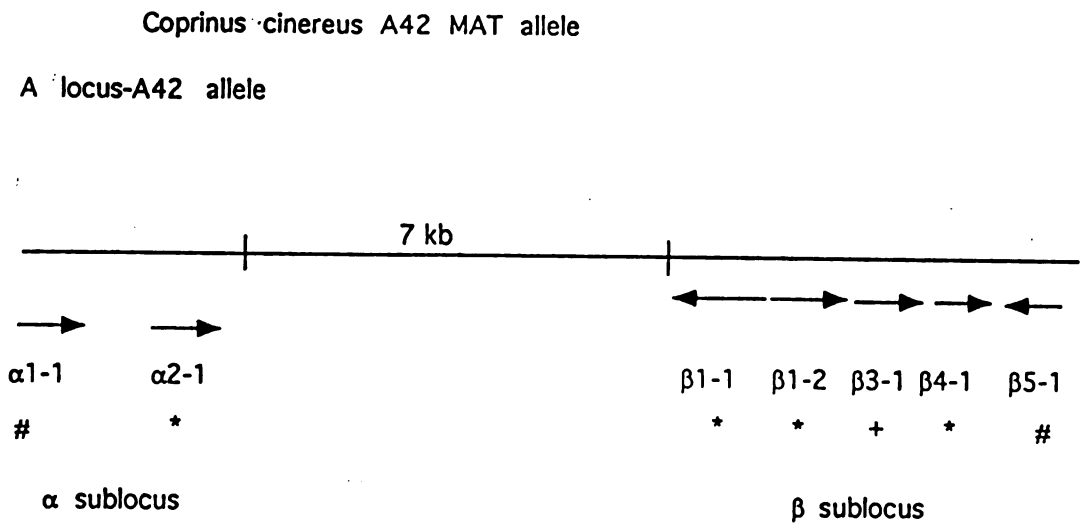

Figure 1-15. Physical map of the *C. cinereus* **A** mating type locus from Kues et al. 1992. Four genes α2-1, β1-1, β1-2, β4-1 are able to confer **A42** mating type specificity individually. The β3-1 gene does not confer **A42** specificity, but is unique to this allele. The remaining two genes shown α1-1 and β5-1 are also unable to confer specificity, and they contain sequences which are common to all **A** mating type alleles (Kues, Richardson et al. 1992).

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                                                    * *  * *
C.cinereus α2-1        LEKYFEYNAYPTAQDRALLARKSMMSARQIEVWFQNHRARARKE
C.cinereus β2-1        LEKYFEYNAYPSARDREWLARKTMMSVRQIEVWFQNHRRRARKE
S.commune α1 HD2       LELYFRFNAYPTYADRRVLAEKTGMLTRQITVWFQNKRRRAK
S.commune α3 HD2       LELYFRFNAYPTFADRRMLAEKTGMQTRQITVWFQNKRRRAK
S.commune α4 HD2       LELEFRFNAYPTYADRRILAEKTGMLTRQITVWFQNKRRRAK
U.maydis bW2           LEQAFKHSPNITPAEKFRLSEVTGLKPKQVTIWFQNRRNRKGKK
U.maydis bW3           LEEAFKHSPNITPAEKFRLSEVTGLKPKQVTIWFQNRRNRKGKK

S.cerevisiae a1        LEQVFRRKQSLNSKEKEEVAKKCGITPLQVRVWFINKRMRSK
S.cerevisiae α2        WFAKNIENPYLDTKGLENLMKNTSLSRIQIKNWVSNRRRRKEKT
S.pombe Pi             WLLLNYDNPYPSNEEFYDLSAATGLTRTQLRNWFSNRRR

U.maydis bE3           HFLHTLENPYPTQEEKEGLVRLTNESVHQLTLWFINARRRSGWS
U.maydis bE5           HFLLTLDNPYPTQEEKQNLVRLTNESVHQLTLWFINARRRSGWS
U.maydis bE3           HFLHTLENPYPTQEEKEGLVRLTNESVHQLTLWFINARRRSGWS
U.maydis bE5           HFLLTLDNPYPTQEEKQNLVRLTNESVHQLTLWFINARRRSGWS
C.cinereus β1-1        WLKDNWYNPYPSGEVRTQIARQTRTSRKDIDAWFIDARRRIGWN
C.cinerus β4-1         WLKDNWYNPYPSPQVRSSIAKQTGASRKDIDAWFIDARKRIGWN
S.commune α3 HD1       WLLQHLHNPYPSKSEKQIIALDTCKTFDDIDRWFSAARIRIGWT
S.commune α4 HD1       WLVNHLDNPYPTKAIKEELLDQARLALGDIDNWFIAARARMGWG
```

Figure 1-16. Comparison of homeodomain motifs from several species. The consensus amino acids found in all homeodomain proteins of multicellular eukaryotes are indicated by an *. The basidiomycetous fungi contain homeodomain motifs which fall into two main classes, WFQN-R and WFIN-R. It is interesting to note that the proteins which are proposed to interact in *U. maydis*, *S. commune*, and *C. cinereus* are pairs made up of one protein from each class (Kues and Casselton 1992, Banuett 1992)).

There is no barrier to fusion of incompatible hyphae, which indicates that self-nonself recognition occurs intracellularly.

***Coprinus cinereus*-MAT loci**

C. cinereus has four multi-allelic loci called ***Aα, Aβ, Bα***, and ***Bβ***, which seem to be functionally redundant since differences at ***Aα*** or ***Aβ*** and ***Bα*** or ***Bβ*** are sufficient for complete sexual development (Refs. May, Chevanton et al. 1991, Mutasa, Tymon et al. 1990, Novotny, Stankis et al. 1991; Figure 1-15). The **A** and **B** loci control separate specific steps in dikaryon differentiation. The **A** locus governs the synchronized division of the mated nuclei. The formation of the clamp cell is also regulated by the **A**. Clamp cell fusion and nuclear migration are controlled by the **B** locus and require different **B** factors. The ***Aα*** and ***Aβ*** loci have been studied in one allele, ***A4***. Seven genes have been identified in the two closely-linked loci, which are separated by 7 kb (Kues, Richardson et al. 1992). **A**-mating type specificity can be determined independently by each of four of these genes, one α and three β, all of which encode homeodomain-related motifs (Figure 1-16). Only one of these four genes is required to be heteroallelic in a cell to trigger **A**-regulated sexual development. The gene products of ***A42α2-1*** and ***A42β2-1*** have similarity to *S. cerevisiae MATa1*, while ***A42β1-1*** and ***A42β4-1*** have similarity with *S. cerevisiae MATα2* (Refs. Kues and Casselton 1992, Banuett 1992, Herskowitz 1992, Egel 1992; Figure 1-16).

Common Themes

The fungi are a diverse group of organisms, and yet there are surprising similarities among the fungi themselves and between fungi and other eukaryotes. Cell type, development, and self-nonself recognition in fungi are dictated by homeodomain proteins located in the mating type locus. Not only do these genes have similarity among the fungi, but they also share similarity with *Drosophila melanogaster* homeotic genes such as *antennapedia, ultrabithorax,* and *fushi tarazu*. Mating type genes containing homeodomains also show analogy to the POU domain. The fungal mating type homeodomain proteins a1 and α2 from *S. cerevisiae* interact in the diploid to form a novel heterodimeric regulatory species which controls a/α cell type. The POU domain is subdivided into DNA binding and dimerization subdomains, and homology to these proteins may suggest analogous functions in the fungal mating type proteins. In *U. maydis* and *S. commune,* one homeodomain-containing gene from each mating type allele is required in the dikaryon to allow further sexual development. The E protein from *U. maydis* and the Z protein from *S. commune,* which fall into one homeodomain class, are proposed to interact with W2 (*U. maydis*) and Y (*S. commune*), both of which fall into a second homeodomain class (Figure 1-16).

Many of the molecules used for cell-cell signaling in fungi are also common to the higher eukaryotes. For example, the mating factor receptors in fungi are similar to the family of seven transmembrane integral membrane receptor proteins in mammals, such as the β-adrenergic, rhodopsin, and muscarinic acetylcholine receptors. The fungal and mammalian receptors are coupled to heterotrimeric G-proteins as part of the signal transduction system. The farnesylation modification, first shown in fungal mating factors, also occurs in mammalian cells on proteins such as *ras* and may contribute to membrane localization. Fungal mating systems are interesting for the diversity with which they handle common biological problems and for the similarities among themselves and other eukaryotes.

CHAPTER 2- DEVELOPING GENETIC METHODS IN CRYPTOCOCCUS

INTRODUCTION

The presence of a sexual cycle in the basidiomycete *C. neoformans* makes it amenable to classical genetic studies. Recent technological developments in *C. neoformans* molecular biology have made it possible to perform genetic studies (for reviews see Kurtz and Scherer 1991 and Whelan 1987). These advances include convenient mutant isolation methods (Jacobson and Ayers 1979), efficient methods for DNA extraction (Restrepo and Barbour 1989), and transformation procedures using a cloned selectable marker (Edman and Kwon-Chung 1990).

C. neoformans is a dimorphic fungal pathogen that is easily propagated on standard liquid or solid yeast media in the haploid yeast phase. Mutant strains with nutritional requirements for amino acid or nucleotide precursors are easily isolated in the haploid yeast. Mating is induced on solid starvation media, followed by limited growth of the dikaryotic hyphal phase, transient diploidy in the basidia and sporulation. Spores are either individually isolated by microdissection or selected on minimal media and analyzed by segregation and recombination tests.

Little is known at a molecular level about the progression of *C. neoformans* through its sexual cycle. In order to begin a molecular and genetic study of mating in *C. neoformans,* a standard set of strains in a common genetic background with positively and negatively selectable markers was required. The isolation, construction, and characterization of such strains will be presented here. The isolation and identification of *HIS3* DNA from *C. neoformans* by complementation in *S. cerevisiae* will also be described.

RESULTS

Starting with the *MATa* strain (JEC20) of the congenic pair, strains with nutritional requirements for amino acid or nucleotide precursors were isolated after UV mutagenesis (Table 2-1).

TABLE 2-1

STRAIN	GENOTYPE	REFERENCE
JEC 20	MATa	Kwon-Chung, Edman, and Wickes, 1992
JEC 21	MATα	Kwon-Chung, Edman, and Wickes, 1992
JEC 30	MATa *lys1*	JEC20, UV mutagenesis
JEC 31	MATα *lys1*	JEC21 X JEC30
JEC 32	MATa *lys2*	JEC20, UV mutagenesis
JEC 33	MATα *lys2*	JEC21 X JEC32
JEC 34	MATa *ura5-1*	JEC20, UV mutagenesis
JEC 35	MATa *ura5-2*	JEC20, UV mutagenesis
JEC 36	MATa *cys1*	JEC20, UV mutagenesis
JEC 37	MATa *glu1*	JEC20, UV mutagenesis
JEC 38	MATa *met1*	JEC20, UV mutagenesis
JEC 39	MATα *cyhR*	JEC21 X JEC60
JEC 40	MATα *canR*	JEC21 X JEC59
JEC 41	MATα *canR ura5*	JEC43 X JEC59
JEC 42	MATα *cyhR ura5*	JEC43 X JEC60
JEC 43	MATα *ura5-1*	JEC21 X JEC34
JEC 44	MATα *lys2-2*	JEC21 X JEC32
JEC 47	MATα Arg5	JEC21 X JEC56
JEC 48	MATα Ade1	JEC21 X JEC57
JEC 49	MATα Ade7	JEC21 X JEC58
JEC 50	MATα *ade2-27*	JEC21 X JEC61

STRAIN	GENOTYPE	REFERENCE
JEC 51	MATα *lys1-2*	JEC21 X JEC31
JEC 52	MATα *ura5 lys1*	JEC30 X JEC41
JEC 53	MATa *ura5 lys1*	JEC30 X JEC41
JEC 54	MATα *cyhR lys1*	JEC30 X JEC42
JEC 55	MATa *cyhR lys1 ura5*	JEC30 X JEC42
JEC 56	MATa Arg5	JEC20, UV mutagenesis
JEC 57	MATa Ade1	JEC20, UV mutagenesis
JEC 58	MATa Ade7	JEC20, UV mutagenesis
JEC 59	MATa *canR*	JEC20,UVmut.,JCEdman,unpublished
JEC 60	MATa *cyhR*	JEC20,UVmut.,JCEdman,unpublished
JEC 61	MATa *ade2-27*	JEC20, UV mutagenesis
JEC 62	MATa *ura5 cyhR*	JEC30 X JEC42
JEC 63	MATα Ade7*canR*	JEC41 X JEC58
JEC 64	MATa Ade7 *canR*	JEC41 X JEC58
JEC 65	MATα Ade1*canR*	JEC41 X JEC57
JEC 66	MATa Ade1*ura5 canR*	JEC41 X JEC57
JEC 67	MATα Ade7*ura5*	JEC41 X JEC58
JEC 68	MATα *ura5* Ade7*canR*	JEC41 X JEC58
JEC 69	MATa Ade7*ura5 canR*	JEC41 X JEC58
JEC 155	MATα *ade2-27 ura5*	JEC34 X JEC50
JEC 156	MATa *ade2-27 ura5*	JEC34 X JEC50
JEC 157	MATα *ade2-27 ura5 lys1*	JEC77 X JEC 156
JEC 168	MATa *lys2 ade2-27 ura5*	JEC44 X JEC 156
JEC 169	MATa *lys1 ade2-27 ura5*	JEC77 X JEC 156
JEC 170	MATα *lys2 ade2-27*	JEC44 X JEC 156
JEC 171	MATa *lys2 ade2-27*	JEC44 X JEC 156

The results of the auxotrophic mutant screen is summarized in Table 2-2. The strains isolated were predominantly lysine (13%), uracil (20%), arginine (16%), and adenine (33%) auxotrophic mutants.

Table 2-2

nutritional requirement	original isolates	petite mutants	revertants	final
lys	14		5	9
met	16		13	3
arg	18	1	7	10
ura	22	1	10	11
cys	3		2	1
his	1		1	0
ade	36	-	-	-

Identification of *ura5* mutants. Strains with mutations in the *ura5* gene orotidine monophosphate pyrophorylase (OMPPase) in a JEC20 derived strain were identified by the following criteria: 1) resistance to 5-fluoro-orotic acid (FOA), a compound which is converted to a toxic metabolite by the *ura5* gene product in *S. cerevisiae*, 2) growth on dihydroorotic acid, orotidine, and orotic acid, but not on ureidosuccinic acid, and 3) conversion to uracil prototrophy when transformed with the cloned *ura5* gene (Ref. Jones and Fink 1982; Figure 2-1).

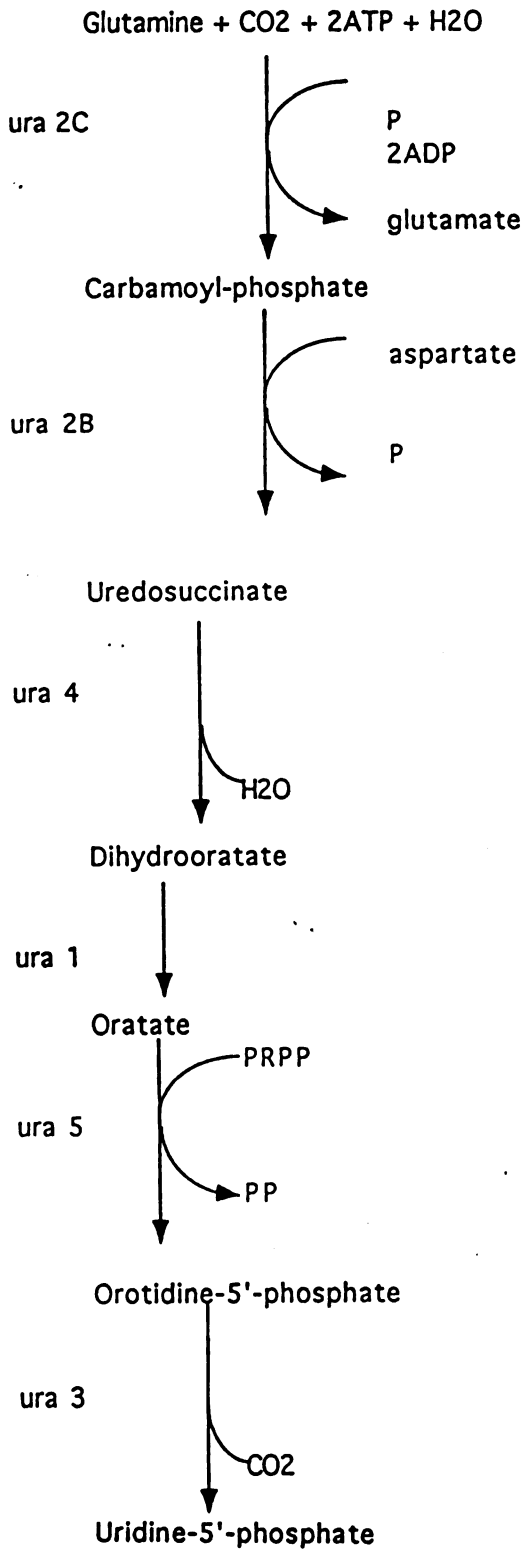


Figure 2-1. A segment of the uracil biosynthetic pathway containing the *S. cerevisiae* gene products of *URA1* through *URA5*. Mutants defective in these genes must salvage uracil from the medium to survive. Cells with mutations in *URA3* or *URA5* are resistant to the antimetabolite effects of 5-fluro-orotic acid (FOA) (Jones and Fink 1982).

Classification of lysine biosynthetic mutants. The lysine auxotrophic mutants were grown on minimal medium containing α-aminoadipic acid, a toxic metabolic intermediate which is used in *S. cerevisiae* to characterize auxotrophic mutants in the lysine biosynthetic pathway (Ref. Jones and Fink 1982; Figure 2-2). *C. neoformans* is not sensitive to α-aminoadipic acid and therefore this classification method could not be used. However, mating among the mutant strains and complementation analysis divided the lysine auxotrophs into two complementation classes, empirically named *lys1* and *lys2*. The *lys1* and *lys2* genes segregate independently (Table 2-1).

Other auxotrophic strains. (See Table 2-1) Strains were constructed for various purposes: to provide mating testers, to assess the genetics of canavanine and cyclohexamide resistance, to transform with selectable markers, and to screen for non-filament forming mutants. Mating pairs JEC31, JEC33, JEC39, JEC40, JEC43, JEC44, JEC47, JEC48, JEC49, JEC50, and JEC51 were constructed from lysine, arginine, uracil, and adenine auxotrophic mutants and also from canavanine-resistant and cycloheximide-resistant mutants. The auxotrophic mutants mate and form hyphae faster and with higher frequency than prototrophic strains, which shortens the time required for mating type analysis by backcrossing. By crossing the strains among themselves, new strains with two or three mutations were constructed (Table 2-1). None of the isolated markers are closely linked to each other or to mating type.

One cross gave some unusual recombinant strains. In a *lys1* X *met1* cross, 20 spores were dissected, 16 germinated (80%) and 5 spores were *lys1 met1* double mutants. All five of the *lys1 met1* strains, however, were self

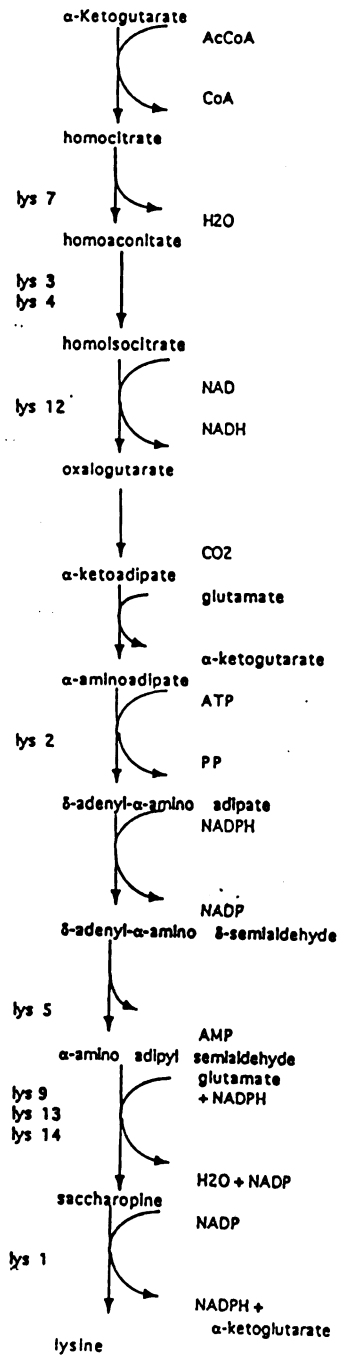


Figure 2-2. Lysine biosynthesis in *S. cerevisiae,* showing the genes and intermediates involved. Lysine auxotrophic mutants in *S. cerevisiae* can be characterized with the compound α-amino-adipic acid (Jones and Fink 1982).

fertile when streaked on either V8 or SD medium. The colony morphology of the yeast colonies on YEPD was rough in appearance compared to the spores which were *lys1* mutants or prototrophs. Diploid strains have been previously reported in *C. neoformans* (White and Jacobson 1985). The self fertile spores may each contain two nuclei.

Drug resistance markers. Strains of both mating types containing nutritional requirements and resistance to cycloheximide or canavanine were constructed to test the genetic behavior of the drug resistance markers (see Table 2-1; Figure 2-3). Canavanine-resistant mutants have been characterized in *S. cerevisiae* and *C. neoformans*. In both, the defect was found to be in the basic amino acid transporter which in *S. cerevisiae* actively transports arginine, lysine, ornithine, canavanine, and probably histidine (Strathern, Jones et al. 1982, Polacheck and Kwon-Chung 1986). The *canR* gene in *C. neoformans* strain JEC59 was behaves as a recessive drug resistance marker, as *can1* does in *S. cerevisiae* (Strathern, Jones et al. 1982). However, in contrast to *S. cerevisiae*, it seems that a canavanine-resistant *C. neoformans* strain cannot salvage lysine, since no *canR lys* double mutant spore colonies were isolated in two dissections, and lysine auxotrophs were under-represented among the spore progeny. The result that canavanine-resistant *C. neoformans* strains cannot salvage lysine may indicate a difference between *C. neoformans* and *S. cerevisiae*, which has a second lysine-specific transporter in addition to the basic amino acid transporter. Cycloheximide resistance in the dikaryon does not seem to follow simple dominant or recessive patterns. Haploid yeast cells are sensitive or resistant to

canR-recessive

	SD	SD+can	YEPD+can
CAN/CAN	hyphae	no growth	yeast, hyphae
CAN/canR	hyphae	yeast	yeast, hyphae
canR/canR	hyphae	hyphae	yeast, hyphae

cyhR-complex

	SD	SD+cyh	YEPD + cyh
CYH/CYH	hyphae	no growth	no growth
CYH/cyhR	hyphae	yeast	yeast
cyhR/cyhR	hyphae	yeast	yeast

Figure 2-3. Determination of the dominance or recessiveness of canavanine-resistant and cycloheximide-resistant mutations (*canR* and *cyhR*) in *C. neoformans*. The *canR* mutant shows simple recessive properties while the *cyhR* mutant displays a more complex pattern of inheritance.

cycloheximide but, dikaryons which are either heterozygous or homozygous for the cyclohexamide resistance gene are both sensitive to cycloheximide.

***HIS3* cDNA isolation and identification.** *S. cerevisiae MATa ste14-2 ade1 trp1 ura3-52 his4 his3* strain (gift of I. Herskowitz), was transformed with a *C. neoformans* cDNA library (prepared by J.C. Edman) in a *S. cerevisiae/E. coli* shuttle vector, pYcDE-Sfi-Not. Plasmid DNA was recovered from transformants prototrophic for histidine and used to transform *E. coli.* Plasmids from twelve of the *E. coli* transformants were analyzed with restriction enzymes, and all contained a *KpnI/EcoRI* insert of approximately 1 kb. The entire insert was subcloned into a Bluescript vector and mapped with several common restriction endonucleases (Figure 2-4). Analysis of the partial sequence of the cDNA using the FASTA program revealed similarity between this sequence and the *S. cerevisiae HIS3* gene product (Figure 2-5). Of the 264 bp fragment that was sequenced, there was 59% amino acid identity to the *HIS3* gene from *S. cerevisiae* in that region. *HIS3* encodes the imidazoleglycerol-P-dehydratase protein, an enzyme required for histidine biosynthesis (Figure 2-6). A *HIS3* cDNA subclone with a 45 bp deletion in the coding region was used to attempt to create a gene disruption. However, this experiment was unsuccessful. None of the 600 transformant colonies screened exhibited histidine auxotrophy. It may be that the gene contains an intron, and a genomic clone is needed to provide enough sequence homology to allow integration.

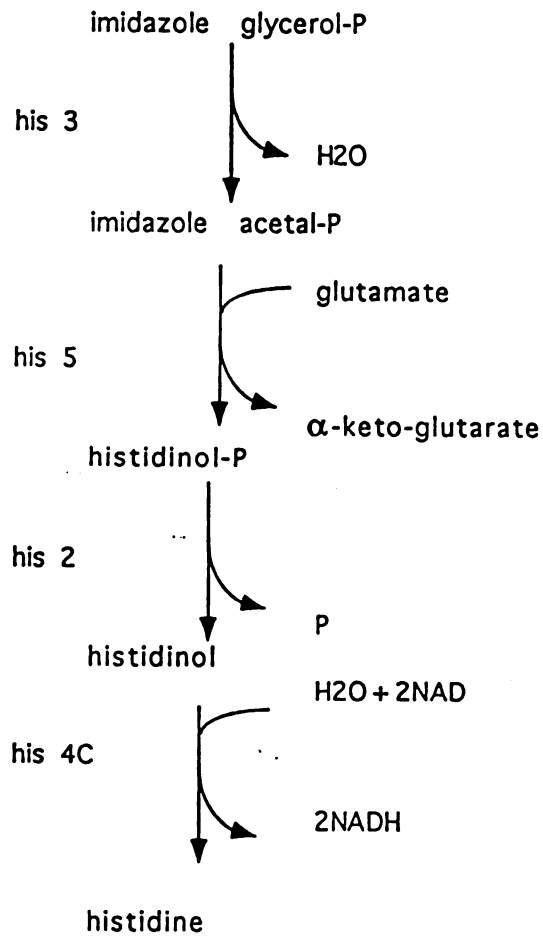


Figure 2-4. A segment of the histidine biosynthetic pathway showing the *S. cerevisiae* genes involved. The reaction catalyzed by the *S. cerevisiae HIS3* gene product is the first step shown. The *C. neoformans HIS3* analog was isolated (Jones and Fink 1982).

Figure 2-5. The restriction map of the *C. neoformans HIS3* cDNA. The mutant used in the attempted knock-out experiments was a *Nde I/Pst I* 45 nt deletion.

```
Sc MTEQKALVKRITNETKIQIAISLKGGPLAIEHSIFPEKEAEAVAEQATQSQVINVH
   | |  | | | | || |   | |   |   |                  | |||
Cn MSERIASVERTTSETHISCTIDLDHIPGVTE-------------------QKINVS

   TGIGFLDHMIHALAKHSGWSLIVECIGDLHIDDHHTTEDCGIALGQAFKEALLARG
   ||||| |||   |||| | ||   | |||||||||| |||  |||  ||
   TGIGFSDHMFYTLAKHGGMSLQLQCKGDLHIDDHHTAEDCALALGEGFKKG

   VKRFGSGF
```

Figure 2-6. Comparison of a portion of the *HIS3* proteins of *S. cerevisiae* and *C. neoformans*. Over the 88 amino acid region, there was a 59% identity.

DISCUSSION

A number of standard laboratory auxotrophic and drug resistant strains were isolated, characterized, and constructed. The *HIS3* cDNA in *C. neoformans* was also isolated and identified.

The discovery of a sexual phase in *C. neoformans* provided the initial impetus for the study of this medically important fungus (Kwon-Chung 1975). *C. neoformans* is an opportunistic pathogen which can cause serious disease predominantly in immunocompromised hosts (Whelan 1987). There are compelling reasons to study this pathogen, the incidence of cryptococcal meningitis has increased in recent years due to the advent of cancer chemotherapy, immunosuppressive regimens and the AIDS epidemic. The lower incidence of severe infection among healthy populations makes *C. neoformans* a safer organism to work with experimentally than many of the true fungal pathogens. Results pertaining to the molecular aspects of virulence in *C. neoformans* may be relevant to the more serious fungal pathogens. *C. neoformans* is a human fungal pathogen, and one of the goals in the study of this organism is ultimately to understand the molecular-genetic basis of virulence. Improvements in the treatment of cryptococcal meningitis will require information of the basic biochemistry of *C. neoformans*.

C. neoformans research at the molecular level has advanced enormously in the past few years. Mutant strains have been isolated and analyzed genetically (Jacobson and Ayers 1979, Kline and Jacobson 1981, Jacobson, Ayers et al. 1982). Mutant strains auxotrophic for methionine, arginine, lysine, cytosine, niacin, and pantothenate, have been isolated

(Jacobson and Ayers 1979). In addition, genes required for capsule formation have been mapped (Still and Jacobson 1983). A method to test for genetic complementation by analyzing strains for growth of heterokaryotic hyphae has been described (Whelan and Kwon-Chung 1986). Protocols for the efficient extraction of DNA have led to the cloning of *C. neoformans* genes, such as the 18S and 25S rDNAs (Restrepo and Barbour 1989). Even more recently the *URA5* gene was isolated and used as a selectable marker to develop a method for transformation of *C. neoformans* with exogenous DNA (Edman and Kwon-Chung 1990). The heterogeneity in the karyotype of isolated *C. neoformans* strains prompted the construction of congenic *C. neoformans* strains (Kwon-Chung, Edman et al. 1992). It is from this congenic pair of strains that all the auxotrophs and drug resistant strains discussed in this chapter were derived (Table 2-1). These strains were isolated and characterized for use in the genetic and molecular analysis of *C. neoformans*. The common background of these strains eliminates strain variation problems from experimental analysis. The *canR* and *cyhR* strains enabled genetic analysis of these genes, which established the recessive nature of *canR* and the complex genetic nature of *cyhR*. The strains provide useful recipients for transformation with *C. neoformans* libraries or plasmids, and parent strains for the isolation of sterile or nonfilamentous mutants to be used in the study of mating. The strains may also be helpful in the development of new and useful techniques such as homologous recombination or the creation of stable diploids.

MATERIALS AND METHODS

Bacterial media and strains. LB or LB containing 100mg/liter ampicillin were used to grow bacterial strains. *E. coli* HB101, JM101 and DH5α strains were used.

***C. neoformans* and *S. cerevisiae* media and strains.** See Table 1 for *C. neoformans* strains. YEPD (1% yeast extract, 2% Bacto-peptone, 2% dextrose, and 2% Bacto-agar) was used as the maintenance medium. Synthetic dextrose (SD) contained 6.7 g/l of yeast nitrogen base without amino acids, 20 g/l of glucose and appropriate amino acid or nucleotide supplements. V8 starvation crossing medium contained 5% V8 juice, 0.5 g/l KH_2PO_4, and 4% agar, adjusted to pH 7.0 with KOH. UV mutagenized *C. neoformans* strain JEC20 (*MATa*) was analyzed for auxotrophic mutants which were unable to grow on minimal media (SD). Master plates of auxotrophs were replica-plated to nine plates each containing a different amino acid/nutrient pool according to published procedures (Sherman, Fink et al. 1986). This procedure was used to identify the specific nutritional requirement of the mutant strains. Strains were further analyzed for reversion frequency by plating 2×10^7 cells on SD medium. The ability to grow on glycerol, a nonfermentable carbon source, was also tested. Lysine auxotrophs were placed into two complementation groups after patch mating on V8 medium. Uracil-requiring mutants were grown on medium containing intermediates in the uracil biosynthetic pathway and FOA. These mutants were also transformed with the cloned *URA5* gene.

To generate strains of both mating types with multiple auxotrophic markers, the isolated mutants were crossed to strain JEC21, followed by microdissection of spore progeny. Parent strains were streaked together on V8 medium, and in four to seven days white filamentous growth, basidia and spores were formed. The spores were transferred to the YEPD dissection plate and individual spores were isolated, and allowed to germinate for three to four days. The resulting colonies were analyzed for nutritional phenotype and mating type.

Isolation of the *C. neoformans* HIS3 gene. The *S. cerevisiae* strain K27-4b (a gift from I. Herskowitz), was transformed with a *C. neoformans* cDNA library in the shuttle vector pYcDE-Sfi-Not (prepared by J.C. Edman), which confers *TRP1* prototrophy in *S. cerevisiae* and ampicillin resistance in *E. coli*. The cDNA was cloned in between the *S. cerevisiae* ADC1 promoter and CYC1 terminator. Transformants were plated to SD medium containing adenine and uracil to select for plasmids with inserts which were able to confer histidine prototrophy. Plasmid DNA was isolated from colonies growing without tryptophan or histidine and used to transform *E. coli* according to published procedures (Maniatis, Fritsch et al. 1981). Clones were analyzed by restriction digestion with *NotI* and *EcoRI* which removes the entire insert. One *EcoRI/NotI* fragment was subcloned into a Bluescript vector and restriction analysis performed. The clone was further subcloned in two fragments, a 209 bp fragment and a 800 bp fragment and sequenced on an ABI automated sequencer. The predicted protein sequence was used to screen the PIR protein data base, which identified a *S. cerevisiae* protein with 59% amino acid identity of the 264 bp portion which was sequenced. A 45 bp

deletion was made in the coding region of the gene and this construct was used to attempt homologous integration into the *C. neoformans* genome to create a mutant strain in which the histidine phenotype could be analyzed.

Genetic behavior of drug resistance in *C. neoformans*. To test the genetic characteristics of resistance to cycloheximide and canavanine in *C. neoformans,* strains were constructed containing several auxotrophic markers in addition to mutations conferring the drug resistance. Drug resistant strains JEC 59 and JEC 60 (from JCE) were mated to several auxotrophic strains to generate multiply marked strains of both mating types (see Table 1). Drug resistance in the homozygous and heterozygous dikaryon was compared on V8 and SD media containing the appropriate drug.

CHAPTER 3 ISOLATION OF THE MATα LOCUS AND THE PHEROMONE GENE FOUND WITHIN

INTRODUCTION

The mechanisms of recognition between *MAT**a*** and *MAT*α cells, and starvation-induced mating in *C. neoformans* have not been identified. Genetic studies in other fungi have demonstrated the role of mating type loci genes in cell-cell recognition, cell fusion, the yeast-hyphal transition, and maintenance of filamentous state (Banuett 1991, Herskowitz 1988, Metzenberg 1990). The role of the genes within the *C. neoformans* mating type loci and their function have yet to be identified. Mating and subsequent developmental changes have been successfully explored in other fungi using mutants in the mating type loci which enable the cloning of these genes through complementation, and functional analysis of each phenotype. Mating type genes have also been isolated by chromosome walking from genes which are linked to mating type. To date, no *C. neoformans* genes have been identified that are linked to mating type and no mutants have been described which map to the *MAT**a*** or *MAT*α loci.

This chapter describes the isolation of the *C. neoformans MAT*α locus by a difference cloning procedure and its identification as a 35 to 45 kb region of nonhomologous DNA present only in *MAT*α strains. Evidence is presented that the locus contains a gene for a peptide mating factor, which induces filament formation when transformed into haploid *MAT**a*** cells.

RESULTS

Isolation of *MATα*-specific DNA. *C. neoformans* does not switch mating type, suggesting a lack of silent copies of the mating type loci. Each mating type should therefore contain unique DNA sequences at the mating type locus responsible for the determination of mating type. This difference was exploited to isolate the α-mating type locus by difference cloning (Kunkel, Monaco et al. 1985), which allows the isolation of DNA sequences present in one DNA preparation but not in another (Figure 3-1). DNA from wild type *MATα* cells (JEC21) was digested with *TaqI* and hybridized to an excess of randomly-sheared DNA from a congenic *MAT**a*** strain (JEC20) using a phenol-enhanced reassociation technique (Table 3-1). During reassociation, DNA fragments unique to *MATα* will re-anneal to form the original *TaqI* fragments with two cohesive ends. The hybridization mixture was ligated to the Bluescript vector which had been linearized with *ClaI*. Transformation of *E. coli* with the ligation mixture selected for fragments with two cohesive ends, as only these would allow the formation of transformation-competent circles. Sixteen plasmid clones were recovered in this way and used to probe genomic Southern blots of *MAT**a*** and *MATα* DNA. One clone, α24, contained a 138 bp insert that hybridized solely to *MATα* DNA and was chosen for further analysis. The remaining plasmid clones did not hybridize to *C. neoformans* DNA or hybridized to repetitive sequences found in both mating types.

Table 3-1: Strains

STRAIN	GENOTYPE	REFERENCE
JEC 20	MAT**a**	(Kwong-Chung, Edman et al. 1992)
JEC 21	MATα	(Kwong-Chung, Edman et al. 1992)
JEC 34	MAT**a** *ura5*	Chapter 2
JEC 50	MATα *ade2*	Chapter 2
JEC 157	MATα *ade2 ura5 lys1*	Chapter 2
JEC 168	MAT**a** *lys2 ade2 ura5*	Chapter 2
JEC 169	MAT**a** *ade2 ura5 lys1*	Chapter 2
JEC 170	MATα *lys2 ade2*	Chapter 2
JEC 171	MAT**a** *lys2 ade2*	Chapter 2

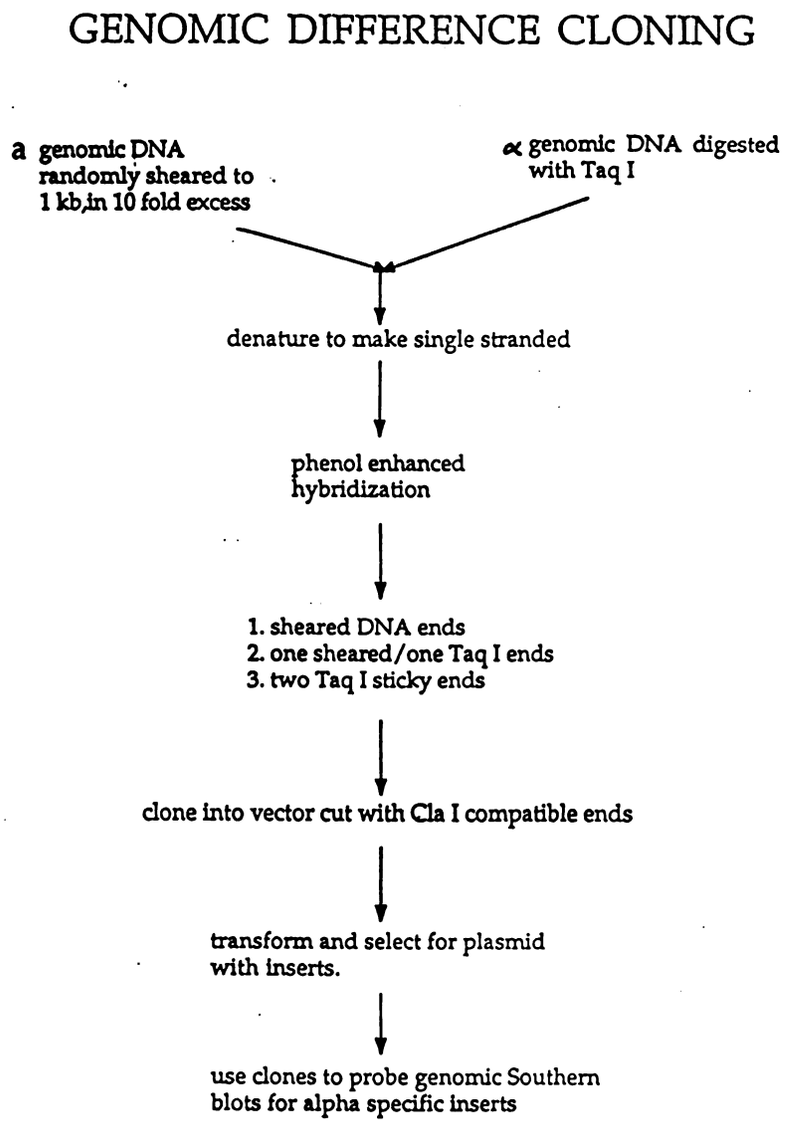
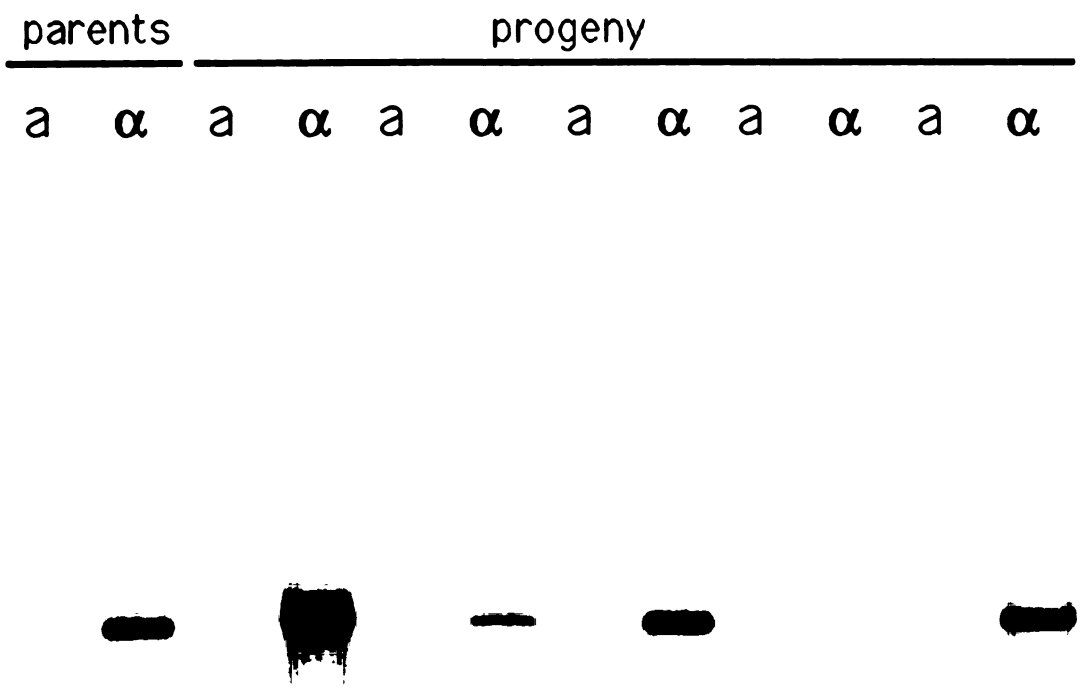


Figure 3-1. Genomic DNA was isolated from both *MATa* and *MATα C. neoformans* strains. The *TaqI* digested *MATα* DNA was mixed with a 10 fold excess of randomly sheared *MATa* DNA 1 kb fragments, denatured, and allowed to reanneal in the presence of phenol. The mixture was ligated to vector cut with *ClaI*, which is compatible with fragments with two *TaqI* cohesive ends. *E. coli* transformed with the ligation mixture were screened for plasmids containing *MATα* specific inserts.

Linkage analysis of α24, the *MATα* specific DNA fragment. To determine the linkage of clone α24 with the *MATα* locus, *MAT**a*** and *MATα* strains were crossed, and ten progeny analyzed for co-segregation of hybridization of clone α24 with mating type. *C. neoformans* strains JEC34 (*MAT**a** ura5*) and JEC50 (*MATα ade2*) were crossed and single basidiospores isolated. Genomic DNA was isolated from both parents and ten progeny (five **a**, five α). A Southern blot of genomic DNA from the parents and progeny was probed with α24 DNA (Figure 3-1). The probe hybridized to a single 12 kb *PstI* fragment in genomic DNA from the *MATα* parent and all *MATα* progeny, but no fragment was detected in DNA from the *MAT**a*** strains. Co-segregation of mating type and the α24 segment suggests that the α24 probe is at least closely linked to the *MATα* locus and may be a portion of the locus itself.

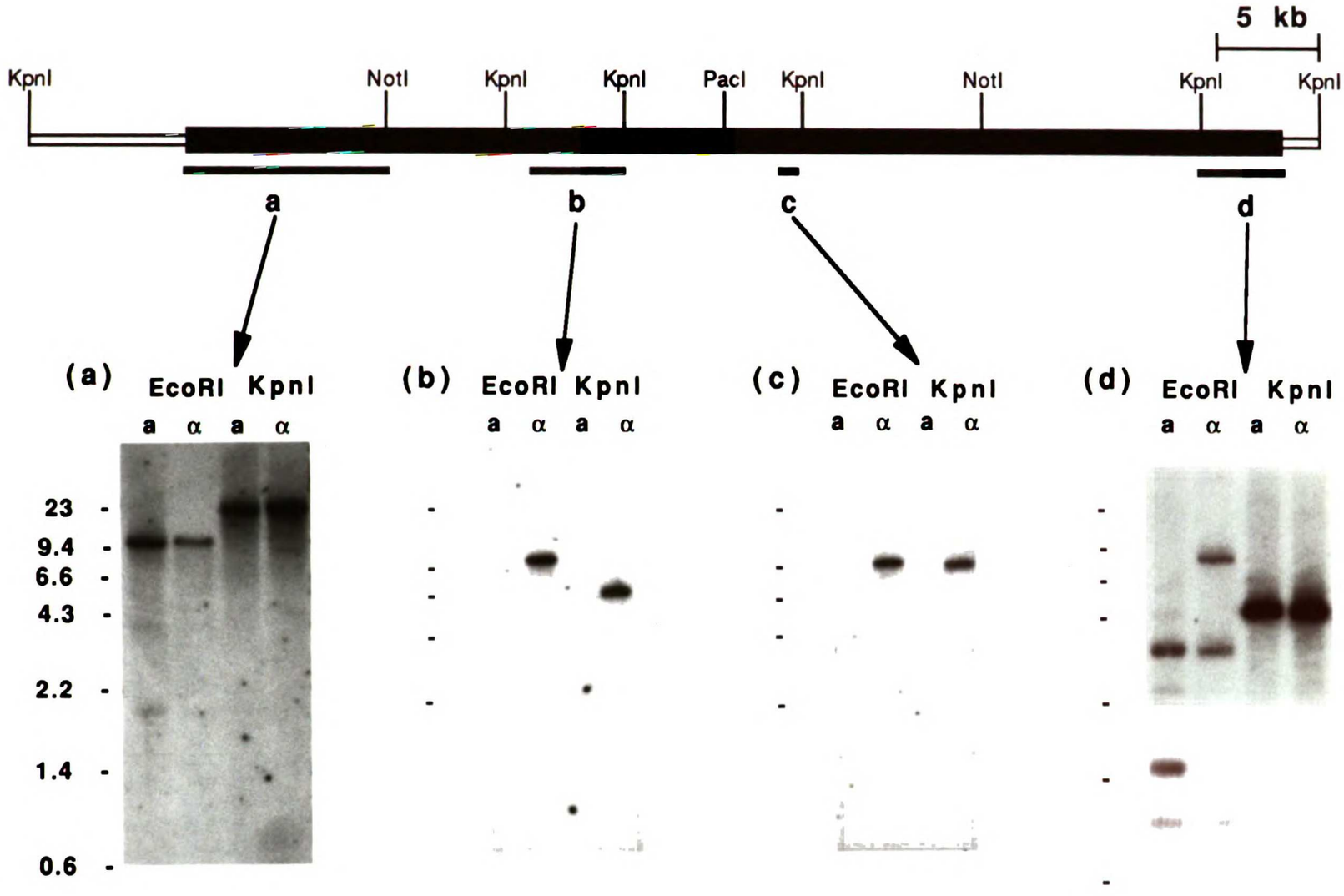
Isolation of the entire *MATα* locus. In order to isolate the entire *MATα* locus, a lambda phage *MATα* genomic DNA library was screened with the α24 probe, and a 2.1 kb α-specific DNA fragment was isolated. This 2.1 kb fragment was used to further screen phage and cosmid *MATα* genomic libraries. Seven overlapping phage and three overlapping cosmid clones were aligned and mapped using restriction enzyme and Southern blot analyses of cloned and genomic DNA.

Figure 3-2. *MAT*α -specific DNA segregates with mating type. Southern blot analysis was performed on *C. neoformans* genomic DNA isolated from the parents and ten progeny of a *MAT***a** *ura5* x *MAT*α *ade2* cross. DNA was digested with *PstI* and hybridized to digoxigenin labeled α24. Lanes 1 and 2, parent strains (1) *MAT***a** *ura5*, and (2) *MAT*α *ade2*. Lanes 3-12, progeny strains (3) *MAT***a** wild type, (4) *MAT*α wild type, (5) *MAT***a** *ade2*, (6) *MAT*α *ade2*, (7) *MAT***a** *ura5*, (8) *MAT*α *ura5*, (9) *MAT***a** wild type, (10) *MAT*α wild type, (11) *MAT***a** *ura5 ade2*, (12) *MAT*α *ade2*.


parents
progeny
a α a α a α a α a α a α

The *MATα* locus was found within five genomic *KpnI* fragments encompassing a region of 64 kb (Figure 3-3). The *MATα* locus itself is 35 - 45 kb in size. The exact size of the locus depends on the precise location of the junction between *MATα*-specific and *MAT*-nonspecific flanking regions within the 6 kb *NotI/KpnI* fragment from the left side and the 4 kb *KpnI* fragment from the right side. The locus contains *MATα* specific-DNA and repetitive DNA which hybridizes to numerous fragments in both *MATa* and *MATα* genomic DNA. The repetitive DNA sequences are dispersed throughout the *MATα* locus (data not shown). The Southern blots probed with two internal non-repetitive DNA fragments (Figure 3-3b and 3-3c) demonstrate mating type-specific hybridization since both fragments hybridized to a single fragment in *MATα* genomic DNA. Fragments from regions flanking the locus showed hybridization which was non-specific to mating type. For example, in Figure 3-3a the probe used was from a region outside the locus and hybridized to *EcoRI and KpnI* fragments of the same size in *MATa* and *MATα* DNA. In Figure 3-3d, however, the probe hybridized to both α-specific and non-specific fragments in a genomic Southern blot. The probe hybridizes to a 5.0 kb *KpnI* fragment present in both mating types. *EcoRI*-digested DNA shows a mixture of mating type-specific fragments (1.5 kb in *MATa* and 9.3 kb in *MATα*) and nonspecific fragments (1.0 and 3.0 kb). This restriction fragment length polymorphism suggests that the probe spans the junction region between the *MATα* locus and the

Figure 3-3. Restriction map of the *C. neoformans* α mating type locus. The thick lines represent the region present in overlapping cosmid and phage clones, thin lines are fragment sizes inferred from genomic Southern analysis. DNA fragments used as probes from regions throughout the locus are underlined. These fragments were used to probe *MAT**a*** and *MAT**α*** genomic DNA digested with *EcoRI* and *KpnI*. The Southern blot corresponding to each probe is shown; (a) 10 kb fragment, (b) 4.5 kb fragment, (c) 138 bp α24 fragment, (d) 4.0 kb fragment.


5 kb
KpnI
NotI
KpnI
KpnI
PacI
KpnI
NotI
KpnI
KpnI
a
b
c
d
(a)
EcoRI KpnI
a α a α
23
9.4
6.6
4.3
2.2
1.4
0.6
(b)
EcoRI KpnI
a α a α
(c)
EcoRI KpnI
a α a α
(d)
EcoRI KpnI
a α a α

flanking DNA outside the locus. These data demonstrated that an extensive *MATα* specific region and homologous flanking regions had been isolated.

Transformation of *C. neoformans* with *MATα* DNA. To identify regions containing genes responsible for mating type behavior, a series of constructs containing various fragments of the *MATα* locus were introduced into *MATa* and *MATα* strains (Figure 3-4). Strains transformed with vector only served as negative controls for the behavior of strains transformed with various fragments. Transformants were mated with MATα *lys2 ade2-27*, and MATa *lys2 ade2-27*. Mating can be assayed by the production of the hyphal phase. By this assay, no fragments resulted in transformants which have shown the ability to mate with both or neither mating type. All show mating behavior identical to their corresponding untransformed haploids. However, when isolated transformants were placed on V8 starvation medium alone, one segment of the locus was found to induce filaments (Figure 3-4). Filaments were formed only when transformants were transferred from SD medium onto V8 starvation plates. Southern analysis demonstrated that the *MATα* DNA was present in the *MATa*, filament-forming transformants and also in the non-filament forming *MATα* transformants (Figure 3-5). The active region was localized to a 2.1 kb *XbaI/HindIII* fragment which caused *MATa*, but not *MATα*, transformants to form hyphal filaments which were visible within 24 to 48 hours after starvation (Figures 3-4, 3-6).

Figure 3-4. Localization of the filament forming activity by transformation of *C. neoformans* with fragments from the *MAT*α locus. The *C. neoformans* strains JEC157 (*MAT*α *ade2 ura5 lys1*) and JEC169 (*MAT***a** *ade2 ura5 lys1*) were transformed by electroporation with various fragments of the *MAT*α locus and evaluated for filament forming activity on V8 starvation media. Fragments that enabled the *MAT**a*** recipients to form filaments are indicated with a (+) , those unable to confer filament forming ability are marked (-). Photographs show (b) a *MAT**a*** strain was transformed with the *MAT*α pheromone gene, and (c) *MAT**a*** cells transformed with pAUcos11 (vector) only. (d) a normal mating (cross of *MAT**a*** *ade2 ura5 lys1* and *MAT*α *lys2*).

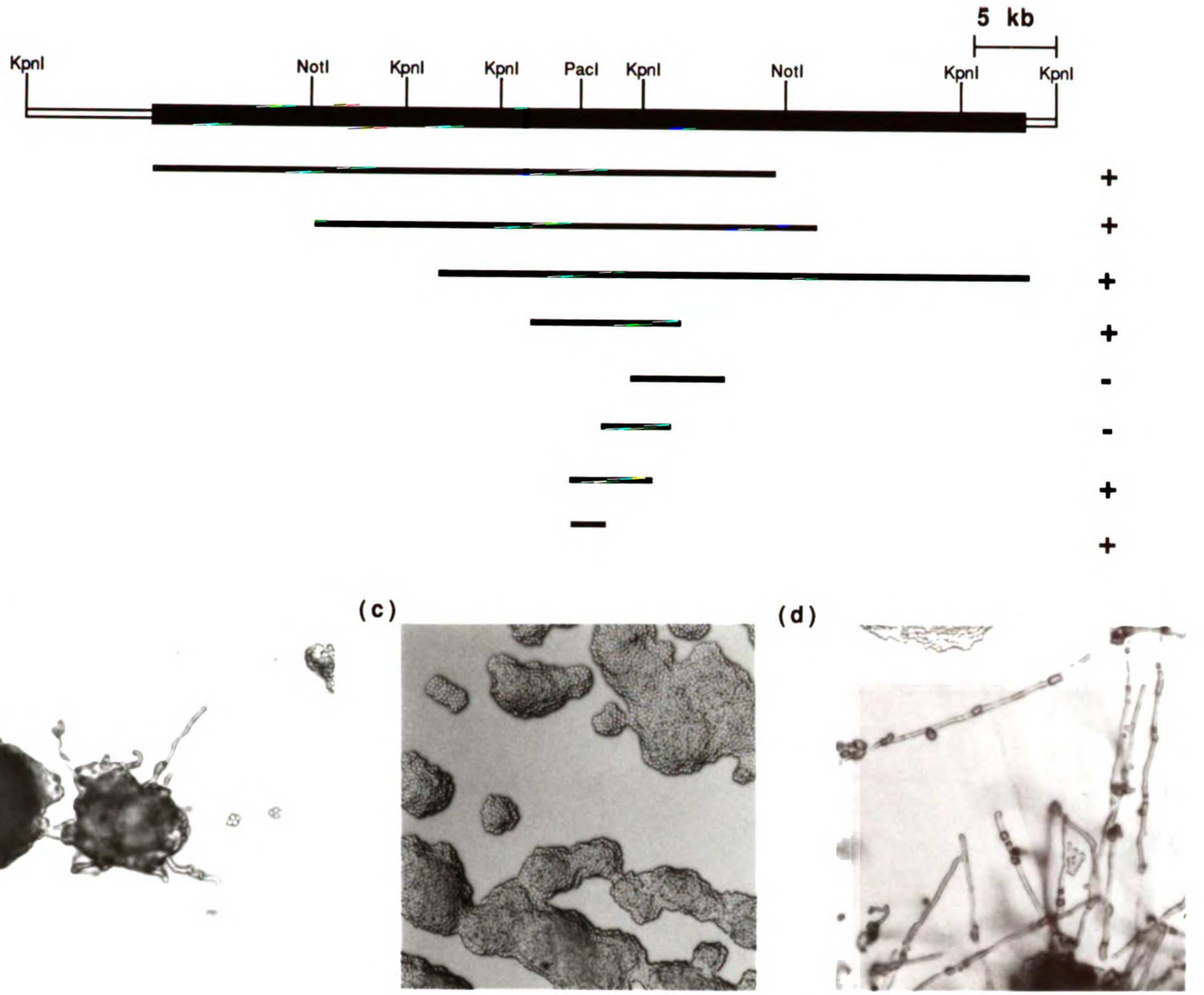
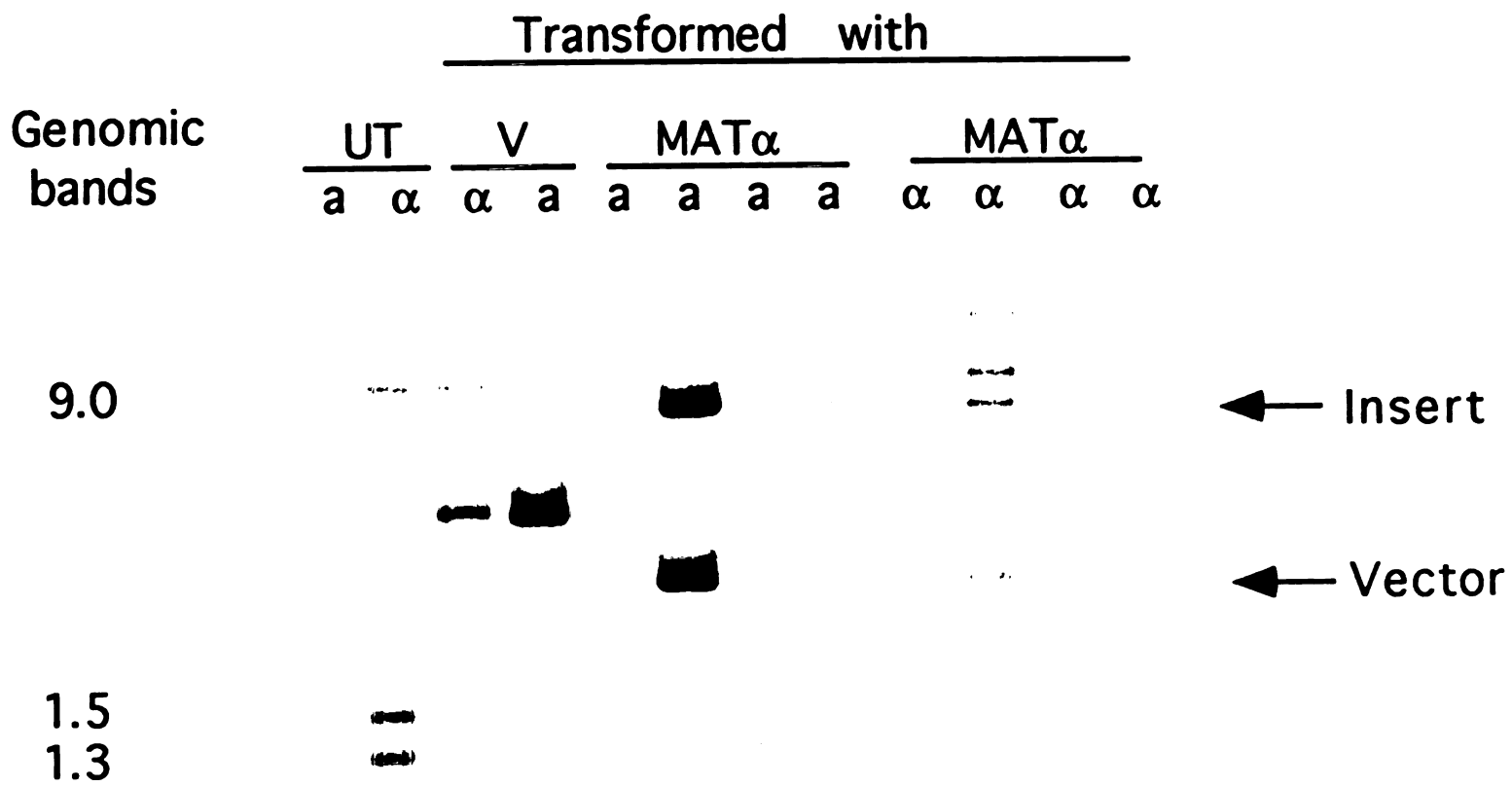

5 kb
KpnI
NotI
KpnI
KpnI
PacI
KpnI
NotI
KpnI
KpnI
+
+
+
+
-
-
+
+
(b)
(c)
(d)

Figure 3-5. Southern analysis of EcoRI digested DNA from *MATα ade2 ura5 lys1* and *MAT***a** *ade2 ura5 lys1* strains transformed with the *MATα* pheromone gene in the pAUcos11 vector. Control lanes show untransformed (UT) and transformed with vector only (V). The probe is a plasmid containing *MATα* specific DNA in a bluescript vector which hybridizes to three genomic EcoRI fragments of 9.0, 1.5, and 1.3 kb in size, plus the bluescript vector sequence contained in the pAUcos11 vector.


Transformed with
Genomic bands
UT
V
MATα
MATα
a α α a a a a a α α α α
9.0
1.5
1.3
Insert
Vector

Molecular analysis of the filament-inducing fragment. The 2.1 kb fragment which was able to direct filament formation was subcloned and sequenced (Figure 3-6). The nucleotide sequence contains a gene with a 114 bp open reading frame (ORF), which encodes a 38 amino acid peptide with similarity to the fungal mating factor precursors found in *Saccharomyces cerevisiae, Schizosaccharomyces pombe,* and *Ustilago maydis,* designated *MF*α (Figure 6; Refs. Bolker, Urban et al. 1992, Brake, Brenner et al. 1985, Davey 1992). All are similar in size with small ORFs encoding peptides between 36 and 44 amino acids in length. An asparagine residue is present at amino acid 24 of *C. neoformans* propheromone and is in a similar position to asparagine residues found to precede the amino terminal cleavage site in *S. pombe* **M**-factor and *S. cerevisiae* **a**-factor. The predicted polypeptide contains the prenylation motif C-A-A-X (where C is Cysteine, A is aliphatic, and X is any amino acid) at the carboxy terminus (Casey, Solski et al. 1989). This motif has been found in *S. cerevisiae* **a**-factors (MFA1 and MFA2), *S. pombe* mating **M**-factors (MFM1 and MFM2), and in the rhodotorucine A precursors, encoding the mating pheromone of *Rhodosporidium toruloides,* all of which are modified by the addition of a farnesyl group on the cysteine residue. The C-A-A-X in the **a**-factor precursor of *S. cerevisiae* is required for farnesylation, which is necessary for biological activity. The *C. neoformans* and *U. maydis* pheromone genes also contain this motif. The peptide pheromones themselves have yet to be isolated. Addition of a farnesyl group occurs in mating pheromones from a variety of fungi including *S. cerevisiae, Rhodosporidium toruloides, Tremella mesenterica, Tremella brasiliensis,* and *S. pombe* (Akada, Minomi et al. 1989, Glass, Vollmer et al. 1988, Ishibashi,

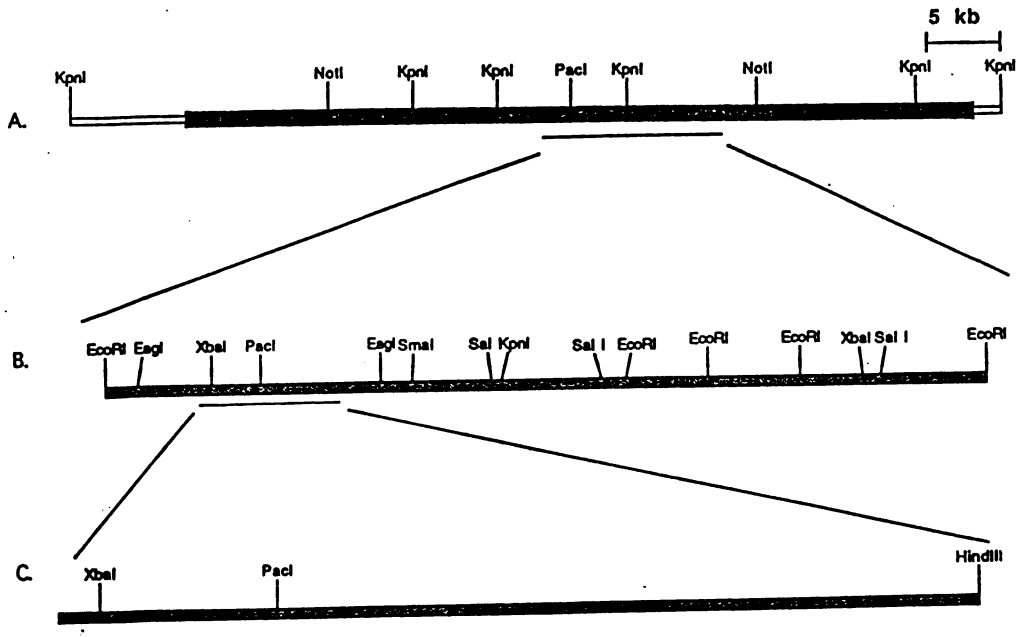


Figure 3-6. Location of the pheromone gene within the *MAT*α locus, (a) entire *MAT*α locus and surrounding cloned DNA, (b) restriction map of a 15 kb region of the *MAT*α locus, and (c) location and orientation of the sequenced region containing the pheromone gene.

```
HindIII
AAGCTTGGATGTGATGCTAAAGTGTCACAGGCGTCACGTCTCAGTCAGCGTACGGCGCTCAACTCGGATGATGTAGGACTCGACAA  -737
TAGAAAGTAAAGGTATCTAGTCTAGCAGAGCTAGATGGAGGAATTGCCAGGCTGACGAAACAGAATCTGGACTTATGAGGAACGAA  -651
TAGCCAGGCCTGAAGGAACAGGAGCTGGACTAATGAGCCTCGAGCTTTTTGTATGCCATTGCACTGTGGTGAGGGCACCCGGTAAT  -561
ACTTTGATGATAATTTAAGAGCTTGCCGAAAAACAATAAAGAACTACATATCCTGCCGGAGAACAATAGAACATTTTGAAAGCCTT  -479
TGCCGATTAAACAAAAAGACACATTAAACAAAAAGACACTCGGCGCTGATGCCAAAGAAACAATGAAAGAGAGAGGCCTTTTACGC  -393
TTTTTGCAGATTCCGCCAAAACCTAAGTTTTCTTTTGTTGTTTCTCCAGCATCAGCGCCGAGTGTCTTTTTGTTTAATCGGCAAAG  -307
GCTTTCAAAATGTTCTATTGTTCTCCGGCAGGATATGTAGTTCTTATTGTTCTTCGGCAAGCTCTCAAATTATCATCAAAGTATTA  -221
CCCGGTGCCCTCACCACAGTGCAATGGCATATAAAAAGCTCGAGGCTTCCCCCTTTTTCTCGCTCATTAGACAGCAACTCAATCCT  -135
CACTACCGACCAGCAACCAACCATCGCTACTCCACAACCAGTTACAATA-                                       -49
ATG GAC GCC TTC ACT GCC ATC TTC ACC ACC TTC ACT TCT GCC GCC ACT TCC TCT TCT               57
Met Asp Ala Phe Thr Ala Ile Phe Thr Thr Phe Phe Ser Ala Ala Thr Ser Ser Ser
GAA GCT CCT CGC AAC CAG GAG GCC CAC CCT GGT GGC ATG ACC CTT TGT GTC ATC GCC              114
Glu Ala Pro Arg Asn Gln Glu Ala His Pro Gly Gly Met Thr Leu Cys Val Ile Ala
TAGACCCAACGTCCCCTCTGCACGCGCGTGTTAGTCGCTATTCTACTTTGCAAAGCATTACGAAGTTGCTCATATTTAATCATAGC  200
TGGTATTCGCATCATATCGCAAGACTAGGTCGAAGCATATCGTCGGACAGATGGTAACGAAGCGTATGGGACTTGTTTAGATGGTA  286
TTGACATCATGTATAAATATAAAGTAGTCCATTTAGACAAATAAGAACGAAAGAAACAGTGGTCCAAAAGCATCATATCTACCATC  372
CAGCAGATCAATCCCTCGGTTTCAGGGAGACAAAATATACAAACAGAATCTAACTTATCTTTATATATTAATATTCCCCGTGTCAC  458
CTTTGAAAATTTCCTCACTTTTCGCCTTCGTTCTCATCCGTGACCCCCACAACCTCCCATTCAACCGCCACCGTTACGTAGGACGA  544
AAATCGTCTCTACATGTCTATACCCTTTTCAGACGTGCATGCATCGAACCATGGACATGAAAAACGTCGCCAAAAAAAATCGTGAC  630
CGTGCCTGAAGGCATGGATTTTTTTTTTTCATATGCATGGCGGATATCGCCGCCGCCCCGGTTTCATTAGGGAAGCTCACGATGCG  716
                                  PacI
ATTTTTTTTCAAAAATCGTCGATTTTTGGCGCTTAATTAACCATCGATTTTCCATCGATTTTTGATCGATTTTTGTTAATAATCCT  802
AATTACAAAAAAATCGATTAAAAAAAAATCGACCTTTTGACATCGATTTTCTATCGATTTTTGCCTGATGTCATGACATGGAACTT  888
CTCGCTTTTTCTGGAGATTTGAACCTCTGGTCATCTCTTTGTAGACGGAGATTTACCTAACTCCGAGGTCCAGAAATCGGTTTGAA  974
ATTATTAACGTCACCTGGGCCACTCACAGCTAAATCGCCGATTTTTATAAAAATCGATTAGAAAAAAAATCGCACCGTGAGCTTCC 1060
                                  XbaI
CTATTCGTCGTCGTTCGTGGAATAACCTTCCGTTTCTAGAACTTCCTTTACGTTTCTTCTTCTGTTATACGTATCTTCTCATGCCA 1146
CGGATTCCTGGACCTTCTACTGCACTATAAACTGCTTTAACT                                               1188
```

Figure 3-7. The nucleotide sequence of the 2.1 kb fragment from the *MATα* locus which induces filament formation in *MATa* cells. The sequence was found to contain an ORF which codes for a 38 amino acid peptide with homology to peptide mating factors in other fungi, and is printed below the nucleotide sequence. Long arrows show the 150 bp inverted repeats, short arrows show 16 bp repeat sequences. The -3 position is occupied by an adenine as is the case in 90% of filamentous fungal genes (Kinghorn 1987).

```
Cn-mfα....MDAFTAIFTTFTSAATSSSEAPR--------N-QEAHPGGMTL-CVIA
Sc-mfa1...MQPSTA--T-AAPKEKTSSEKKD--------N-YIIKGVFWDPACVIA
Sc-mfa2...MQPITTASTQATQKDK-SSEKKD--------N-YIIKGLFWDPACVIA
Sp-MFm1...MDSMANSVSSSSVVNAGNKPAQTLNKTVK--N-YTPKVPYM---CVIA
Sp-MFm2...MDSIATNTHSSSIVNAYNNNPTDVVKTGNIKN-YTPKVPYM---CVIA
Um-mfa1...MLSIFAQ-TTQTSASEPQQPTAPQGR-----N-DNGSPIGYSS-CVVA
Um-mfa2...MLSIFETWAAAAPVTVAETQQAS--------N-NENRGQPGYY-CLIA
```

Figure 3-8. Amino acid alignment of the *C. neoformans* putative pheromone precursor with the precursor peptides of *U. maydis, S. pombe,* and *S. cerevisiae*. *S. pombe* M-factor and *S. cerevisiae* a-factor each have two genes encoding the pheromone and are shown. The single copy pheromone genes from both the a1 and a2 mating types of *U. maydis* are shown. The CAAX prenylation motif and the conserved asparagine are shown in bold.

Sakagami et al. 1984, Kamiya, Sakurai et al. 1978, Sakagami, Yoshida et al. 1981).

Putative promoter elements TATAAA and CAAAT of the pheromone gene are shown in Figure 3-7. The region upstream of the ORF contains a pair of large (150 bp) inverted repeats, and three shorter repeats (16 bp). The shorter repeats in *C. neoformans* contain sequences which are similar to the 8 bp repeats found in the pheromone genes recently isolated from *U. maydis,* (ACAAAGGG) and may be involved in transcriptional regulation of the pheromone genes in response to opposite mating type pheromone or nutritional factors (Bolker, Urban et al. 1992).

Analysis of the 2.1 kb fragment containing the pheromone gene showed no other likely open reading frames. There were no large open reading frames and the small open reading frames did not have any similarity to fungal pheromone precursor genes.

DISCUSSION

Structure of fungal mating type loci. The mating type loci of fungi are complexes of genes with related function. The alternative alleles or complexes are not homologous in sequence or physical size and their structure has been compared to the mammalian immunoresponse loci (Egel 1992, Weissman and Singer 1991). The ascomycete *S. cerevisiae* has the best characterized of the fungal mating type loci. *S. cerevisiae* has a single locus with two alleles, **a** and α, which are co-dominant (Herskowitz 1988). The **a**

and α alleles code for the transcription factors at the top of the regulatory cascades which control cell type and mating functions.

Basidiomycete mating type loci structure and function. Higher basidiomycetes usually have two or more mating type loci, each containing more than one gene. In general, at least one allele at each locus must be nonidentical between the mating pair to maintain the dikaryon and progress through the sexual cycle. Each locus controls a specific set of developmental changes associated with mating or the dimorphic change (Banuett 1992, Metzenberg 1990). *Schizophyllum commune* has two loci, **A** and **B**, each containing two genes with multiple alleles. The **A** locus is responsible for nuclear pairing, conjugate nuclear and cell division, clamp connections, and cell septation, whereas the **B** locus controls nuclear migration and septal dissolution (Giasson, Specht et al. 1989). The A locus of *Coprinus cinereus* has recently been shown to be composed of two subcomplexes which contain a total of seven genes, four of which contain homeodomain-related motifs and are known to determine A-mating type specificity (Kues and Casselton 1992). The hemibasidiomycetous corn pathogen *Ustilago maydis* has two loci, **a** and **b**, but only the **b** locus has several alleles (thirty) and contains homeodomain related proteins. The two **a** alleles encode proteins directly involved in cell signaling and are proposed to control cell fusion, while the **b** alleles contain genes for the two homeodomain proteins which regulate the self/non-self recognition system, pathogenicity, and tumor production. Both loci are involved in maintenance of the filamentous state (Akada, Minomi et al. 1989, Bolker, Urban et al. 1992, Gillissen, Bergemann et al. 1992). *C. neoformans* appears to have a genetically less complex mating type system than the basidiomycetes discussed above, with a single mating type locus and

only two known alleles for that locus (Kwon-Chung 1976). However the large size of this locus suggests that many genes may reside within the region, and it may be comparable to both loci of *U. maydis*. The yeast-like cells of *C. neoformans* form a filamentous hyphal phase only after mating of opposite mating type cells.

***C. neoformans MATα* locus.** Several observations confirm that the *MATα* locus of *C. neoformans* has been isolated. The DNA is present in a single copy in *MATα* but not *MATa* strains and co-segregates with α phenotype in a cross (Figure 3-2). Southern analysis demonstrated the lack of any silent copies of alternate mating type information in the genome, which explains lack of mating type switching in *C. neoformans*. Subfragments of the region induce filaments only in *MATa* cells consistent with the introduction of *MATα* specific information (Figures 3-4, 3-6). The *C. neoformans MATα* allele is much larger, 35 to 45 kb (Figure 3-3), than previously isolated mating type alleles from other fungi, such as *S. cerevisiae* (**a**, 640 bp and α, 750 bp; Ref. Nasmyth and Tatchell 1980), *U. maydis* (**a1**, 4.5 kb and **a2**, 8 kb; Ref. Bolker, Urban et al. 1992) , *N. crassa* (**A**, 5.3 kb and **a**, 3.2 kb; Refs. Glass, Grotelueschen et al. 1990, Staben and Yanofsky 1990), and *S. pombe* (**P** and **M**, 1.1 kb; Ref. Kelly, Burke et al. 1988).

Filament forming activity in the *MATα* locus. To begin to elucidate the functions of specific genes within the *MATα* locus of *C. neoformans* and their role in the mating and filament formation, DNA fragments from the *MATα* locus were used to transform haploid cells of the opposite mating type. A 2.1 kb fragment from the *C. neoformans MATα* locus, after transformation into *MATa* cells, caused the transformants to produce filaments. This activity seems to be under appropriate control as filament production occurs only

when these transformants are starved. Presumably, other regions of the *MAT*α locus or complete diploidy are required to complete the entire sexual cycle as the filaments do not grow to normal lengths, produce basidia or form spores.

A pheromone gene in the *MAT*α locus. The 2.1 kb fragment that induces filament formation was found to contain a small ORF (114 bp) encoding a peptide with similarity to other fungal mating factors (Figures 3-7, 3-8). Pheromones have been described in a variety of fungi including *S. cerevisiae* , *S. pombe*, *U. maydis* , *R. toruloides*, and *Tremella* species (Abe, Kusaka et al. 1975, Bolker, Urban et al. 1992, Davey 1992, Duntze, MacKay et al. 1970, Ishibashi, Sakagami et al. 1984, Kamiya, Sakurai et al. 1978, Sakagami, Yoshida et al. 1981) . The two types of pheromone found in *S. cerevisiae*, **a**-factor and α-factor, are quite different in their gene structure, processing, and secretion. The mature *S. cerevisiae* **a**-factor is a farnesylated dodecapeptide which is required for mating (Michaelis and Herskowitz 1988) and thought to be transported out of the cell via a peptide export protein (STE6) with similarity to bacterial permeases, the mammalian multi-drug resistance protein and cystic fibrosis gene product (Michaelis, Chen et al. 1992, Raymond, Gros et al. 1992). The peptide encoded by the ORF found in the *C. neoformans* *MAT*α locus is similar to the *S. cerevisiae* **a**-factor and other fungal mating factors (Figure 3-8). The similarity includes the small size of the predicted peptide (all between 36 and 44 amino acids), the abundance of threonine, alanine and serine in the presumptive propheromone coding region, and a Cys-A-A-X motif (where A is aliphatic, and X is any amino acid) at the carboxyterminus (Casey, Solski et al. 1989). The last 4 amino acids of the predicted pheromone sequence are identical to those in *S. cerevisiae* and *S.*

pombe. This motif is the prenylation signal for a variety of proteins (Ras oncoproteins and the γ-subunit of trimeric G-proteins) and peptides (lipoprotein pheromones) (Schafer, Trueblood et al. 1990). Farnesylation, as directed by the C-A-A-X motif in *S. cerevisiae* **a**-factor precursor, is important for membrane localization, further propheromone processing, biological activity, and export (Schafer, Kim et al. 1989, Schafer, Trueblood et al. 1990). *S. cerevisiae* **a**-factor, *S. pombe* **M**-factor, and rhodotorucine A, are each modified by farnesylation, carboxymethylation, and N-terminal truncation. The tremerogens of *T. mesenterica* and *T. brasiliensis* are also carboxymethylated and farnesylated (Ishibashi, Sakagami et al. 1984, Sakagami, Yoshida et al. 1981). The peptide mating factor itself has not been isolated from *C. neoformans*, but processing and the structure of the mature pheromone can be predicted by analogy with the *S. cerevisiae* pheromone (Figure 3-9).

Control of pheromone gene expression. The pheromone genes in *S. cerevisiae* and *S. pombe* are not present within the mating type loci, and every cell has the genes for both sex-specific pheromones as would be required for a homothallic species. The synthesis of these pheromones is therefore under mating type control. *R. toruloides* has two mating types **A** and **a**, and, although it is not known if the three genes encoding rhodotorucine A are located in the mating type locus, the genes are present only in **A** strains (Akada, Minomi et al. 1989). In *C. neoformans*, as in **a** locus of *U. maydis*, a pheromone gene is located in the mating type locus and therefore present only in a single cell type. It is possible that pheromone gene control is under mating type and/or nutritional control.

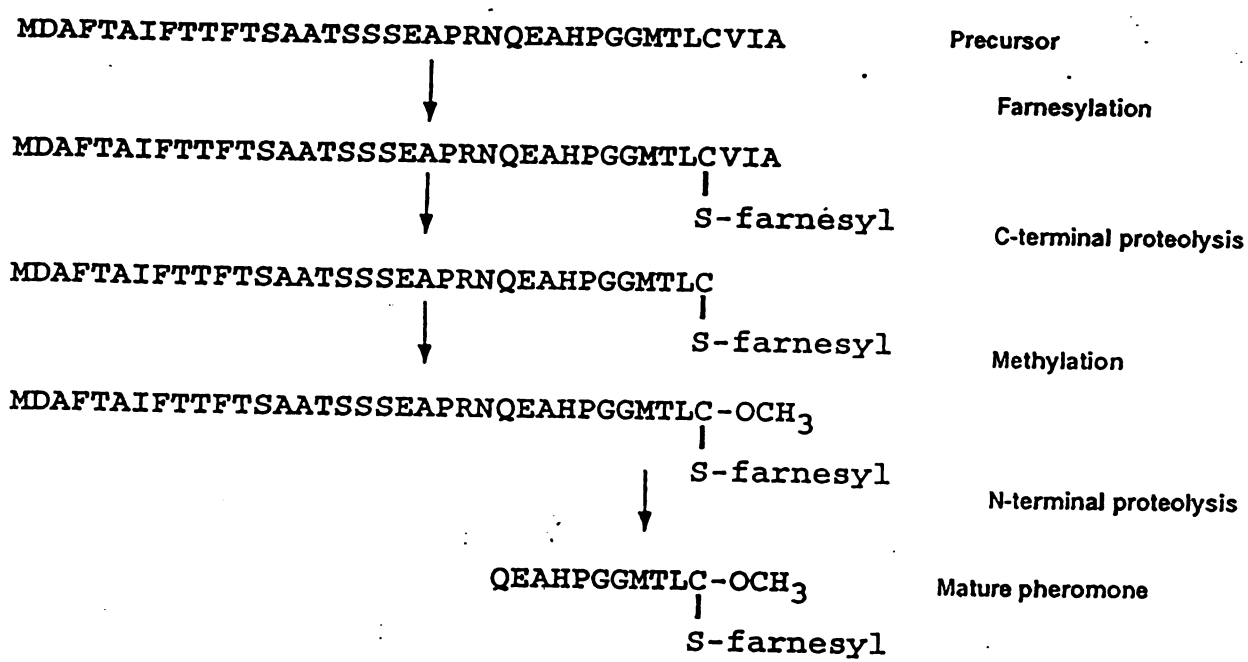


Figure 3-9. Predicted processing of the *C. neoformans* mating factor. The processing of the *C. neoformans* pheromone precursor gene is predicted by comparison to the genes and mature peptide pheromones of *S. cerevisiae* a-factor (2 genes) and *S. pombe* M-factor (2 genes). The carboxy-terminal processing shown here also occurs in *R. toruloides*.

Mating factor activities. Binding of mating factors to their receptors on cells of the opposite mating type in *S. cerevisiae* causes the cells to shmoo, exhibit morphological alterations, agglutinate, undergo G1 arrest, and induce genes required for cell and nuclear fusion (Marsh, Neiman et al. 1991). Upon exposure to its mating factor, the cells of *R. toruloides* form conjugation tubes oriented toward the opposite mating type cell (Kamiya, Sakurai et al. 1978). Tremerogens from *T. mesenterica* or *T. brasiliensis* **A** and **a** strains induce mating tube formation in the opposite mating type strain of the same species. *C. neoformans* has not been observed to undergo any of these responses as part of its normal mating process. The filament formation seen in the transformants may be an uncharacteristic block at an intermediate step.

Models for pheromone action in *C. neoformans*. It is possible that the filaments seen in *MAT**a*** *C. neoformans* cells transformed with the *MF*α gene are conjugation tubes, since closely related fungi such as *R. toruloides*, *T. mesenterica* and *T. brasiliensis* and *Ustilago violacea* form conjugation tubes in response to pheromone production by opposite mating type cells (Flegel 1981). Another possibility is that the production of the alpha-mating factor by transformed *MAT**a*** cells allows the cells to mate and fuse with each other but that the subsequent steps in filament production are blocked. In this case, it may be that heterozygosity of other mating type gene(s) is required for further filament production. *U. maydis* requires different alleles of some genes at both the **a** locus and the **b** locus for filament formation to occur. In this respect, *C. neoformans* may be similar to *U. maydis* but require heterozygous alleles at more than one gene in its single mating type locus. It may also be that the pheromone is involved in post-fusion events such as filament formation, and the haploid transformants respond weakly but need other

areas of the *MAT*α locus or complete dikaryosis to maintain filamentous growth. The *U. maydis* pheromone is expressed in the diploid cell, and its continued production is required for maintenance of filamentous growth. A pheromone receptor mutant in *U. maydis* cannot mate if it is a haploid, and cannot make filaments as a diploid (Bolker, Urban et al. 1992). The exact role that MFα plays in mating and/or filament formation in *C. neoformans* can be addressed with the identification of its gene and mature product.

Differences in mating type locus size and control. Many fungal mating type loci commonly encode regulatory proteins (Kues and Casselton 1992). In *S. cerevisiae*, *S. pombe*, and *N. crassa*, genes involved in mating are controlled by transcription factors encoded within their mating type loci. Because the smaller single mating type loci of both *S. cerevisiae* and *N. crassa* encode regulatory proteins, a relatively small mating type locus is able to control mating and cell type through the transcriptional regulation of a large set of genes dispersed throughout the genome. *U. maydis* has two loci. The **b** locus, similar to loci in the higher basidiomycetes *S. commune* and *C. cinerus*, encodes homeodomain-containing proteins which regulate self/nonself recognition. The **b** locus contains two multi-allelic genes, each of which contain variable N-terminal domains, constant C-terminal domains and homeodomain-related motifs (Gillissen, Bergemann et al. 1992). The **a** locus contains genes involved in cell signaling components and has only two alleles. These alleles contain genes for mating pheromones and the receptor for the opposite mating type pheromone (Bolker, Urban et al. 1992). For example, the **a1** allele codes for the **a1** pheromone and the receptor for the **a2** pheromone. Sex-specific transcription of these structural genes involved in mating depends solely on the presence or absence of the gene within each cell

type. This type of regulation is in contrast to *S. cerevisiae* which uses peptide pheromones and receptors to identify partners of the opposite mating type, but the genes for each pheromone and their receptors are present in both mating types and subject to mating type control. In *S. cerevisiae,* mating type-specific expression of these genes is controlled by the transcription factors encoded by the mating type alleles.

Unusually large mating type allele in *C. neoformans*. The *C. neoformans MAT*α allele encodes at least one gene whose product is directly involved in filament formation, a pheromone. This type of organization, where the sex-specific genes are found within the mating type locus, is similar to that of the **a** locus of *U. maydis*. *C. neoformans,* however, has only one locus controlling mating type, possibly the equivalent of a joined *U. maydis* **a**-**b** locus. It is highly probable that the unusually large *C. neoformans MAT*α locus contains more than the pheromone precursor gene identified here. It is likely to contain genes for a pheromone receptor, as well as genes for a transcription factor, or other genes involved in mating and the dimorphic change.

MATERIALS AND METHODS

C. neoformans* strains and media.** (See Table 3-1). The initial *MAT**a auxotrophic mutants were isolated from UV-mutagenized cells. Subsequent *MAT**a*** and *MAT*α strains were constructed by crossing auxotrophic strains and dissecting spore progeny with a micromanipulator. YEPD (1% yeast extract, 2% Bacto-peptone, 2% dextrose) was used as the maintenance medium. Synthetic dextrose (SD) contained 6.7 g/l of yeast nitrogen base

without amino acids, 20 g/l of glucose and appropriate amino acid or nucleotide supplements. V8 starvation crossing medium contained 5% V8 juice, 0.5 g/l KH_2PO_4, 4% agar adjusted to pH 7.0 with KOH.

Difference cloning of *MATα* specific DNA. Difference cloning was performed using the phenol-enhanced reassociation technique (PERT) and molecular cloning of specifically-reassociated molecules (Kunkel, Monaco et al. 1985). Genomic DNA was isolated from congenic wild type strains JEC20 (*MAT**a***) and JEC21 (*MATα*) (Edman 1992). The *MAT**a*** DNA was sheared to an average size of 1000 bp by sonication for 15 seconds of a solution containing 100 μg of genomic DNA in 500 μl. *MATα* DNA (5 μg) was digested to completion with *TaqI* restriction endonuclease at 65°C for 1 hour. After digestion, 2μl of 0.5 M EDTA, and 1 μl of 10 mg/ml proteinase K was added to the 20 μl digestion mixture, and incubated at 65°C for 30 min. For the reassociation reaction, 25 μg of sonicated *MAT**a*** DNA was mixed with 0.125 μg *MATα* DNA and heated to 100°C for 5 minutes, cooled on ice, and added to a final reaction volume of 250 μl in 7% phenol/ 1.25 M $NaClO_4$/0.12 M $NaPO_4$. The reassociation mixture was vortexed for 37 hours. After reassociation, the mixture was extracted twice with chloroform, dialyzed against TE (10 mM Tris HCl, pH 8.0 and 1 mM EDTA), ethanol precipitated, and resuspended in 10 μl TE.

Ligations were carried out in a 10 μl reaction volume containing 2 μl of the PERT-reassociated DNA, 25 ng of Bluescript digested with *ClaI*, and 1 unit of T4 DNA ligase (BM) at 25°C for 30 minutes and 4°C for 15 hours. JM101 electroporation competent cells were prepared and transformed according to published procedures (Dower, Miller et al. 1988). The cells were found to have a transformation efficiency of 1.0×10^9 transformants/microgram of DNA.

Preparation of *C. neoformans* DNA. *C. neoformans* genomic DNA was isolated from 100 ml cultures grown in SD containing adenine, uracil or both. The cells were collected by centrifugation and resuspended in 20 ml 1M sorbitol, 1% beta-mercaptoethanol, and 1 mg/ml lytic enzymes (Sigma), and incubated for 30 to 60 minutes. The spheroplasts were collected by centrifugation and lysed by incubation at 65° C in 10 ml of 50mM EDTA, 1% SDS, and 100 μg/ml RNase A for 30 minutes. Proteinase K was added to 167 μg/ml and the incubation continued for another 30 minutes. A one-third volume of 5M potassium acetate was added and the mixture was kept on ice for 30 minutes, clarified by centrifugation, and the supernatant precipitated with isopropanol. The DNA was resuspended in TE, heated to 65° C for 15 minutes, and precipitated with ethanol and 1/10 volume 7.5M ammonium acetate. Approximately 10 μg of genomic DNA was recovered per ml of culture.

Southern analysis. For Southern analysis, genomic DNA (5μg) was digested to completion with restriction enzymes (Boehringer Mannheim and New England Biolabs) and run on a 0.8% agarose gel, transferred to a nylon membrane and fixed by UV-crosslinking (Stratagene, Stratalinker). All membranes were hybridized with digoxigenin-labeled probes labeled according to manufacturer's instructions (Genius, Boehringer Mannheim). Blots were prehybridized at 42° C for two hours in 5X SSC, 50% formamide, 0.2% SDS, 5% Genius blocking reagent, and 0.1% N-lauroylsarcosine. Blots were hybridized overnight at 42° in the same mixture with the addition of the probe. All blots were washed according to the manufacturer's instructions and visualized using the chemiluminescent substrate AMPPD (Tropix).

Screening of phage and cosmid libraries. A *C. neoformans MATα* genomic library with 5 - 10 kb inserts prepared in lambda ZAP II (Edman and Kwon-Chung 1990) was screened using digoxigenin-labeled *MATα* specific probes. Phagemid were rescued from all libraries by coinfection of *E. coli* XL-1B with 10^7 PFU of lambda phage and 10^{10} PFU of R408 helper phage in 10 ml LB. After 6 hours shaking at 37°C, the culture was heated to 70° C for 30 min., clarified by centrifugation, and the supernatant containing rescued phagemid was saved. One ml of XL-1B cells were infected with 2 µl of the rescue supernatant. Plasmid DNA was harvested from the resulting colonies by boiling 5 hour cultures, and the resulting supernatant was used to transform HB101 competent cells.

A cosmid library was prepared by partial digestion of genomic *MATα* DNA with *XbaI, SpeI, NheI,* and *AvrII,* selection for 30 kb fragments on a sucrose gradient, and insertion into the *Xba I* site of pAUcos11, which contains sequences from Bluescript, the *C. neoformans ADE2* and *URA5* genes and a cos fragment (prepared by Dr. Haydee Torres-Guerro). Approximately 15,000 colonies were grown on 100 mm LB ampicillin (100 µg/ml) plates at 37°C until the colony diameter was 0.2 mm. Replica filters were prepared by pressing the lifted filter to a clean filter between filter paper and glass plates. The filters were placed colony side up onto LB ampicillin (100 µg/ml) plates and grown until the colonies were approximately 0.5 mm in diameter. The two replica filters were then placed colony side up onto chloramphenicol (250 µg/ml) plates for 20 hours to amplify the DNA. The filters were denatured, neutralized, washed in 2x SSC, and UV-crosslinked (Stratagene Stratalinker). The fixed filters were washed at 65° C in 0.1 X

SSC/0.1% SDS for 30 minutes, and then probed and developed as with the Southern blots.

Electroporation of *C. neoformans*. Strains JEC157 and JEC169 were grown overnight in YEPD at 30° C with shaking at 300 rpm. The cells were diluted 1 to 50 in fresh YEPD and then grown for an additional five hours at 30° C. The cells were washed in EB (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 270 mM sucrose) plus 1 mM DTT and resuspended in 0.5 ml EB. DNA constructs were linearized with *RsrI* or *PacI* prior to transformation. In the linear construct, the insert DNA is flanked by the markers *ADE2* and *URA5*. Prior to electroporation, 50 µl of cells were mixed with 100 to 500 ng sample DNA and 660 ng of salmon sperm DNA. The mixture was transferred to an 0.2 mm electroporation cuvette, and electroporated at 470 volts and 25 µF. Cells were plated immediately to SD plus lysine. Transformants were isolated on SD plus lysine to confirm adenine and uracil prototrophy and then used for further analysis.

Filament formation assay. Constructs containing *MAT*α DNA and vector alone as a negative control were transformed into *MAT**a** ade2 ura5 lys1* strains and streaked onto V8 medium and starved for 24 to 48 hours. These cells were then examined with a Zeiss microscope for the production of filamentous projections. All constructs were also transformed into *MAT*α *ade2 ura5 lys1* strains as a negative control.

DNA sequencing. Subclones used for sequencing were created as nested deletions or restriction fragments (Maniatis, Fritsch et al. 1981). Nested deletions were made by digestion with exonuclease III followed by mung bean nuclease treatment. Deletion clones were constructed in both directions (Stratagene). DNA sequencing was performed on double-stranded templates on an ABI model 470 DNA sequencer (operated by R. Chadwick) or with ^{35}S-dATP using a Sequenase kit.

CHAPTER 4 IDENTIFICATION OF GENES NECESSARY FOR MATING OR FILAMENT FORMATION IN *C. NEOFORMANS*

INTRODUCTION

One way to study mating in *C. neoformans* is to identify individual components of that system, the genes involved in conjugation and sexual dimorphism. Sterile or non-mating mutants are a means of isolating those genes which are involved in cell-cell recognition and conjugation (MacKay and Manney 1974, a and b). Mutant strains which do not undergo the dimorphic change upon mating are useful to identify genes necessary for this transition (Banuett 1991). Sterile mutants isolated in *S. cerevisiae* have enabled extensive analysis of the pathways involved in its mating process (Marsh, Neiman et al. 1991). The *S. cerevisiae* sterile mutants have been particularly important in delineating the pheromone response pathway (For reviews see Kurjan 1992, Marsh, Neiman et al. 1991), Herskowitz 1988). Non-filament producing haploid and diploid strains have been isolated from *U. maydis* (Banuett 1991). Diploid Fuz- strains, which are unable to form the hyphal phase, have helped in determining the roles of both mating type loci in filament formation (Banuett and Herskowitz 1989). Similarly haploid Fuz- mutants have been used in the identification of three genes distinct from the mating type loci that are required for filament formation in *U. maydis* (Banuett 1991). It is expected that sterile and non-filament producing mutants isolated in *C. neoformans* will be members of three genetic classes: 1) mutations in the mating type loci, 2) mutations in genes whose products are required for mating in one mating type only (mating type specific), and 3) mutations in genes whose products are required for mating or filament formation in both mating types (nonspecific). Examples of nonspecific gene products are those which are involved in a signal transduction pathway

Figure 4-1. A petri dish showing several crosses on V8 agar. White fuzz is the characteristic sign of the hyphal phase produced after mating. White patches show crosses in which the parents were able to mate and complement to form prototrophic dikaryotic hyphae. Non-filamentous patches show crosses incompatible in either mating type or auxotrophic mutations.

MATα lys2 X

MATa lys1 ura5

MATa lys1

MATa lys2

MATα lys1

MATa ade2

MATa ura5

common to both cell types, or more directly involved in formation of filamentous structures.

A genetic screen was devised to isolate non-mating and non-filamentous mutants (Nmf) in *C. neoformans*. Twenty-seven Nmf mutants of both mating types were isolated and 4 have undergone progeny analysis. Nmf mutants were transformed with the *MAT*α locus and analyzed either for complementation of the sterile defect (*MAT*α cells) or for production of filaments in the haploid when transformed with the pheromone gene (*MAT**a*** cells).

RESULTS

Auxotrophic strains were analyzed for mating frequency. The *lys1, lys2, ura5, met1,* and *arg1* strains, which can complement in the heterokaryotic hyphal phase, were mated by streaking together on V8 media in all possible combinations (see Table 4-1, Figure 4-1). The mated patches were compared in terms of the time for the hyphal phase to appear and the frequency of mating by the cells. Using this method, the best mating strains (fastest and highest frequency) were *lys1* strains mated with *lys2* strains. A defined minimal mating medium was then formulated using the *lys1, lys2* pair to assay mating on medium containing various amounts and types of carbon sources, nitrogen sources, vitamins, and minerals (Table 4-2). The resulting formulation of synthetic mating media (SMM) contains 2 grams maltose, 1.6 grams yeast nitrogen base without nitrogen or amino acids, 0.05 grams ammonium sulfate and 40 grams Bacto agar per liter of media. Mating was

induced on SMM medium between auxotrophic haploid cells and allowed complementation in the heterokaryotic hyphae at a high frequency. Mating and complementation by replica plating on the minimal medium was optimized to occur in 100% of the plated colonies (Figure 4-2).

TABLE 4-1 STRAINS

STRAIN	GENOTYPE	REFERENCE
JEC 30	MAT**a** *lys1*	chapter 2
JEC 31	MATα *lys1*	chapter 2
JEC 32	MAT**a** *lys2*	chapter 2
JEC 33	MATα *lys2*	chapter 2
JEC 50	MATα *ade2-27*	chapter 2
JEC 52	MATα *ura5 lys1*	chapter 2
JEC 53	MAT**a** *ura5 lys1*	chapter 2
JEC 61	MAT**a** *ade2-27*	chapter 2
JEC 155	MATα *ade2-27ura5*	chapter 2
JEC 113	MAT**a** *nmf1 lys1*	
JEC 114	MAT**a** *nmf3 lys1*	
JEC 115	MAT**a** Nmf57 *lys1*	
JEC 116	MAT**a** *Nmf79 lys1*	
JEC 117	MAT**a** Nmf85 *lys1*	
JEC 118	MAT**a** Nmf91 *lys1*	
JEC 119	MAT**a** Nmf96 *lys1*	
JEC 120	MAT**a** *nmf2 lys1*	
JEC 121	MAT**a** Nmf108 *lys1*	
JEC 122	MATα Nmf1α *lys2*	
JEC 123	MATα Nmf3α *lys2*	
JEC 124	MATα Nmf5 *lys2*	
JEC 125	MATα Nmf7 *lys2*	
JEC 126	MATα Nmf13 *lys2*	

Unless otherwise noted the strains in this table come from the work discussed in this chapter.

STRAIN	GENOTYPE	REFERENCE
JEC 127	MATα Nmf16 *lys2*	
JEC 128	MATα Nmf21 *lys2*	
JEC 129	MATα Nmf22 *lys2*	
JEC 130	MATα Nmf25 *lys2*	
JEC 131	MATα Nmf27*lys2*	
JEC 132	MATα Nmf31 *lys2*	
JEC 133	MATα Nmf36 *lys2*	
JEC 134	MATα Nmf51*lys2*	
JEC 135	MATα Nmf64*lys2*	
JEC 136	MATα Nmf66 *lys2*	
JEC 137	MATα Nmf74 *lys2*	
JEC 138	MATα Nmf75 *lys2*	
JEC 139	MATα Nmf80 *lys2*	
JEC 140	MATa *nmf1 lys1 ura5*	
JEC 141	MATa *nmf3 lys1 ura5*	
JEC 142	MATa Nmf57 *lys1 ura5*	
JEC 143	MATa Nmf79 *lys1ura5*	
JEC 144	MATa Nmf96 *lys1 ura5*	
JEC 145	MATa *nmf2 lys1 ura5*	
JEC 146	MATa Nmf108 *lys1 ura5*	
JEC 147	MATα Nmf5 *lys1 ura5*	
JEC 148	MATα Nmf21 *lys1 ura5*	
JEC 149	MATα Nmf31*lys1 ura5*	
JEC 150	MATα Nmf36*lys1 ura5*	
JEC 151	MATα Nmf64 *lys1 ura5*	
JEC 152	MATα Nmf66 *lys1 ura*	

Unless otherwise noted the strains in this table come from the work discussed in this chapter.

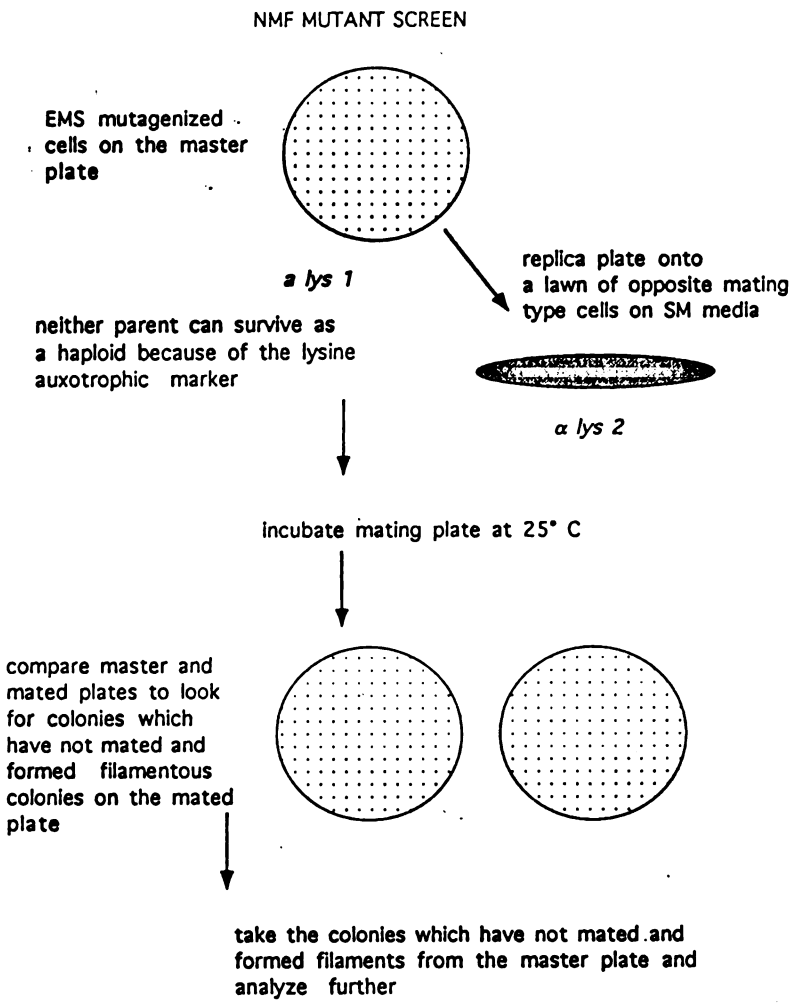


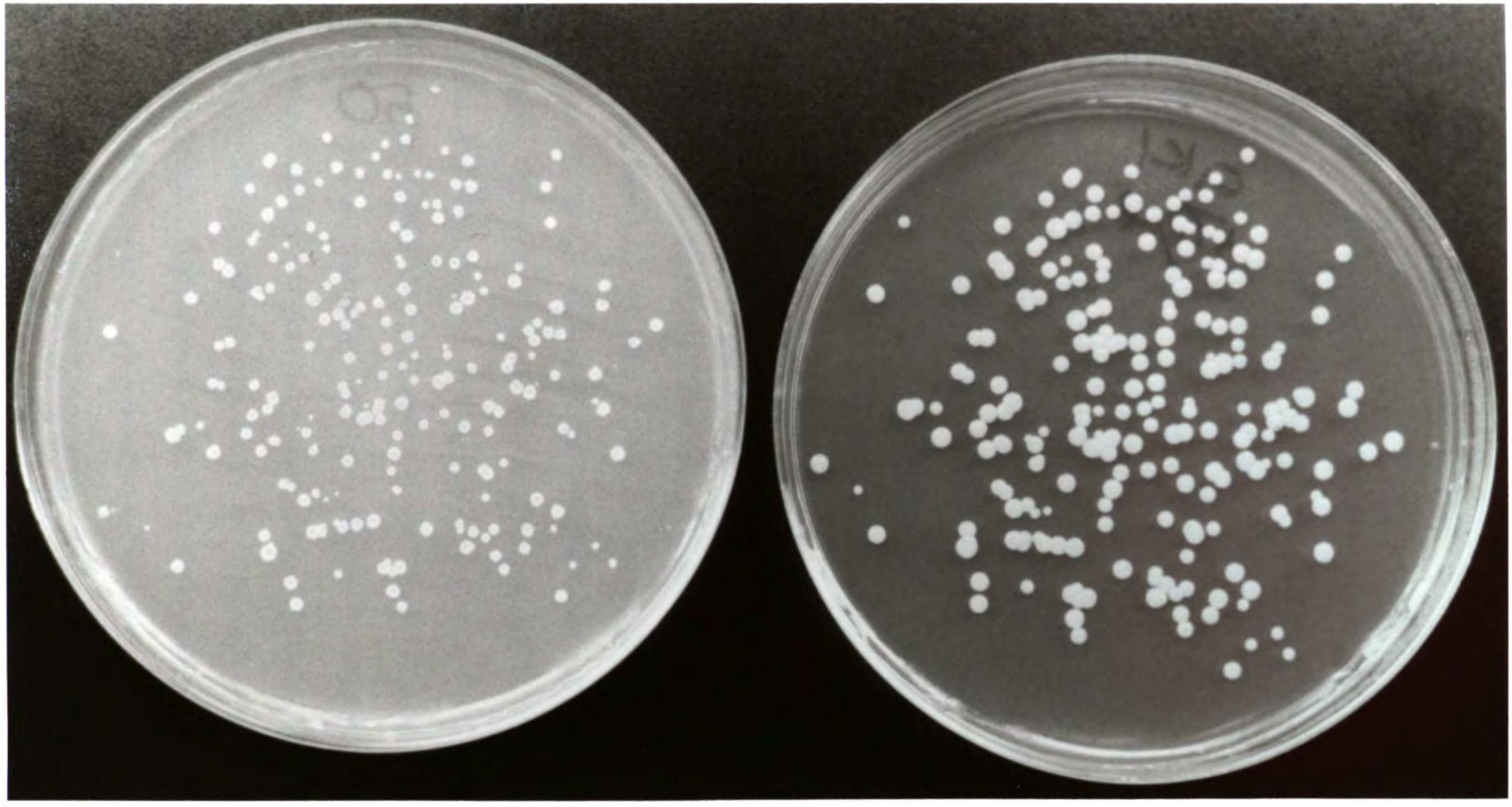
Figure 4-2. Isolation of *C. neoformans* nmf mutants. This genetic selection method identifies mutant strains which cannot produce prototrophic dikaryotic filaments when mated with a complementing strain of the opposite mating type. In the diagram a *MATa lys1* strain was mutagenized, and replica plated onto a lawn of *MATα lys2* cells on synthetic mating medium. The plates were incubated at 25 °C, and compared to the master plates to find colonies which have not mated or are not making filaments (nmf mutants). These colonies were analyzed further.

TABLE 4-2 MEDIA FORMULATION

Carbon sources	Nitrogen sources	Misc.
0.2% or 2%	0.005% or 0.05%	+ or -
Dextrose	Asparagine	vitamins
Glycerol	Glycine	trace minerals
Potassium Acetate	Ammonium Sulfate	
Sucrose		
Maltose	0.2% or 0.4%	
Starch	Yeast nitrogen base	

A genetic screen was used to isolate nmf mutants (Figure 4-2). A master plate containing colonies of EMS mutagenized *MATa lys1* cells was replica-plated onto a lawn of *MATα lys2* cells on mating media. For hyphal growth to occur, mating must take place between the mutagenized cells from the master plate and the lawn cells to form prototrophic heterokaryotic hyphae (figure 4-3). Colonies unable to mate or form filaments subsequent to mating were identified by the lack of a fuzzy white colony on the mating plate and removed from the master plate for further analysis. Those which retained the parental nutritional phenotype, did not revert with detectable frequency, and did not possess a petite phenotype were defined as non-mating or non-filamentous mutants (Nmf). An assay to quantitate the relative mating efficiency of the Nmf mutants was devised. The putative Nmf mutants were mixed in liquid culture with an excess of a mating tester strain. The mixture was plated to a SMM plate to quantitate mated hyphal colonies and also to a

Figure 4-3. Control plate showing the replica plate technique used to isolate nonmating and nonfilamentous mutants. Presence of a white fuzzy colony indicates that mating and formation of a prototrophic dikaryotic hyphae has occurred. This is a cross of *MAT**a** lys1* cells with an *MATα lys2* lawn.



titer plate which allowed growth of the nmf parent strain only. Using the replica plate selection method a total of 40,000 mutagenized colonies derived from both *MATa lys1* and *MATα lys2* strains have been screened, and twenty-seven nmf mutants have been characterized with mating efficiencies of less than 10% of the nonmutagenized control strain from which that mutant was isolated. (see Table 4-1).

Progeny analysis of the Nmf mutants. Analysis of spore progeny phenotype segregation was used to distinguish genetic defects which reside in the mating type locus from those outside the locus. Progeny analysis also distinguishes mutant genes which are mating type specific from mating type nonspecific mutations. Once identified, transformation of the mutants with phenotypes of interest with a *C. neoformans* library or mating type locus should allow recovery of the mutated gene by complementation.

The strains used to isolate the original sterile mutants each contained a single auxotrophic mutation in either the *LYS1* or *LYS2* gene. *C. neoformans* strains mate with higher frequency if the parent strains form prototrophs only when mated. Since the parents had a single nutritional defect, the spore progeny were all prototrophic, which makes the analysis of mating type and fertility undependable. For this reason, the original sterile mutants were plated to FOA media to select for mutations in the uracil biosynthetic pathway. Initial attempts at progeny analysis were unsuccessful in determining the mating phenotype of the spore colony isolates using the patch method of mating on V8 medium. Using this method, it was not possible to distinguish reliably the control strains which were known Nmf mutants or wild type maters. There is a quantitative difference in the mating frequency, but this difference is difficult to quantitate with this method and

will varies significantly. A second technique, the 24-well tissue culture plate method, was developed which was able to distinguish nmf from wild type mating strains. Using this method, 100% of the wild type strains formed filamentous colonies after mixture with the opposite mating type strain, either *MATα ade2-27* or *MATa ade2-27*. However, nmf mutant strains did not form hyphal colonies with either mating type tester using this method (figure 4-4). The spotting method was used for all future analysis. After mating the nmf strains to wild type mating strains (NMF), *MATa ura5 lys1* or MATα *ura5 ade2-27* hyphal mass suspensions were plated to minimal media, which allowed only recombinant *ura5* spores to grow.

A total of 96 recombinant spore progeny from each of four Nmf mutants have been analyzed to date (table 4-3). One mutant, *nmf1*, produces no progeny which mate as *MATa* and therefore appears to be in or closely linked to the *MATa* locus. The *nmf2* defect causes the Nmf phenotype in *MATa* cells only (*MATa* specific). The *nmf2* mutant crossed to a *NMF* strain results in 50% *MATα* progeny, 25% *MATa* progeny, and 25% *nmf* progeny. The last two mutants, *nmf3* and Nmf64 are mutations in genes which are nonspecific to mating type. It has not yet been determined if *nmf3* and Nmf64 are in the same or different complementation groups.

In the progeny analysis described above the spores plated onto media grew partly as yeast. However, some of the Nmf mutants did not produce all normal recombinant *ura5* spore progeny (JEC141, JEC143, JEC145, JEC148, JEC150, JEC151). Instead a proportion of spore colonies were crystalline in appearance and seemed to be filamentous and could not be analyzed for segregation patterns. These strains may produce a high frequency of diploid or dikaryotic spores.

Figure 4-4. The 24 well tissue culture plates filled with SMM medium (see materials and methods) used to assay Nmf phenotype and mating type. This assay was used to analyze the spore progeny of nmf mutants. Shown here is analysis confirming the nmf phenotype of the *MAT**a** nmf1* mutant, JEC141, after transformation studies in which the mutant formed filaments in response to transformation with the *MAT*α pheromone gene. One plate is a control cross of JEC53 (*MAT**a** lys1 ura5*) with *MAT**a*** and *MAT*α *ade2* strains. The other plate is the test cross of JEC141 (*MAT**a** lys1 ura5 nmf1*) with *MAT**a*** and *MAT*α *ade2* strains.

MATα ade2-27

MATa ade2-27

TABLE 4-3 PROGENY ANALYSIS

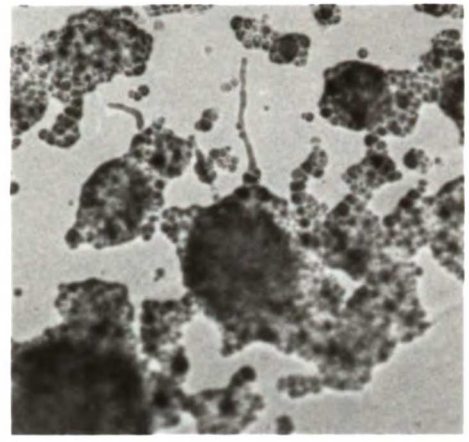
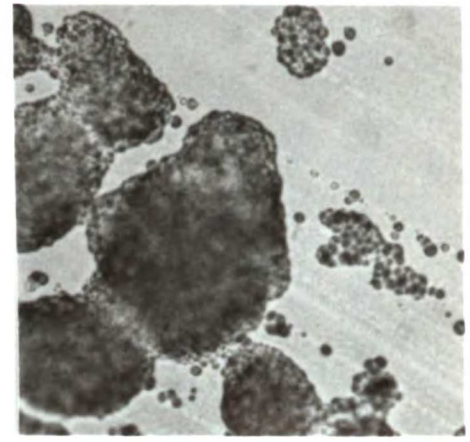
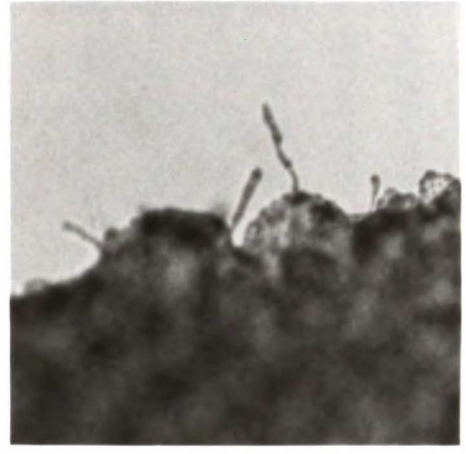
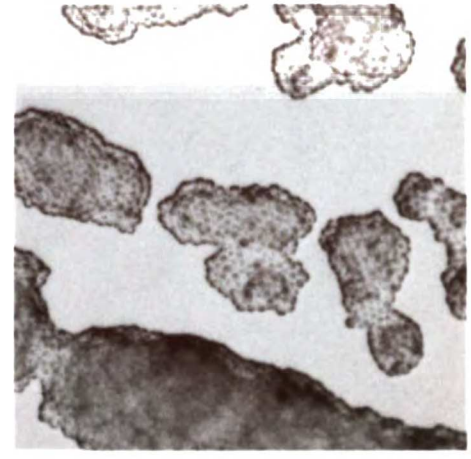
	ura5 spore progeny			
	MATa	MATα	NM	SF
MATα ura5 ade2-27 **x**				
MATa nmf1 lys1 ura5	0	67	56	0
MATa nmf3 lys1 ura5	30	18	45	3
MATa nmf2 lys1 ura5	27	50	22	1
MATa lys1 ura5 **x**				
MATα Nmf64 *lys2 ura5*	24	20	43	9

SF- self-fertile, NM- non-mater

Transformation studies with the nmf mutants. The nmf mutant hunt was originally intended to be used as a method for isolating the mating type loci. However, since the *MATα* locus was isolated by difference cloning, the locus and smaller segments containing the pheromone precursor gene could be used to transform the *MATα* nmf mutants to look for complementation (table 4-4). None of the *MATα* nmf mutants transformed to date were complemented by *MATα* DNA.

The *MATα* pheromone gene directs starvation-induced filament formation when transformed into haploid *MATa* cells, but not *MATα* cells. This result suggests that the machinery of pheromone maturation and secretion may be similar in the two mating types. The *MATa* nmf mutants

Figure 4-5. Transformation of *MAT**a** nmf1 lys1 ura5* with the *MAT*α pheromone and subsequent starvation induced filament formation by the transformed cells. *MAT**a** lys1 ura5* cells transformed with the pheromone gene are also shown. Cells transformed with vector alone, and starved do not form filaments.

	pheromone gene	vector
MAT**a** lys1 ura5 nmf1		
MAT**a** lys1 ura5		

were transformed with *MAT*α pheromone gene DNA fragments and the ability of haploid transformants to form filaments upon starvation was assessed (table 4-5). The *MAT**a** nmf1* mutant strain, forms filaments when transformed with the pheromone gene as does the wild type strain. It is interesting to note that although the *nmf1* strain cannot mate and form dikaryotic hyphae in the 24 well plate mating assay, it does respond to *MAT*α pheromone and is able to form the filament structures when transformed with the *MAT*α pheromone gene. Since the cell is responding to the opposite mating type pheromone introduced by transformation, it is likely that the mutant is not defective in the mating factor receptor or signal transduction machinery coupled to the receptor. Since the *nmf1* mutant can produce and secrete mature *MAT*α pheromone it is possible that there is so defect in the maturation or secretion of its own pheromone. It could be that the *nmf1* mutant strain has a defect in its own pheromone. The mutation could also be in a gene required in the fused cytoplasm of the dikaryotic cell to signal hyphal growth.

The remainder of the *MAT**a*** Nmf mutant strains were unable to form filaments following transformation with the pheromone gene (table4-5). A Nmf mutant strain unable to form filaments may lack a molecule which interacts with the pheromone, such as the *MAT*α pheromone receptor. A non-filament forming mutant may lack signal transduction machinery coupled to or downstream from the receptor for the *MAT*α pheromone which transduces the signal through the cell membrane. The defect may also be in a gene required to construct hyphal structures or maintain filamentous growth.

The nmf mutants were also analyzed for growth characteristics. Two MATα strains, JEC148 and JEC150, exhibit inhibited growth in liquid culture. These two strains have been resistant to transformation perhaps due to the growth defect.

TABLE 4-4 TRANSFORMATIONS WITH MATα LOCUS FRAGMENTS

	MATα complementation		
	v	2.1	4
MATα Nmf5 *lys2 ura5*	-	-	-
MATα Nmf31 *lys2 ura5*	-	-	-
MATα Nmf66 *lys2 ura5*	-	-	-

TABLE 4-5 TRANSFORMATIONS WITH THE MATα PHEROMONE GENE

	Filament formation
MATa nmf1 lys1 ura5	+
MATa nmf3 lys1 ura5	nd
MATa Nmf57 lys1 ura5	-
MATa Nmf79 lys1 ura5	-
MATa Nmf96 lys1 ura5	-
MATa Nmf2 lys1 ura5	-
MATa Nmf108 lys1 ura5	-

DISCUSSION

A genetic screen was used to isolate non-mating and non-filament forming mutants. Twenty-seven nmf mutants of both mating types were collected and partially characterized. All have mating efficiencies less than 10% of the parent strain from which they were derived. One mutation, *nmf1*, is in or very tightly linked to the *MAT**a*** locus. Another mutation, *nmf2*, is specific for mating and filament formation in *MAT**a*** cells. Two other mutants have defects which are nonspecific to mating type, (*nmf3* and Nmf64). Other nmf mutants were partially characterized by transformation with the *MAT*α locus in general and the *MAT*α pheromone gene in particular.

The initial nonmating or sterile mutants in the ascomycete *S. cerevisiae* were isolated by selecting for unmated haploids using a recessive canavanine resistance marker in the mutagenized parent strain (MacKay and Manney 1974, a and b). Mating type specific and nonspecific mutants of single copy genes were isolated. Mutants in pheromone genes were not isolated and it was later found that these pheromone precursor genes are present in multiple copies. Two mating type specific genes, *STE2* and *STE3*, were isolated, and found to encode the pheromone receptors of *MAT**a*** and *MAT*α cells respectively (Sprague, Jensen et al. 1983, Hagen, McCaffrey et al. 1986, Nakayama, Miyajima et al. 1987). Two mating type nonspecific mutants, *ste4* and *ste5*, which are part of the common signal transduction pathway, were also isolated. The *STE4* gene product has similarity to the mammalian G-protein beta subunit. Studies suggest that *ste5* may act downstream of the G-protein/receptor complex (MacKay and Manney 1974 a, Whiteway, Hougan et al. 1989, Hartwell 1980). Other sterile mutants were isolated in *S. cerevisiae* by

selecting for mutants which are unresponsive to pheromone induced growth arrest (Hartwell 1980). In this study, sterile mutants *ste2*, *ste4*, and *ste5* were re-isolated, in addition to *ste7*, *ste8*, *ste9*, *ste11*, and *ste12*. Subsequently isolated sterile mutants include three *MAT**a*** specific genes. The first, *STE6*, encodes a protein which has similarity to bacterial permeases and the mammalian multidrug resistance receptor, and may be responsible for pumping **a**-factor out of the cell (Rine 1979, Kuchler, Sterne et al. 1989). The other two are required for **a**-factor processing. *STE14* catalyzes the addition of a carboxymethyl group, and *STE16* is responsible for the addition of a farnesyl moiety to the **a**-factor precursor (Blair 1979, Wilson and Herskowitz 1987, Hrycyna and Clarke 1990). The *STE13* gene encodes a dibasic amino acid aminopeptidase involved in alpha factor processing. Another nonspecific sterile gene isolated in *S. cerevisiae*, *STE18*, has similarity to the mammalian G-protein gamma subunit. The sterile genes have been important in the elucidation of the cell-cell signaling mechanisms and dissection of the signal transduction pathway which is activated following pheromone binding.

Nonfilamentous or Fuz- mutants have been isolated in the basidiomycete *U. maydis*. The diploid Fuz- mutants were used to demonstrate the importance of heterozygosity at the **a** locus and to reconfirm the role of the **b** locus in the maintenance of the filamentous phase in this dimorphic fungus (Banuett and Herskowitz 1989). Haploid *U. maydis* Fuz- mutants were used to identify three new genes which are either distinct from the mating type loci (*Fuz1*, *Fuz2*), or linked but separable from the **b** locus (*rtf1*), and are required for filament formation (Banuett 1991). The two alleles of the **a** locus in *U. maydis* contain the genes for mating factors and the receptors for the

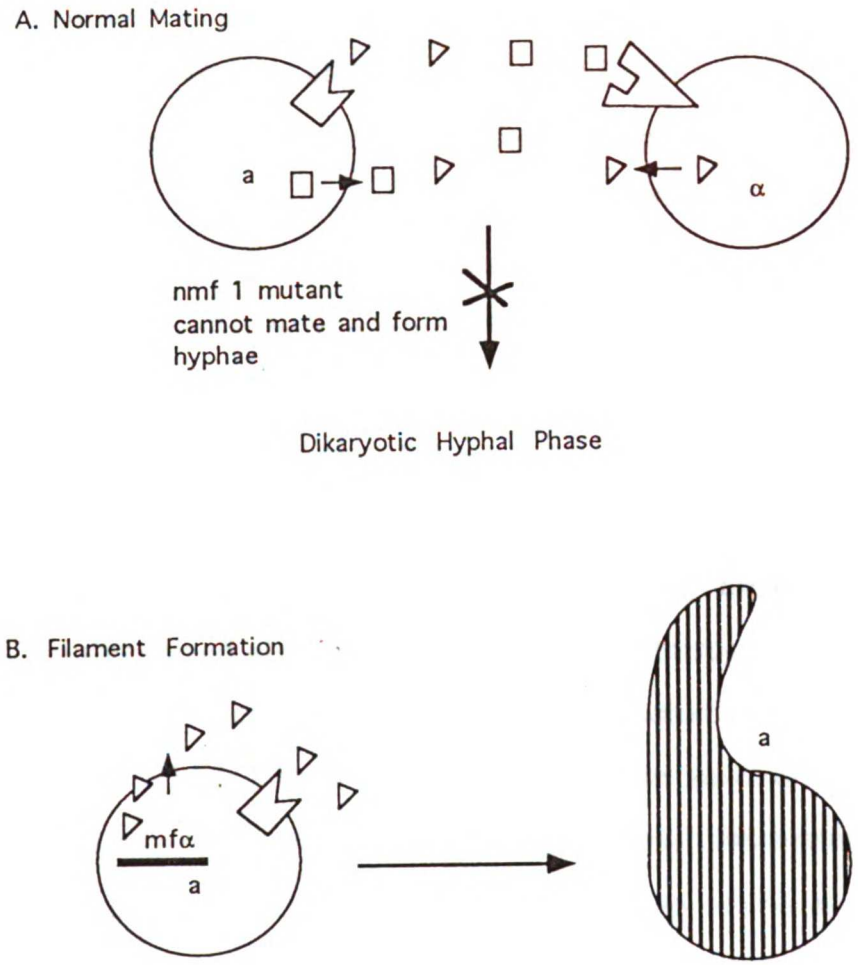


Figure 4-6. The phenotype of a nmf1 mutant. The mutant is unable to mate and form prototrophic hyphae (A). Progeny analysis has shown this mutant to contain a defect which segregates with the *MAT**a*** locus (see Table 4-3). A model of the transformation results in which he nmf1 mutant is able to produce, secrete and respond to *MATα* pheromone (B).

opposite mating type factor. Continuous stimulation of the pheromone receptors is required in *U. maydis* for maintenance of the filamentous state after mating (Bolker, Urban et al. 1992). It has not been rigorously proven that the pheromone or receptor are required for mating and fusion as well as maintenance of the filamentous state in *U. maydis* but it seems likely. Recently the *MAT*α locus in *C. neoformans* was isolated and found to contain a gene with similarity to mating factor genes in other fungi such as *S. cerevisiae, Schizosaccharomyces pombe,* and *U. maydis* (Chapter 3).

The screening process used here to isolate Nmf mutants in *C. neoformans* involves replica plate mating of opposite mating type strains with complementing auxotrophic markers on a newly formulated defined medium. Unmutagenized strains mate and form prototrophic heterokaryotic hyphae at 100% efficiency, forming white fuzzy colonies. This procedure identifies mutants which either cannot mate or after mating are unable to undergo the dimorphic change and form hyphal filaments. The *C. neoformans* mutants isolated were named nonmating or filament forming (nmf) mutants. The purpose for the isolation of these mutants is to study mating in *C. neoformans*. Several nmf mutants were isolated by this procedure. As is the case in the *S. cerevisiae* sterile mutants, the strains will mate and sporulate at greatly reduced frequencies, allowing the progeny of a Nmf^-/Nmf^+ cross to be analyzed.

One mutant, *nmf1*, appears to be in the *MAT**a*** locus or a very tightly linked mating type specific gene. The *nmf1* mutant phenotype segregates with the *MAT**a*** phenotype in a cross with a fertile strain. The *MAT**a*** spores may have a lower viability, since the *MAT*α spores account for slightly more than

50% of the spore isolates. The *nmf1* mutant strain forms filaments when transformed with the pheromone gene from the *MAT*α locus, the same gene that causes wild type haploid *MAT**a*** cells to form filaments when starved. The *MAT**a*** mutant strain can apparently produce, secrete, and respond to the *MAT*α pheromone This suggests that the defect is not in the pheromone receptor, response pathway, maturation process, or secretion process. It is possible that the mutation is in the *MAT*α pheromone gene itself, an observation that if found to be true would point to a single pheromone gene in *C. neoformans MAT**a*** strains (figure 4-5). The fact that the opposite mating type cell produces the pheromone suggests similar pheromone processing and secretion methods in both mating types.

The *nmf2* mutant was mating type specific. This mutant has a defect in a gene which appears from the progeny analysis to be required for mating and filament formation in *MAT**a*** but not *MAT*α cells, and is not linked to mating type. The *nmf2* mutant was also unable to form filaments in after transformation with the *MAT*α pheromone gene. Two other nmf mutants (*nmf3*, Nmf64) had mutations nonspecific to mating type, and their Nmf phenotype segregated into progeny of both mating types. The nonspecific Nmf mutants were unable to form filaments when transformed with the *MAT*α pheromone gene. The nonspecific Nmf mutants may have a defect in a common signal transduction pathway coupled to the pheromone receptor and are unable to respond to exposure to pheromone. Alternatively, the gene may be more directly involved in the formation of hyphal structures.

None of the *MAT*α nmf mutants which have been transformed with fragments from the *MAT*α locus including the pheromone gene have had their Nmf phenotype complemented.

A number of the *nmf* mutants produce fuzzy/crystalline spore colonies. In usual random spore isolation, the recombinant spores are isolated by plating the hyphal suspension on media which selects against the parental auxotrophic phenotype. In the case of JEC141, JEC143, JEC145, JEC148, JEC150, and JEC151, instead of isolating single yeast-like colonies, many of the colonies were crystalline looking and filamentous in nature. This spontaneous formation of hyphae on SD (minimal media), but not starvation medium suggests that the strains are not mating, which requires starvation, before forming filaments. This may indicate an unusually high proportion of diploid or dikaryotic spores which are filamentous upon germination. The nmf parent does not have a constitutive fuzzy phenotype, which suggests a problem in spore formation rather than filament control. It also may be that if the spores do contain two nuclei, the mutant gene or absence of the wild type gene somehow relieves the usual requirement of starvation before the filamentous phase is formed. Diploid or self-fertile spores have been described in *C. neoformans* (White and Jacobson 1985). *U. maydis* requires charcoal media to mate and form filaments in vitro. Constitutive fuzzy spore segregants which do not have this requirement have been described in *U. maydis*, but the mechanism is unknown (Banuett 1991).

A method for the isolation and identification of Nmf mutants was able to select several interesting mutants. The further characterization of these mutants and the isolation of other mutants should provide insights into the mating process of *C. neoformans*.

MATERIALS AND METHODS

Strains and media. See Table 4-1 for strains. YEPD (1% yeast extract, 2% Bacto-peptone, 2% dextrose) was used as the maintenance medium. Synthetic dextrose (SD) contained 6.7 g/l of yeast nitrogen base without amino acids, 20 g/l of glucose and appropriate amino acid or nucleotide supplements. V8 starvation crossing medium contained 5% V8 juice (Campbell soup company), 0.5 g/l KH_2PO_4, and 4% agar adjusted to pH 7.0 with KOH. Synthetic mating media (SMM) contained 2 grams maltose, 1.6 grams yeast nitrogen base without amino acids, 0.05 grams ammonium sulfate and 40 grams Bacto agar per liter of media.

Synthetic mating medium. A synthetic medium was devised that would support mating of *C. neoformans* and complementation of the mating strains. The medium contains 2 grams maltose, 1.6 grams yeast nitrogen base without nitrogen or amino acids, 0.05 grams ammonium sulfate and 40 grams Bacto agar per liter of medium. The SM medium was formulated by testing mating pairs on several mixtures which varied with respect to type and amount of carbon and nitrogen sources. The pH was also varied, as was the amount of vitamins and minerals.

EMS mutagenesis. 50 ml cultures of JEC30 (alys1) or JEC44 (αlys2) were grown for two days at 30 ° C in a shaking incubator, and harvested by centrifugation at 5 K for 5 minutes. The harvested cells were washed once in 50 ml Phosphate buffered saline (PBS), resuspended in 5 ml of PBS and titered by

diluting and plating the suspension to YEPD plates. The mutagenesis reaction contained 1.4 ml of the cell suspension, 2.0 ml of PBS, and 0.1 ml of EMS. The mixture was contained in a 12 ml screw cap tube and incubated in a shaking incubator at 30° C. After 1.5, 2.0, 2.5, and 3.0 hours one ml aliquots were taken and added to 40 ml of $Na_2S_2O_3$. The mutagenized cells were harvested by centrifugation at 5 K for ten minutes, resuspended in 10 ml sterile water, and titered for percent cells killed. Titer plates were replica plated from rich to minimal media to assess the percent induced auxotrophic mutation frequency. The time points used varied between 2 and 3 hours, which exhibited cell killing at 90 to 98 percent and auxotrophic mutation frequencies of 2% to 7%.

Nmf mutant isolation procedure. EMS mutagenized cells were plated to YEPD media at densities of 100 cells per plate in a volume of 100 microliters and incubated at 30° for 15 hours. Lawn cells were grown in YEPD for 15 hours at 30° C in a shaking incubator. The lawns were prepared by plating 100 microliters of the culture to a 100mm petri plate of synthetic mating media (SMM) and then inverting and pressing the plate onto the replica-plating block three times, rotating the plate 120° each time. The master plates were then replica-plated onto the lawn of the complementary auxotrophic strain and opposite mating type first, and then onto a YEPD plate. Master and replica YEPD plates were incubated at 30°C while the mating plates were incubated at 25°C. After 2 to 3 days, the mating plates were compared to the YEPD plates to look for non-filamentous colonies which failed to mate and form a prototrophic filamentous colony. Control strains, *MAT**a** lys1* or *MAT*α *lys2* identical except that they have not undergone mutagenesis, mated at 100

percent efficiency under these optimized conditions. Non-filamentous colonies were removed from the YEPD plate and analyzed further for petite mutants, auxotrophic mutants and switched mating type, and retested for sterility on V8 media (the standard undefined mating media). After these tests, remaining strains were assayed using the mating efficiency assay.

Mating efficiency assay. Cultures of 1 ml of each sterile to be tested, the positive control and the tester strain, *MATα ade2-27* or *MATa 1ade*, were grown 15 hours in YEPD, in a 30°C shaking incubator. The steriles and positive control were diluted 1:200. Five microliters of the diluted samples were mixed with 0.5 ml of the mating tester strain in a 6 ml culture tube, vortexed to mix, and 10 microliters were spread to SMM and SD+lys plates. The mated colonies on the SMM plate were counted and divided by the number of colonies on the SD+lys titer plates to give the percent of sample cells which have mated. Strains which mated with less than 10% of the unmutagenized strains were used for further analysis.

Nmf mutant progeny analysis. Analysis of the segregation of the progeny of the Nmf mutants was undertaken to determine if the mutations isolated were specific or nonspecific to mating type, and if the mutations were linked to mating-type. *C. neoformans* must be starved to mate, and even then the mating reaction is far from vigorous. The strains used for the isolation were complementing auxotrophic mutants and resulted in unusually vigorous mating. However, difficulties arose when trying to analyze the mating phenotype of the spore progeny of Nmf mutants. For this reason, FOA was used to induce a Ura- mutation in the Nmf strains. The Nmf mutants were

plated onto FOA media +ura +lys (1 gram FOA, 6.7 gram yeast nitrogen base w/o amino acids, 20 grams dextrose, and 20 grams Bacto agar per liter of media), which selects for Ura- mutants. Of the twenty-eight Nmf mutants isolated, 6 **a** strains and 7 α strains were made Ura-. The newly created *MAT**a** lys1 ura5 nmf* strains were mated with an *MAT α ura5* strain to produce hyphae, basidia, and spores. A portion of the hyphal mat was removed from the plate and resuspended in 0.5 ml of sterile water, and 0.1 ml of the mixture was plated to SD+ura to select for the ura- spores only. From the Ura- spore progeny, 96 isolated colonies were selected and grown overnight at 30°C in 0.2 ml SD+ura liquid media in a 96 well tissue culture plate. Each spore culture was then mixed 1:1 with the mating testers *MAT**a** ade2-27* and *MAT α ade2-27*, and 5 microliters of each mixture spotted onto one well in a 6 well tissue culture plate which had been filled 1/2 full of SMM. The plates were incubated at 25°C for 2 days and then analyzed to note if they had mated, and if so, what mating type they were.

Electroporation of *C. neoformans*. Strains JEC157, JEC169, and all Nmf strains were grown overnight in YEPD at 30° C with shaking at 300 rpm. The cells were diluted 1 to 50 in fresh YEPD and then grown for an additional five hours at 30° C. The cells were washed in EB (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 270 mM sucrose) plus 1 mM DTT and resuspended in 0.5 ml EB. DNA constructs were linearized with *RsrI* or *PacI* prior to transformation. In the linear construct, the insert DNA is flanked by the markers *ADE2* and *URA5*. Prior to electroporation, 50 µl of cells were mixed with 100 to 500 ng sample DNA and 660 ng of salmon sperm DNA. The mixture was transferred to an 0.2 mm electroporation cuvette, and electroporated at 470 volts and 25 µF. Cells were plated immediately to SD plus lysine. Transformants were

isolated on SD plus lysine to confirm adenine and uracil prototrophy and then used for further analysis.

Filament formation assay. Constructs containing *MAT*α DNA, the *MAT*α pheromone gene, or vector alone were transformed into *MAT***a** *ade2 ura5 lys1* and nmf strains. Transformed cells were streaked onto V8 medium and starved for 24 to 48 hours and the cells examined with a Zeiss microscope for the production of filamentous projections. All constructs were also transformed into *MAT*α *ade2 ura5 lys1* strains as a negative control.

CHAPTER 5 SUMMARY AND PROJECTIONS

SUMMARY

Cryptococcus neoformans is a basidiomycetous human fungal pathogen. Mating type in *C. neoformans*, a heterothallic yeast, is controlled by one locus with two alleles, *MAT**a*** and *MAT**a***. Little is known on a molecular level about the progression of *C. neoformans* through its sexual cycle. *MAT*α strains occur more frequently in nature and are more virulent than *MAT**a*** strains. To begin a molecular and genetic study of mating in *C. neoformans*, a standard set of auxotrophic and drug-resistant strains in a common genetic background was constructed. The *MAT*α locus was isolated to identify the genetic differences between mating types and their contribution to virulence. The locus was found to contain a gene, MFα1 encoding a pheromone precursor similar to the lipoprotein mating factors found in *Saccharomyces cerevisiae*, *Ustilago maydis* and *Schizosaccharomyces pombe*.

The mating type loci of fungi are complexes of genes with related function. The alternative alleles are not homologous in sequence or physical size between mating types and these limited genomic differences have been compared to the more expansive genomic differences of sex chromosomes (Egel 1992). The ascomycete *S. cerevisiae* has the best characterized of the fungal mating type loci. *S. cerevisiae* has a single locus with two alleles, **a** and α, which are co-dominant (Herskowitz 1992). The **a** and α alleles code for the transcription factors at the top of the regulatory cascades which control cell type and mating functions. Because the smaller single mating type loci of *S. cerevisiae* encode regulatory proteins, the relatively small mating type locus is able to control cell type and mating functions through the transcriptional regulation of a large set of genes dispersed throughout the genome. Many

basidiomycetes have two multiple mating type loci, each which can contain more than one gene. In general, at least one allele at each locus must be nonidentical between the mating pair to maintain the dikaryon and progress through the sexual cycle. *Schizophyllum commune* has two mating type loci, each of which controls a specific set of developmental changes associated with mating or the dimorphic change. The **A** locus is responsible for nuclear pairing, conjugate nuclear and cell division, clamp connections, and cell septation, whereas the **B** locus controls nuclear migration and septal dissolution (Casselton 1978). Another basidiomycete, the corn pathogen *Ustilago maydis* has two loci, **a** and **b**. The **a** locus encodes cell signaling components proposed to control cell fusion; the **b** locus contains genes regulate self/non-self recognition and pathogenicity (Banuett 1991). The **b** locus contains two genes, each encoding a protein with a variable N-terminal domain, a constant C-terminal domain and a homeodomain-related motif (Gillissen, Bergemann et al. 1992). Models have been proposed for both *U. maydis* and *S. commune* in which a homeodomain protein encoded by the mating-type locus of each partner is required to form a heteromultimeric regulatory species which regulates the developmental events associated with mating (Herskowitz 1992, Kues and Casselton, 1992). This is analogous to the **a1**-α2 heterodimer which is formed after mating in *S. cerevisiae* and controls diploid cell type. It is interesting to note that only the diploid *S. cerevisiae* cell is able to form pseudohyphae in response to starvation. The situation is different at the **a** locus in *U. maydis* where the two alleles contain genes for mating pheromones and the receptor for the opposite mating type pheromone. For example, the **a1** allele codes for the **a1** pheromone and the receptor for the **a2** pheromone (Bolker, Urban, *et al.*, 1992). Sex-specific

transcription of these genes which are directly involved in mating behavior depends solely on the presence or absence of the gene within the genome of each cell type. *S. cerevisiae* has similar peptide pheromones and receptors which are used to identify partners of the opposite mating type, but the genes for the pheromones and receptors are present in the genomes of both mating types. In *S. cerevisiae,* mating type specific expression of the pheromone and receptor genes is controlled by the transcription factors encoded by the mating type alleles. *C. neoformans* appears to have a genetically less complex mating type system than the basidiomycetes discussed above, with a single mating type locus and only two known alleles for that locus.

Several observations confirm that the *MAT*α locus of *C. neoformans* has been isolated. The DNA is present in a single copy in *MAT*α but not in *MAT**a*** strains and co-segregates with α phenotype in a cross. Southern analysis demonstrates that silent copies of alternate mating-type alleles are not present within the genome. The lack of archival mating type loci can explain the failure to observe mating type switching as is seen in *S. cerevisiae.* Subfragments of the region induce filaments only in *MAT**a**,* cells consistent with the introduction of *MAT*α-specific information. The *C. neoformans* *MAT*α allele is much larger, 35 to 45 kb, than previously isolated mating type alleles from other fungi, such as *S. cerevisiae* (**a**, 640 bp and α, 750 bp; ref. Nasmyth and Tatchell, 1980), *U. maydis* (**a1**, 4.5 kb and **a2**, 8 kb; ref. Bolker, Urban, *et al.*, 1992) , *N. crassa* (**A**, 5.3 kb and **a**, 3.2 kb; ref. Glass, Grotelueschen, et al., 1990, Staben and Yanofsky, 1990), and *S. pombe* (**P** and **M**, 1.1 kb; ref. Kelly, Burke, et al., 1988).

The *C. neoformans* *MAT*α allele encodes at least one gene whose product is directly involved in filament formation, a pheromone. To begin to

elucidate the functions of specific genes within the *MAT*α locus of *C. neoformans* and their role in mating behavior and filament formation, DNA fragments from the *MAT*α locus were used to transform haploid cells of the opposite mating type. A 2.1 kb fragment from the *C. neoformans MAT*α locus, after transformation into *MAT**a*** cells, caused the transformants to produce filaments. This activity seems to be under appropriate control as filament production occurs only when these transformants are starved. The 2.1 kb fragment that induces filament formation contains a small ORF (114 bp) encoding a peptide with similarity to other fungal mating factors. The last four amino acids are identical to *S. cerevisiae* and *S. pombe,* where they function as a farnesylation signal. Pheromones have been described in a variety of fungi including *S. cerevisiae* , *S. pombe, U. maydis* , *R. toruloides,* and *Tremella* species (Brake, Brenner et al. 1985, Kurjan and Herskowitz 1982, Julius, Brake et al. 1984, Davey 1992, Bolker, Urban, *et al.*, 1992, Akada, Minomi et al. 1989, Glass, Vollmer et al. 1988, Ishibashi, Sakagami et al. 1984, Kamiya, Sakurai et al. 1978, Sakagami, Yoshida et al. 1981) .

The two types of pheromone found in *S. cerevisiae* , **a**-factor and α-factor, are quite different in their gene structure, processing, and secretion. The mature *S. cerevisiae* **a**-factor is a farnesylated dodecapeptide which is required for mating (Michaelis and Herskowitz, 1988). This peptide is thought to be transported out of the cell via a peptide export protein (STE6) with similarity to bacterial permeases, the mammalian multi-drug resistance protein and cystic fibrosis gene product (Michaelis, Chen et al. 1992, Raymond, Gros, et al. 1992). The peptide encoded by the ORF found in the *C. neoformans MAT*α locus is similar to the *S. cerevisiae* **a**-factor and other fungal mating factors. The similarity includes the small size of the peptide (all between 36

and 44 amino acids), the abundance of threonine, alanine and serine in the presumptive propheromone coding region, and a Cys-A-A-X motif (where A is aliphatic, and X is any amino acid) at the carboxylterminus (Casey, Solski, et al. 1989). In fact, the last four amino acids of the predicted *C. neoformans* peptide are identical to those in the *S. cerevisiae* and *S. pombe* precursors. This motif is the prenylation signal for a variety of proteins (Ras oncoproteins and the γ-subunit of trimeric G-proteins) and peptides (lipoprotein pheromones) (Schafer, Trueblood, et al. 1990, Clarke 1992). Farnesylation, as directed by the C-A-A-X motif in *S. cerevisiae* **a**-factor precursor, is important for membrane localization, further propheromone processing, biological activity, and export (Schafer, Kim, et al. 1989, Schafer, Trueblood, et al. 1990,). *S. cerevisiae* **a**-factor, *S. pombe* **M**-factor, and rhodotorucine A, have been shown to be modified by farnesylation, carboxymethylation, and N-terminal truncation. The tremerogens of *T. mesenterica* and *T. brasiliensis* are also undergo these hydrophobic modifications (Ishibashi, Sakagami et al. 1984, Sakagami, Yoshida et al. 1981).

Binding of mating factors to their receptors on cells of the opposite mating type in *S. cerevisiae* causes the cells to shmoo, agglutinate, undergo G1 arrest, and induce genes required for cell and nuclear fusion (Marsh, Neiman, et al. 1991). Upon exposure to its mating factor the cells of *R. toruloidies* form conjugation tubes toward the opposite mating type cell (Kamiya, Sakurai et al. 1978). Tremerogens specific for *T. mesenterica* or *T. brasiliensis*, and for **A** or **a** strains induce mating tube formation in the opposite mating type strain of the same species. It is possible that the filaments seen in *MATa C. neoformans* cells transformed with the *MFα1* gene are conjugation tubes, since closely related fungi such as *R. toruloidies*,

T. mesenterica and *T. brasiliensis* and *Ustilago violaia* form conjugation tubes in response to pheromone production by opposite mating type cells (Flegel 1981). Peptide pheromone genes have been isolated in both *U. maydis* and *C. neoformans,* but no morphology changes have been observed in either organism after exposure to opposite mating-type cells (Bolker, Urban, *et al.,* 1992). It may be that heterozygosity of other mating type gene(s) are required for further filament production. Presumably, other regions of the *MAT*α locus or two nuclei of opposite mating types are required to complete the entire sexual cycle as the filaments do not grow to normal lengths, produce basidia or form spores. In contrast to *S. cerevisiae,* the *U. maydis* pheromone continues to be expressed in the diploid cell and its production is required continually for filamentous growth. A pheromone receptor mutant in *U. maydis* cannot mate if it is a haploid, and cannot make filaments as a diploid (Bolker, Urban, *et al.,* 1992). The exact role that the pheromone plays in mating and/or filament formation in *C. neoformans* is not understood. The result that *MAT***a** cells can transcribe, modify, and secrete the *MAT*α pheromone in response to starvation suggests that gene products which are common to both mating-type cells are involved in these processes. These findings also suggest that the production of pheromone is under a type of nutritional control which is common to both cell types.

The pheromone genes in *S. cerevisiae* and *S. pombe* are not present within the mating type loci. Each cell of either mating-type contains the genes for both sex-specific pheromones, however, the synthesis of these pheromones is under mating type control. Since the *C. neoformans* pheromone gene is located in the mating type locus and therefore present only in a single cell type, it could be under mating type and/or nutritional

control. This type of organization, where the sex specific genes are found within the mating type locus is similar to the **a** locus of *U. maydis*. *C. neoformans*, however, has only one locus controlling mating type. It is possible that the single large locus of *C. neoformans* is functionally analogous to a fusion of both the **a** and **b** loci of *U. maydis*. In any event it is likely that the *C. neoformans MATα* locus contains more than the pheromone precursor gene identified here. The unusually large size of the *C. neoformans* α-mating type allele suggests that additional genes required for determining cell type and mating behavior may be encoded directly in the mating type locus, possibly a pheromone receptor, transcription factor, or other genes.

To identify genes in *C. neoformans* involved in mating and the subsequent dimorphic change, non-mating and non-filament forming (Nmf) mutants were isolated. The replica plate method identified strains derived from EMS mutagenized auxotrophic parents which are unable to mate and form prototrophic dikaryotic hyphae. Using this method, twenty-seven Nmf mutants of both mating-types were isolated. Of the mutants in which progeny have been analyzed, *nmf1* has a defect in the *MAT**a*** locus, another is defective in a gene which causes a Nmf phenotype only in *MAT**a*** cells (*nmf2*). Another produces a Nmf phenotype in either mating-type (*nmf3*, Nmf64). The *nmf1* mutant was able to form filaments following transformation with the *MATa* pheromone gene. This result indicates that the *nmf1* mutant is able to produce and secrete mature pheromone, respond to the pheromone, and form the filament structures. Therefore, the defect is not in the peptide processing enzymes, the receptor, or the secretory machinery. The receptor for the *MAT**a*** pheromone and its coupled signal

transduction apparatus are also functional. These characteristics are consistent with a defect in the *MAT**a*** pheromone gene.

The *MAT*α locus was isolated to begin to study mating in *C. neoformans* and the contribution of the locus to virulence. The unexpectedly large locus was found to contain a putative pheromone gene. This discovery provides new information about the organization of the mating-type locus and cell-cell signaling in *C. neoformans*. The locus is expected to contain more genes, which as they are identified will provide more insight into both these processes. It is too early to tell if mating type genes will form a focus for therapeutic research, but the identification and study of the genes within *MAT*α locus should allow the analysis of the mechanisms of cell-type specific expression in *C. neoformans* as well as insight into mechanisms of virulence in cryptococcal meningitis.

FUTURE DIRECTIONS

Other *MAT*α locus genes. The *MAT*α locus of *C. neoformans* is approximately 40 kb, and the pheromone gene has been identified. It is highly probable that other genes are present within the locus and it may be possible to identify one or more of these by transforming *MAT*α locus fragments which do not contain the pheromone gene into *MATa* strains and observing the transformants for filament production. Both the receptor and pheromone genes in the *U. maydis* a1 and a2 alleles were identified by the same filament forming response when transformed into strains unable to mate. Either the receptor or the pheromone gene were sufficient to confer a mating behavior upon a strain containing the opposite *MAT* allele. Another approach to identifying genes in the *MAT*α locus is to look for homeobox-containing genes which may be within the *MAT*α locus. Degenerate nucleotides modeled on the consensus basidiomycete homeodomain sequences can be used to probe the cloned *MAT*α locus.

Isolation and structure identification of the *MAT*α pheromone. To study the mature structure of the pheromone and its effects on cells of the opposite mating type, it would be useful to isolate the peptide pheromone itself. The native pheromone may be accessible to purification from overproducing *C. neoformans* strains. Alternatively, the effects of pheromone may be studied using chemically synthesized pheromone, designed using the consensus modification sites which are known in *S. cerevisiae, S. pombe, R. toruloides,* and *Tremella* species.

Isolation of the *MATa* locus. Fungal mating type loci can be physically defined as regions of nonhomologous DNA sequences flanked by homologous regions. Now that the entire *MAT*α locus and flanking regions have been isolated, the *MATa* locus can be isolated simply by using the homologous flanks as probes. This would enable a comparison to be made between the mating types. It will be interesting to see if the same transformation assay which was used to identify the *MAT*α pheromone gene will be useful in isolating analogous genes from the *MATa* locus. The mating type alleles of the a locus in *U. maydis*, **a1** and **a2**, were found to be symmetric in that either allele produced an analogous response when transformed into appropriate strains. It may also be possible to complement the *MATa* mutant, *nmf1* and isolate the *MATa* pheromone gene.

More genes involved with mating. The partially characterized nmf mutants may be used in several ways to study mating. One mutant appears to be in the *MATa* locus, which could be exploited to identify a *MATa* specific gene by complementation. The other mutants, which appear to be mating type nonspecific, could also be transformed with genomic or cDNA libraries to isolate and identify the exact genes that are mutated, and are required for mating and/or filament formation.

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