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# GENETICS OF *CRYPTOCOCCUS NEOFORMANS*

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■ **Abstract** *Cryptococcus neoformans* is a pathogenic fungus that primarily afflicts immunocompromised patients, infecting the central nervous system to cause meningoencephalitis that is uniformly fatal if untreated. *C. neoformans* is a basidiomycetous fungus with a defined sexual cycle that has been linked to differentiation and virulence. Recent advances in classical and molecular genetic approaches have allowed molecular descriptions of the pathways that control cell type and virulence. An ongoing genome sequencing project promises to reveal much about the evolution of this human fungal pathogen into three distinct varieties or species. *C. neoformans* shares features with both model ascomycetous yeasts (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*) and basidiomycetous pathogens and mushrooms (*Ustilago maydis*, *Coprinus cinereus*, *Schizophyllum commune*), yet ongoing studies reveal unique features associated with virulence and the arrangement of the mating type locus. These advances have catapulted *C. neoformans* to center stage as a model of both fungal pathogenesis and the interesting approaches to life that the kingdom of fungi has adopted.

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## INTRODUCTION

*Cryptococcus neoformans* is a fungal pathogen that infects the central nervous system to cause meningoencephalitis that is uniformly fatal if untreated [reviewed in (18, 111, 126)]. The organism is largely an opportunistic pathogen but can also be a primary pathogen in a small cohort of patients with no apparent immune system defects. The majority of afflicted patients have compromised immune function as a consequence of HIV infection, steroid therapy, cancer, chemotherapy, or therapy to suppress rejection of transplanted solid organs. The existing arsenal of antifungal agents is limited, and toxic side effects and the emergence of drug resistance are significant impediments to effective therapy.

*C. neoformans* is a basidiomycete fungus, and is therefore quite distinct in evolution from ascomycete fungi such as the model yeast *Saccharomyces cerevisiae*, the fission yeast *Schizosaccharomyces pombe*, and many other common human fungal pathogens, including *Candida albicans*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Coccidioides immitis*, and *Pneumocystis carinii*. Instead, *C. neoformans* is more similar to other basidiomycetes, including the corn and grain plant fungal pathogens *Ustilago maydis* and *Ustilago hordei*, the *Tremella* wood-rotting jelly fungi, and the model mushrooms *Coprinus cinereus* and *Schizophyllum commune*. *S. cerevisiae* and *C. neoformans* have been diverging for ∼500 million years, and thus, studies have the potential to reveal molecular principles conserved between these two divergent fungi as well as those that make them unique.

*C. neoformans* has a defined life cycle that involves vegetative growth as a budding yeast combined with the ability to undergo filamentous dimorphic transitions [reviewed in (3)]. The organism exists in the environment as a haploid budding yeast with two mating types: $\alpha$ and **a**. Under appropriate nutrient-limiting conditions and in response to mating pheromones, the two mating partners produce conjugation tubes, and the cells fuse (46, 95, 96, 154). In contrast to model yeasts in which nuclear fusion occurs immediately (150), karyogamy is delayed in the basidiomycetes, and the resulting heterokaryon adopts a filamentous state. Ultimately, basidia are formed, and it is in these structures that nuclear fusion and meiosis occur. Spores are then produced on the surface of the basidium. Cells of the $\alpha$ mating type can also respond to nitrogen limitation, desiccation, and MF**a** pheromone signals and differentiate by a process known as haploid fruiting, which also involves filamentation and sporulation (183, 190).

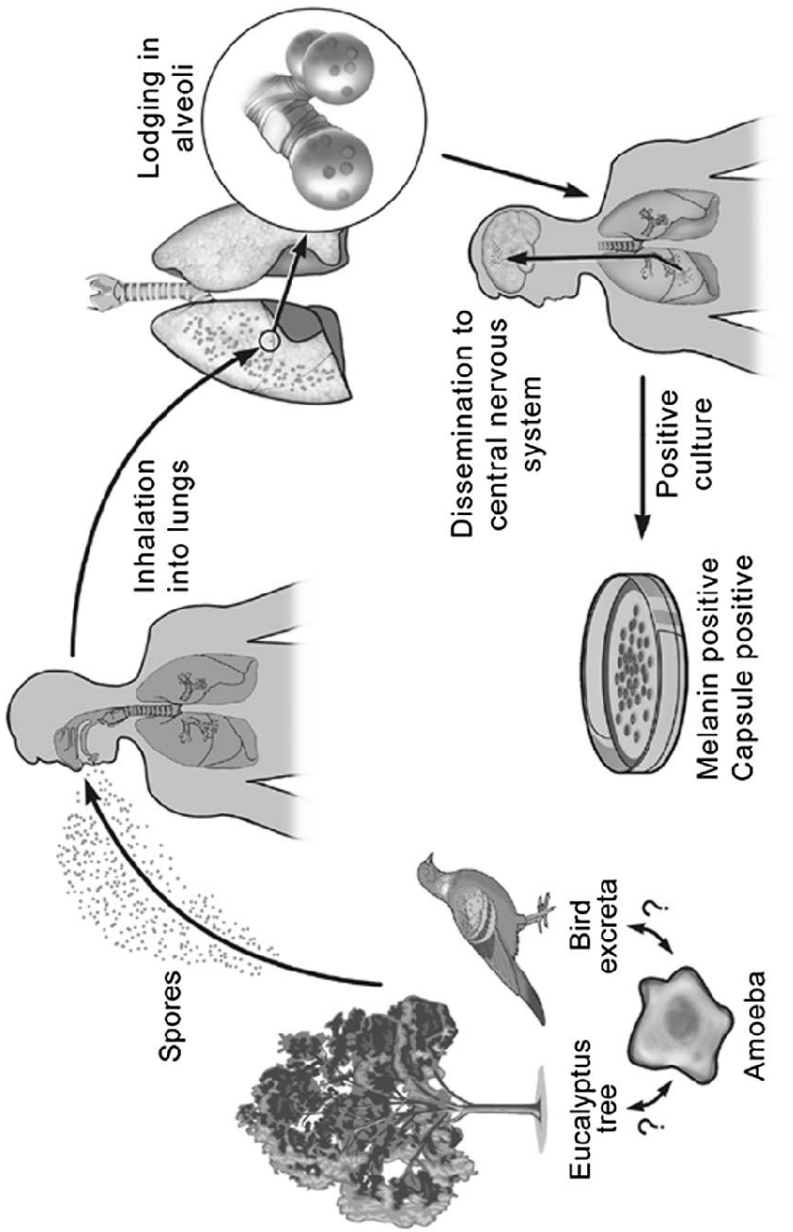
*C. neoformans* is ubiquitous in the environment and is most commonly found in association with pigeon guano or certain tree species (18). The organism exists

in two distinct groups, one found in temperate climates (serotypes A and D) that accounts for the vast majority of human infections, and one (serotypes B and C) that is restricted to tropical regions, associates with *Eucalyptus* trees, and infects immunocompetent individuals. Humans are exposed to the organism via inhalation, and the basidiospores that are produced by mating or haploid fruiting are thought to represent the infectious propagule because their size (1–2 $\mu$) is optimal for deposition in the alveoli of the lung (Figure 1). Experimental studies suggest that basidiospores may be up to 100$\times$ more infectious than vegetative yeast form cells, which are larger (4–10 $\mu$) and often encapsulated (165). Recent evidence suggests that infection usually occurs early in life, and can either be rapidly cleared or establish a dormant infection from which activation can occur later in life in response to waning immunity (52, 65, 70). The initial pulmonary infection spreads via the bloodstream to the central nervous system, the most common site of clinical presentation. Although *C. neoformans* grows in a filamentous form in vitro during mating, haploid fruiting, and diploid filamentation, the predominant form associated with clinical infection is the budding yeast. There is little evidence that the filamentous dimorphic transitions play a role in the infectious cycle beyond their potential to produce the highly infectious basidiospores. Instead, in response to host conditions *C. neoformans* produces two specialized virulence factors that are essential for infection: the polysaccharide capsule and the pigment melanin [reviewed in (15)].

One of the most interesting features of *C. neoformans* is that its prevalence in the environment, differentiation pattern, and virulence phenotype have each been linked to the $\alpha$ mating type. The majority of environmental isolates are of the $\alpha$ mating type, virtually all clinical isolates are $\alpha$, and in studies of congenic strains in a murine model $\alpha$ strains were found to be more virulent than **a** strains (99, 101). One hypothesis for this $\alpha$ prevalence is that $\alpha$ cells gain an advantage in the environment via their ability to undergo haploid fruiting, and the resulting production of $\alpha$ basidiospores leads to more clinical infections by this mating type (190). However, in a murine tail vein injection model in which the lung stage of the infection is bypassed, an $\alpha$ strain was intrinsically more virulent than a congenic **a** strain (101), suggesting that the *MAT*$\alpha$ allele of the mating-type locus makes additional contributions to virulence.

Several features and recent advances have led to the development of *C. neoformans* as an excellent genetic model of fungal pathogenesis. These include the following:

- The organism has a defined sexual cycle that can be manipulated for classic genetic studies.
- The organism is predominantly haploid, facilitating genetic analysis.
- Congenic $\alpha$ and **a** strains have been constructed.
- Gene disruption by biolistic transformation and homologous recombination is now routine, and nearly 100 genes have been analyzed by such approaches.



- Both auxotrophic (*URA5*, *ADE2*) and dominant selectable markers (hygromycin, nourseothricin) are available. The *URA5* gene can be both positively and negatively selected using 5-fluoro-orotic acid.
- Stable, congenic diploid strains have been identified that enable studies of the life cycle and analysis of essential genes and regions of the genome.
- Several different and robust animal models have been implemented that allow the study of each phase of the infectious cycle and direct analyses of mutant strains and candidate therapies.
- Episomal linear telomeric plasmids have been developed that can be shuttled between bacterial and fungal cells. These plasmids allow genes to be identified by complementation or overexpression and can be readily rescued from fungal cells.
- A meiotic recombination map of the genome has been created for studies of genome structure and quantitative trait mapping.
- The *MAT**a*** and *MATα* alleles of the mating type locus have been identified, enabling molecular studies of mating type that led to the discovery of sterile interspecies *α*AD**a** and **a**AD*α* hybrid strains and serotype A strains of the **a** mating type, which had been thought to be extinct.
- A genome sequencing project is ongoing for the serotype D laboratory-adapted reference strains JEC21 and B3501, and for the serotype A pathogenic isolate H99. Comparative genomic analysis is beginning for the divergent serotype B variety *gattii* strain WM276.

It is now possible in *C. neoformans* to identify a gene of interest, to disrupt this gene by transformation and homologous recombination, and to reintroduce the gene ectopically or at its normal chromosomal locus. It is then possible to test the effects of these genetic manipulations on the physiology of the organism both in vitro and in vivo with several different animal models. The application of these approaches has recently yielded insights into the genetic control of development and virulence and the role of the mating-type locus in the control of cell fate and identity. These studies also illustrate both conserved and organism-specific pathways that enable fungi to respond to challenges in their environment, including the ability to survive within and infect a mammalian host [reviewed in (109)].

←

**Figure 1** The *C. neoformans* infectious cycle. *C. neoformans* resides in the environment and has been found associated primarily with pigeon droppings (serotypes A and D) and *Eucalyptus* trees (serotypes B and C). *C. neoformans* may also have interactions in the soil with amoebae. It is thought that infection of humans generally occurs when basidiospores produced by *C. neoformans* in nature are inhaled into the lungs. Inhaled spores are deposited into the alveoli and germinate to establish a dormant infection or disseminate to the central nervous system. Once dissemination has occurred, viable cells can be cultured from the cerebrospinal fluid of infected individuals and confirmed as *C. neoformans* by assaying for melanin and capsule production.

## LIFE CYCLE AND SEXUAL CYCLE

*C. neoformans* is isolated from the environment and infected patients as a haploid, budding yeast. In growing cultures, the typical pattern of division by budding is observed, with larger mother cells and smaller daughter cells. Direct microscopic observations reveal that budding occurs predominantly in a bipolar fashion (J.A. Alspaugh, personal communication). As *C. neoformans* cells enter stationary phase, growth arrest occurs in both the G1 and the G2 phases of the cell cycle (167). This is in contrast to the budding yeast *S. cerevisiae*, in which nutrient limitation and stationary phase impose a G1 cell cycle arrest and is more similar to the fission yeast *S. pombe* in which a similar G2 arrest occurs when nutrients are exhausted. The rationale is that organisms that have evolved to exist predominantly as haploids, including *C. neoformans* and *S. pombe*, arrest in G2 rather than G1 to ensure that an extra copy of genomic information is available for repair.

*C. neoformans* can also adopt a filamentous growth form during several phases of its life cycle (Figure 2). These include the filamentous heterokaryon produced during mating, filamentation that occurs during haploid fruiting, and the temperature-controlled filament formation observed with diploid isolates (95, 96, 155, 190). Each of these filamentation pathways is described below.

### Mating

The discovery of the *C. neoformans* sexual cycle was the key event in developing a workable genetic system for the organism and has resulted in an explosion of information about the virulence attributes, life cycle progression, and phylogenetic relationships among *C. neoformans* isolates. In 1975, Kwon-Chung identified the sexual state of *Cryptococcus neoformans* (for which the teleomorphic designation is *Filobasidiella neoformans*) by culturing different clinical isolates in pairwise combinations on various sporulation agars and scoring hyphae formation (95). After three weeks of incubation, a subset of strain combinations formed hyphae with clamp connections (associated with mating in other basidiomycetes). Careful observations revealed that the hyphae differentiate into basidia with spore chains similar to, but not identical to, members of the genus *Filobasidium*. This realization led Kwon-Chung to place the sexual state of *Cryptococcus neoformans* in the family Filobasidiaceae and name this sexual state *Filobasidiella neoformans*. Her subsequent cytologic work characterized the morphological events associated with basidiospore formation, and her pioneering genetic experiments revealed a bipolar mating system (two mating types, **a** and $\alpha$) in which meiotic recombination occurs between strains.

Kwon-Chung also described differences in mating among the different serotypes of *C. neoformans*. She observed that mating was easily detected with strains of serotypes A and D but not with B and C. This prompted her to investigate serotype B and C matings and led to the finding that B and C strains can mate under certain conditions and form basidia and spores (97). These spores, however, were different

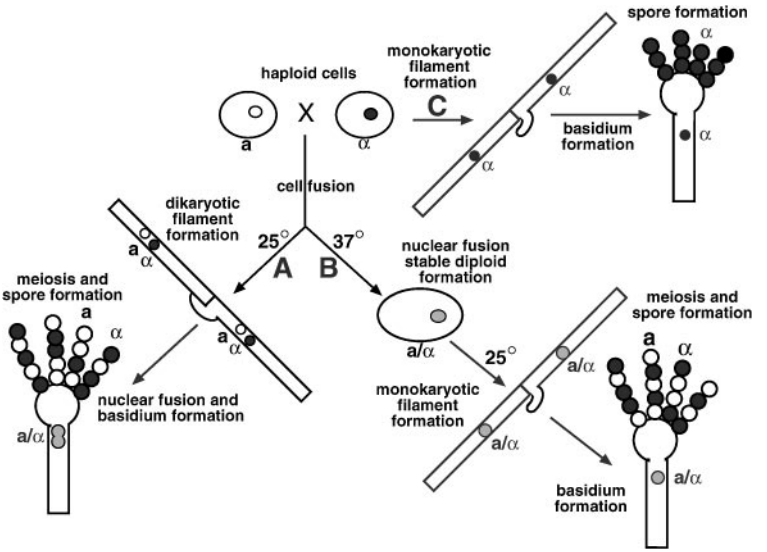



**Figure 2** Phases of the *C. neoformans* life cycle. There are three developmental pathways for *C. neoformans* cells. The first pathway, known as mating (*A*), begins when two haploid cells of opposite mating types fuse and are maintained at 25°C. The haploid cells fuse and grow as filaments with distinct nuclei (dikaryons) and special clamp cells that are fused to the filaments. In response to unknown signals, the dikaryon produces a specialized sporulation structure called a basidium. It is in this basidium that nuclear fusion and meiosis take place. Many rounds of duplication and mitosis lead to the production of haploid **a** and $\alpha$ spore products that form long chains on the basidial head. The second developmental pathway, known as diploid filamentation (*B*), occurs when cells of the opposite mating type fuse and are maintained at 37°C. In this case, the haploid cells and their nuclei fuse to create a yeast form, diploid cell that grows as a yeast at 37°C. In response to a decrease in temperature (25°C), this diploid differentiates to form monokaryotic filaments with unfused clamp cells, basidia, and haploid **a** and $\alpha$ spores similar to those of mating filaments. The third possible fate occurs primarily in the $\alpha$ cell type. This pathway is known as haploid fruiting (*C*) and occurs when $\alpha$ cells are grown under nutrient limited, desiccating conditions. In this pathway, haploid $\alpha$ cells form monokaryotic filaments with unfused clamp cells, basidia, and haploid $\alpha$ spores. In each case the resulting spores are competent to germinate and grow as haploid cells or initiate one of the three developmental pathways again.

from those formed in A and D matings, and Kwon-Chung designated the sexual state of serotypes B and C as *Filobasidiella bacillispora* to reflect the elongated bacillus form spores produced by this species.

The identification of different *C. neoformans* mating types allowed Edman et al. to develop congenic serotype D strains through a series of ten backcrosses (79, 101).

The two parental strains for the crosses were NIH12, a *MATα* clinical isolate from a patient who presented with osteomyelitis caused by *C. neoformans* and NIH433, a *MAT**a*** strain that was isolated from the environment (pigeon guano) in Denmark. Importantly, the ultimate congenic strains are the products of backcrossing to the F2 generation and thus are not congenic with either NIH12 or NIH433 and share, on average, half their genomes with each parent. The resulting congenic strain pair (JEC20/B4476 and JEC21/B4500) was then used in controlled experiments both in vivo and in vitro. The availability of easily manipulated strains has proven invaluable; however, one serious drawback to these laboratory-adapted serotype D strains is that they are significantly attenuated in animal models, and this attenuation hampers analysis of virulence attributes. On the other hand, JEC20 and JEC21 represent an exceptional resource for conducting genetic analyses in this system. A variety of mutant and auxotrophic derivatives are currently available, and the use of these strains has led directly to a better understanding of the life-cycle processes (like mating) that are crucial to *C. neoformans* growth.

An important step in creating useful strains was refining the conditions under which mating can occur. Efficient mating requires strains of opposite mating types and a nitrogen-limited growth medium or a medium containing a plant extract. A common and efficient mating medium is solid agar containing V8 juice (100). V8 juice (and carrot juice) media are limiting for several amino acids and nucleotides, but also contain a water-soluble, heat-stable, small-molecular-weight component that stimulates mating (J.A. Alspaugh & J. Heitman, unpublished observations). Mating occurs most efficiently on V8 medium at room temperature in the dark. When strains of the opposite mating type confront each other under these conditions, $\alpha$ cells make conjugation tubes, and **a** cells expand to form round, enlarged cells. The **a** and $\alpha$ cells fuse with one another to form elongated cells that contain two distinct nuclei. These dikaryons grow as filaments until they differentiate to form a specialized cell called a basidium. It is in this basidium that nuclear fusion and meiosis occur. The meiotic products are repeatedly duplicated mitotically, packaged into spores, and moved to the surface of the basidium to form long chains of randomly ordered basidiospores extending from the basidial head (95, 98).

The signals that govern the stages of this differentiation process are suspected to mirror those of other basidiomycetes such as *C. cinereus* and *S. commune*, but in fact very little is known about the gene products that control these events in *C. neoformans*. What is clear is that *C. neoformans* develops quite differently from other model fungi. For example, in contrast to its ascomycete relatives (like *S. cerevisiae*), the spores of this basidiomycete are not packaged in an ascus. In the case of *C. neoformans*, they are loosely attached to the basidium and free to dislodge and be carried away to another venue (such as the lung of a human host). There are several other major differences between the mating systems in *S. cerevisiae* and *C. neoformans*. Unlike in *C. neoformans*, mating in the ascomycete *S. cerevisiae* takes place under nutrient-replete conditions. The diploid state is stable, and meiosis and sporulation take place under low nutrient conditions (81). In this system the term "mating" has traditionally been meant to include only the initial steps of cell and nuclear fusion to form a stable diploid. In *C. neoformans*,

however, the predilection of heterokaryons and diploids to grow filamentously has led the term "mating" to include the processes of cell fusion and filament formation. Mating assays to date assess the ability of a strain to form filaments and do not speak directly to the issue of partner cell fusion. As a result, strains that fuse with a mating partner but fail to make filaments have generally been scored as sterile. More recent experiments have addressed the issue of cell fusion using assays to score the production of prototrophic dikaryons following fusion of two auxotrophic strains (26, 154, 198).

These mating and filamentation assays are very similar to those carried out in a relative of *C. neoformans*, the plant pathogen *U. maydis* (94). In *U. maydis* dikaryotic filaments are formed when compatible mating partners come into contact and fuse with one another. Mating in this system is also generally scored as the ability to form dikaryotic filaments. The dikaryon is infectious to corn plants and can differentiate only in planta to form the black spores that are characteristic of this smut fungus. Although the spores of *C. neoformans* are thought to be the infectious propagule in cryptococcosis, there is no evidence that the diploid or filamentous state is required for any part of pathogenesis within the human host.

## Haploid Fruiting

The second phase of the *C. neoformans* life cycle that can lead to filamentation is known as haploid or monokaryotic fruiting (Figure 2). This phase involves filamentous differentiation that occurs when cells of the $\alpha$ mating type are grown under conditions of desiccation and severe nitrogen limitation (190). Haploid $\alpha$ cells produce filaments that are monokaryotic and septated, but the filament cells have unfused clamp connections. Basidia are produced that often yield four short chains of basidiospores. Robust blastospores (yeast-form cells) are produced along the filaments via budding from the clamp cell, and proliferation of these cells gives rise to chains of microcolonies that have a beads-on-a-string appearance. Haploid fruiting has been proposed as a mechanism by which the organism could forage for nutrients, in a manner analogous to the pseudohyphal filamentous differentiation that occurs in *S. cerevisiae* in response to nitrogen limitation (67). Fruiting might also promote survival by producing spores that could survive nutrient-poor conditions. As discussed further below, haploid fruiting may play a role in generating the infectious propagule, the basidiospore. Recent observations indicate that haploid fruiting is dramatically stimulated by mating partner cells, and thus likely functions in the early steps of mating, allowing $\alpha$ cells to detect **a** cells at a distance (183). The mating partners might then come into contact via filamentation, or the dispersal of spores from aerial basidia. Haploid fruiting of $\alpha$ cells driven by **a** cells might also provide a selective pressure to maintain both mating types in the environment without a strict requirement for mating.

The process of haploid fruiting is thought to be asexual and to involve mitotic production of spores. However, it has not been examined experimentally whether meiosis occurs during haploid fruiting. In *S. cerevisiae*, meiosis is normally restricted to diploid cells that are heterozygous at the *MAT* locus (**a**/$\alpha$)

(124). However, **a**/**a** and $\alpha/\alpha$ yeast strains that lack the Rme1 repressor of meiosis (*rme1/rme1*) can undergo meiosis and sporulate (125). Thus, by analogy *C. neoformans* $\alpha$ haploid cells might undergo fusion to produce $\alpha/\alpha$ homozygous strains that then undergo meiosis.

Two other conditions have recently been discovered that dramatically stimulate haploid fruiting: darkness and pheromone secreted by **a** cells (154, 183). Limited haploid fruiting of the standard haploid $\alpha$ lab strain JEC21 typically occurs in 2 to 4 weeks when incubated on SLAD (nitrogen-limiting) medium at room temperature. Under the same conditions, cultures incubated in the dark undergo much more robust haploid fruiting in less than 1 week. This finding came as a surprise, given that *S. cerevisiae* has no apparent light-sensing mechanisms. However, many other fungi, including the light-entrained clock-containing *Neurospora crassa*, clearly sense light [reviewed in (113)]. The mechanisms by which *C. neoformans* senses and responds to light, or even the wavelength detected remain to be explored.

A second signal that robustly stimulates haploid fruiting of $\alpha$ cells is the presence of factors secreted by **a** cells (183). This was discovered during efforts to develop confrontation assays to examine the morphological actions of pheromones during mating. When a line of $\alpha$ cells is streaked in close proximity to but not touching a line of **a** cells, both cell types respond to factors secreted by the opposite cell type. Initially, both cell types produce short conjugation tubes. The filaments produced by the **a** cells fail to elongate and instead enlarged, round cells are observed. In contrast, the $\alpha$ cells produce conjugation tubes that dramatically extend and then haploid fruit, producing basidia and basidiospores. Haploid fruiting of $\alpha$ cells is stimulated by **a** cells at a distance with no requirement for contact between opposite cell types. Stimulation of $\alpha$ haploid fruiting by **a** cells can also occur through a dialysis membrane. Finally, recent studies have revealed that haploid fruiting is intimately controlled by pheromones (154). When the gene encoding the MF**a** pheromone that is normally produced by **a** cells is transformed into $\alpha$ cells, haploid fruiting is dramatically stimulated in an autocrine signaling fashion. In addition, overexpression of the MF$\alpha$ pheromone in $\alpha$ cells also enhances haploid fruiting, and deletion of the *MF$\alpha$1,2,3* genes results in a pheromoneless triple mutant strain that exhibits reduced haploid fruiting. These observations provide evidence that the pheromones of *C. neoformans* function in both paracrine signaling between cells of the opposite mating type and autocrine signaling pathways that act on the pheromone producing cells themselves.

Recent observations suggest that **a** strains can also undergo haploid fruiting under certain conditions. The original studies that demonstrated that $\alpha$ strains undergo haploid fruiting while **a** strains do not included a genetic analysis showing that the ability to haploid fruit and the $\alpha$ mating type cosegregated in genetic crosses (190). In more recent studies involving stimulated haploid fruiting (darkness, confrontation with **a** cells), haploid fruiting remains restricted to cells of the $\alpha$ mating type. However, an unusual isolate of the serotype D *MAT**a*** strain B3502, one of the ancestral strains of the JEC20/JEC21 congenic strain series undergoes robust haploid fruiting on SLAD or V8 medium (K. Lengeler & J. Heitman, unpublished observations). These findings suggest that mutations might enable **a** strains to haploid fruit

under certain conditions. Similarly, a recently developed improved medium (sucrose-proline agar) supports haploid fruiting of the serotype D *MAT**a*** strain JEC20 (K.J. Kwon-Chung, personal communication). These findings suggest that there is a relative but not absolute difference between $\alpha$ and **a** strains in their potential to undergo haploid fruiting. This also suggests that genes within the *MAT* region likely work in concert with nonmating type-specific genes, such as elements of the MAP kinase cascade, to promote fruiting in both $\alpha$ and **a** cells. The conditions under which fruiting of **a** cells is physiologically relevant remain to be defined.

The realization that both $\alpha$ and **a** cells of *C. neoformans* can undergo haploid fruiting under different conditions is analogous to the recent discovery that haploid cells of *S. cerevisiae* can undergo pseudohyphal differentiation [reviewed in (109)]. Previously, pseudohyphal growth was thought to be restricted to only diploid cells, to function in foraging for nutrients, and to not involve the pheromones, pheromone receptors, or the coupled heterotrimeric G-protein (67, 115). Yet recent studies reveal that haploid yeast cells respond to low doses of mating pheromone by switching from axial to unipolar budding, elongating, and invading the growth substrate, all features of pseudohyphal differentiation (58, 146). The revised view is that filamentous growth allows diploid yeast cells to forage for nutrients and allows haploid yeast cells to search for mating partners. Pseudohyphal growth of haploid yeast cells in response to pheromone requires the mating pheromones, their receptors, and the coupled G-protein. By analogy, *C. neoformans* $\alpha$ cells may haploid fruit in response to MF**a** mating pheromone to search for mating partners. In contrast, **a** cells have not yet been observed to filament in response to a mating partner but rather in response to different environmental conditions or in mutant strain backgrounds. Thus, in a manner similar to *S. cerevisiae*, the functions of filamentous growth and sporulation may differ between the two cell types of *C. neoformans*.

Some serotype A strains have been reported to haploid fruit (190) but this has not been widely observed for most serotype A strains under a variety of conditions. These include altered growth mediums, incubation in the dark, and in confrontation with serotype A or D strains. It is likely that serotype A strains will fruit more readily under certain conditions that remain to be defined, but currently, this poses a dilemma for the theory that haploid fruiting represents the mechanism by which the infectious basidiospores are produced, since most clinical infections are produced by serotype A strains.

## Diploid Filamentation

The third and final phase of the *C. neoformans* life cycle in which a filamentous transition occurs is during the growth of diploid strains (Figure 2). *C. neoformans* is thought to exist predominantly as a haploid budding yeast in which the diploid stage of the life cycle is transient and unstable. However, the dissection of basidiospores during standard genetic crosses results in a small number of unusual self-filamentous segregants (187, 188). Sia et al. characterized these isolates and found that they grow as budding yeasts with smooth, uniform colonies when cultured at 37°C, yet when grown at 24°C these isolates spontaneously filament and

sporulate on a variety of media (155). Upon further examination, these strains were found to be uninucleate but with nuclei larger than the nuclei of the haploid parental strains. FACS analysis revealed that these strains were diploid, and PCR analysis showed that these strains were heterozygous at the mating-type locus (**a**/$\alpha$) (155). When diploid strains that were heterozygous for several genetic markers were sporulated and dissected, the resulting progeny were haploid and exhibited meiotic segregation of genetic markers. These congenic diploid strains can be transformed and used to study key features of cell identity and to establish that genes are essential (43, 83). The filaments produced by these congenic serotype D diploid strains share some features with haploid fruiting filaments and other features with the filaments produced during mating (155). For example, the diploid filaments are monokaryotic and have unfused clamp connections, similar to haploid fruiting. But like mating, the diploid filaments produce abundant basidia with long chains of basidiospores.



## Serotype AD Diploids

The relevance of the diploid state in nature has also come to the fore in recent years with the discovery of naturally occurring diploid strains in the patient population. These strains are known as AD hybrids and appear to be the result of a fusion event between a serotype A strain and a serotype D strain. Serotype A strains are the predominant form of *C. neoformans* infection. ~95% of all *C. neoformans* infections are caused by serotype A isolates, and >99% of infections in AIDS patients are serotype A (18). In some regions of the world, such as Europe, serotype D strains are more common and have been thought to represent up to 30% of infections (51). Even in New York City up to 12% of infections may be caused by serotype D (159). Recent studies, however, indicate that many of these strains in fact represent unusual serotype AD hybrid strains (11).

Early speculation was that AD strains might represent unusual mutants of the enzymes that produce the capsular polysaccharide antigens. This speculation may have been fueled by the finding that strains that initially typed as AD often reverted to only serotype A or D with passage. However, a variety of studies suggested that the serotype AD strains might represent unusual hybrid strains (10, 12, 179). Following the rediscovery of congenic serotype D diploid strains by Sia et al., Lengeler et al. applied molecular methods to analyze a collection of serotype AD strains (108). These strains were found to be aneuploid or diploid by FACS analysis, to be uninucleate, to often contain both serotype A and D alleles of several genes, and to be heterozygous at the mating-type locus (**a**/$\alpha$). A few serotype AD strains were found to be self-filamentous, but the spores produced germinated poorly, and those spores that did germinate gave rise to isolates that were usually still diploid and serotype AD. Thus, meiosis fails to occur properly in AD hybrid strains, likely as a result of genome differences between the original serotype A and D parental strains.

The AD hybrid strains fall into two classes (108). In one type, the *MAT*$\alpha$ allele was inherited from the serotype A parent, and the *MAT****a*** allele was inherited from

the serotype D parent. In the second type, the *MAT**a*** allele was inherited from the serotype A parent, which, until recently, had been thought to be extinct, and the *MATα* allele was inherited from a serotype D parent. We call these two classes of AD hybrid strains the *α*AD**a** and the **a**AD*α* strains to reflect their distinct origins. Somewhat surprisingly, from the analysis of 10 serotype AD strains, four were found to be of the atypical **a**AD*α* origin. This suggests that serotype A *MAT**a*** strains might still be common in some unique environmental niche that remains to be discovered. Alternatively, the serotype A *MAT**a*** strains might have begun to disappear after the time of origin of the AD hybrids, which has been estimated from population genetic studies to be recent but could still be as long as several million years ago (194).

Several complementary studies come to similar conclusions but by different approaches. Boekhout et al. have used AFLP (amplified fragment length polymorphism) analysis to derive evolutionary trees for a vast collection of environmental and clinical isolates of *C. neoformans* serotype A, D, B/C, and AD strains (11). First, they find evidence that serotype A and D strains are quite distinct from serotype B/C strains, leading to suggestions that these represent different species, as discussed further below. Second, they find that serotype AD strains are hybrids containing AFLP banding patterns that are a superimposition of the patterns found in haploid serotype A and D strains. Third, they find that many European strains reported to be serotype D are in fact serotype AD hybrid strains. These findings support the hypothesis that serotype AD strains are intervarietal or interspecies hybrids between parental A and D haploid strains, and they suggest that serotype A strains and the intervarietal AD diploid strains are more virulent than haploid serotype D strains. The virulence of serotype D strains may be enhanced by fusion with more pathogenic serotype A strains, and the molecular analysis of AD hybrid strains might represent one avenue to analyze the molecular determinants of virulence on a genome-wide level.

Cogliati et al. (29) recently analyzed a large series of serotype AD strains by molecular approaches similar to those employed by Lengeler et al. (108). Additional strains were analyzed, and the analysis included both the *STE20α/**a*** genes and the mating pheromone genes. The conclusions are strikingly similar: Namely, the serotype AD strains represent hybrids between parental A and D haploid strains and occur in the two general classes of the *α*AD**a** and **a**AD*α* types. That two independent approaches with two different strain collections reached concordant conclusions provides strong support for the hypothesis that the serotype AD strains originated via hybridization. In addition, the studies of Lengeler et al. and Cogliati et al. in which the serotype A *MAT**a*** locus was found to be present in the unusual class of **a**AD*α* hybrid strains both served as the impetus to search for and independently discover the first two haploid serotype A *MAT**a*** strains (described further below). Finally, by population genetic approaches using AFLP analysis, Xu and colleagues provide evidence that the serotype AD hybrid strains arose via several independent origins, fully consistent with the previous finding that there are two distinct classes of AD hybrids (*α*AD**a** and **a**AD*α*) (194). In a recent study,

Yan et al. examined the distribution of the mating-type locus by PCR analysis and mating assays with a collection of 358 strains (197). Their analysis revealed no additional serotype A *MAT**a*** strains from 324 different serotype A strains, and provided independent confirmation that two classes of serotype AD hybrids exist.

In addition, Lengeler et al. found that AD hybrid strains could be generated readily in the laboratory with defined serotype A and D parental strains (108). In these studies, genetically marked auxotrophic serotype A H99–derived and serotype D JEC20–derived strains were co-cultured under mating conditions, and prototrophic strains resulting from cell-cell fusion were isolated. These strains are diploid by FACS analysis, and by molecular analysis using PCR, these strains are diploid and heterozygous for both the mating-type locus and the serotype A and D alleles of several genes. Curiously, most of these AD hybrid strains do not serotype as AD strains; instead the majority type as serotype D. Perhaps the serotype D phenotype is dominant [as is the case in progeny of serotype B by D crosses (100)] and as these diploid AD hybrids become aneuploid upon passage, they become serotype AD by standard antibody tests. As the resulting serotype AD strains become further aneuploid, they have frequently been observed to type as only serotype A or D. Thus, careful analysis of environmental and clinical isolates is necessary to ascertain whether strains are haploid or hybrid.

Xu and colleagues conducted a similar series of laboratory serotype A by D crosses and observed that mitochondria are inherited in a uniparental fashion in the resulting AD hybrid progeny (193). They employed RFLP markers in the mitochondrial genome and found that in 570 progeny of six A by D crosses, all progeny inherited the mitochondrial pattern from the serotype D parent. This led to an initial suggestion that the mitochondria from serotype D might be dominantly inherited. To address this hypothesis, Xu and colleagues recently analyzed the pattern of mitochondrial inheritance in serotype AD hybrid strains (192). To their surprise, they found that some serotype AD hybrid strains inherited the mitochondria from the serotype A parent, whereas others inherited the mitochondria from the serotype D parent. These findings again reflect a uniparental inheritance of mitochondria, but underscore that it is not determined by the serotype from which they originate.

The finding that the behavior of $\alpha$ and **a** cells differs during mating provides one possible explanation. In response to mating pheromones, the $\alpha$ cells produce filaments that extend and search out **a** mating partner cells. In turn, the **a** cells dramatically enlarge to form large swollen cells that serve as ready targets for the filaments of $\alpha$ cells. Perhaps following fusion of the tip of an $\alpha$ filament with a swollen **a** cell, the nucleus from the $\alpha$ partner migrates through the filament into the **a** cell, the two nuclei pair, and dikaryotic filaments are then launched from the swollen **a** cell as the base. In this scenario, the mitochondria that migrate into the dikaryotic filament cell might be preferentially derived from the **a** cell parent, rather than from the $\alpha$ cell. Additional mechanisms could also function to destroy the mitochondria from one parent in the cross, analogous to the nucleolytic

mechanisms that destroy chloroplasts derived from one parent in the green alga, *Chlamydomonas reinhardtii* (173).

## Serotype A *MATa* Strains

In addition to information about mitochondrial inheritance, the studies of AD hybrids provided the impetus to explore the issue of the elusive serotype A ***MATa*** strains. As mentioned previously, until recently, no serotype A ***MATa*** strain had been identified and it was thought that this mating type might have become extinct, possibly reflecting an evolution of serotype A strains toward an asexual life cycle. Two unusual serotype A ***MATa*** strains have been discovered recently, again forcing a reevaluation of the life cycle and the role of the sexual cycle in this organism.

During an examination of clinical isolates from Tanzania, Lengeler et al. identified an unusual isolate, strain 125.91, that typed as serotype A but lacked several genes encoded by the *MATα* allele of the mating type locus (112). Two hypotheses were proposed: either this strain contained a large deletion of the *MATα* allele, or the strain represented a serotype A ***MATa*** isolate. Using primers to the *STE20**a*** gene encoded by the serotype D ***MATa*** mating-type allele, they amplified a serotype A-specific *STE20**a*** allele by PCR, proving that strain 125.91 represents an elusive serotype A ***MATa*** strain. Lengeler et al. showed that this strain is haploid by FACS analysis, and the strain contains serotype A-specific genes and lacks serotype D-specific genes by PCR analysis. Moreover, using two novel transposable elements (T1 and T2) that are common in serotype D but rare or absent in serotype A (M.C. Cruz & J. Heitman, in preparation) as probes, strain 125.91 exhibited the serotype A transposon fingerprint (112). Strain 125.91 was sterile when crossed with the standard serotype A *MATα* strain H99 under a large variety of conditions. Conditions have recently been discovered that support mating of this unusual serotype A ***MATa*** strain (K. Nielsen & J. Heitman, unpublished results), and the entire ***MATa*** allele for this strain has been cloned and sequenced (110), opening the door to further genetic analysis in the predominant serotype causing infection.

The serotype A ***MATa*** strain 125.91 is a clinical isolate from a Tanzanian AIDS patient with cryptococcal meningitis, and is therefore pathogenic. In the murine tail vein injection model, the serotype A ***MATa*** strain 125.91 is less virulent than the serotype A *MATα* strain H99, suggesting that the *MATα* allele might also be linked to virulence potential in the serotype A clinical isolates. A second serotype A ***MATa*** strain has recently been reported, IUM96-2828, an environmental isolate from the soil in Italy (178). The IUM96-2828 strain has been reported to be fertile in crosses with serotype A *MATα* strains. 125.91 and IUM96-2828 represent the only two serotype A ***MATa*** strains that have been identified from a search of ∼1500 strains in several labs worldwide (197; T. Boekhout, personal communication; W. Meyer, personal communication; K.J. Kwon-Chung, personal communication; K. Lengeler & J. Heitman, unpublished results). Thus, serotype A ***MATa*** strains are rare but not extinct, and exist as about 0.1% of the serotype

A population compared to the serotype D population in which **a** strains occur at about 2% of the population (99).

The discovery of two serotype A *MAT**a*** strains is an exciting breakthrough because these strains provide the platform to construct congenic serotype A strains. Once congenic strains become available, much of the genetic work in this organism should shift to studies in the more pathogenic serotype A isolates. Reasons for switching to serotype A backgrounds include that serotype A strains represent the overwhelming majority of clinical isolates, and that most serotype A strains are more virulent than most serotype D strains, facilitating genetic analysis of virulence properties. Finally, an increasing number of mutants exhibit differences in phenotypes between the serotype A H99 strain background and the serotype D JEC20/JEC21 congenic strain series (26, 40, 182, 198).



## IDENTIFICATION AND CHARACTERIZATION OF THE *MAT* LOCUS

Mating type in fungi is controlled by the information encoded by the mating-type or *MAT* locus. This region of the genome is special because homologous chromosomes contain nonhomologous information that specifies the genetic differences between cell types. In ascomycete fungi, this region has been studied extensively, particularly in *S. cerevisiae* [reviewed in (81, 90)]. In *S. cerevisiae* information at *MAT* is different between **a** and $\alpha$ cells. *MAT**a*** encodes the transcriptional regulator **a**1, and *MAT*$\alpha$ encodes the transcriptional regulators $\alpha$1 and $\alpha$2. The region of difference between the two chromosomes containing *MAT* is about 700 base pairs. The size and composition of this locus is common for ascomycetes, which generally encode the key transcriptional regulators of cell type at a single *MAT* locus of relatively small size. In basidiomycetes, there are usually two, unlinked *MAT* loci; one encodes pheromones and pheromone receptors, while the other encodes homeodomain transcriptional regulators. Both *MAT* loci are necessary to specify cell type. The basidiomycete *MAT* loci are typically much larger than the ascomycete *MAT* loci, encode more than one gene product, and exist in multiple alleles, giving rise to thousands of different mating types [reviewed in (20, 94)].

Given the intriguing relationship in *C. neoformans* between the $\alpha$ mating type and prevalence in patients and the environment (99), an early effort was made to clone the *MAT* region from $\alpha$ cells. Moore & Edman used a difference cloning technique to identify regions of the $\alpha$ genome that were not present in the **a** genome (129). They identified an approximately 35-kb region that was present in only $\alpha$ cells. They also defined a 2-kb fragment of this region that when transformed into **a** cells conferred the ability to form conjugation tubes (mating structures) in response to conditions that support mating (V8 medium). The sequence of this fragment revealed an open reading frame with strong similarity to the mating pheromones of other fungi. This gene has subsequently been shown to encode one of three $\alpha$ pheromones used for signaling to a mating partner in *C. neoformans* (46, 154).

To understand the basis of filament production in *C. neoformans*, Wickes et al. carried out a screen to identify genes that when overexpressed enhance the production of filaments by $\alpha$ cells in the absence of an **a** mating partner (189). A genomic library of $\alpha$ DNA fragments in a telomeric vector was transformed into $\alpha$ cells, which were then tested for filament formation on filament agar. One consistently hyperfilamentous strain was selected for characterization, and the plasmid insert was recovered. The insert contained a gene with extensive similarity to the *STE12* gene found in other fungi. Ste12 in other fungi is a transcription factor that activates the expression of many different genes in response to signals from conserved MAP kinase cascades regulating mating and filament formation.

Preliminary experiments in *C. neoformans* suggested roles consistent with those in other fungi; namely, Ste12 induces the expression of at least one mating-type gene, the pheromone gene *MF$\alpha$1*, and can control gene expression in both **a** and $\alpha$ cells. Expression of the *CNLAC1* gene encoding the melanin biosynthetic enzyme laccase was found to be induced upon overexpression of *STE12* in **a** and $\alpha$ cells. This established the first molecular connection between a mating pathway component and a virulence factor in *C. neoformans* (even though *ste12* mutants have no melanin synthesis defect). These expression experiments also revealed perhaps the most interesting feature of the *STE12* gene: This allele of *STE12* in *C. neoformans* is specific to the $\alpha$ cell type. That is, Southern and northern blot analyses showed hybridization in only the $\alpha$ mating type in several different backgrounds, and no signal in **a** cells of the same background was detected. This intriguing finding suggested that unlike *STE12* in other organisms, the *C. neoformans STE12$\alpha$* was carrying out a function specific to the $\alpha$ cell type, like traditionally recognized components of the *MAT* locus. However, *STE12$\alpha$* did not reside in the 35-kb region of the *MAT* locus originally identified by Moore & Edman, and probes to the gene hybridized to a previously unidentified cosmid of $\alpha$-specific DNA. Identification and localization of another $\alpha$-specific gene, *STE20$\alpha$* revealed that it also did not map to the original 35-kb region of *MAT$\alpha$* (112, 182). These findings suggested that the *MAT* locus was larger than previously realized.

To understand the organization of *MAT$\alpha$* further, characterization of the *MAT$\alpha$* region was carried out by Karos et al. in which they identified cosmid clones containing $\alpha$-specific DNA (91). Endpoints were defined using probes to both **a** and $\alpha$ cells to identify 5–7-kb boundaries of an approximately 50-kb MAT$\alpha$ region. This analysis confirmed the suspicion that the locus was larger than that identified by Moore & Edman, placed several previously identified $\alpha$-specific genes in the locus (*STE11$\alpha$*, *STE20$\alpha$*, *STE12$\alpha$*), and identified several unexpected genes in the locus including a myosin homolog (*MYO2$\alpha$*), an apparent translation initiation factor (*PRT1$\alpha$*), two additional pheromone genes (*MF$\alpha$2* and *MF$\alpha$3*), and a pheromone receptor gene (*STE3$\alpha$* a.k.a. *CPR$\alpha$*). This expanded locus was unusual in its size and architecture, but additional experiments revealed that it was still not the complete *C. neoformans MAT* locus.

Recent work by Hull et al. in which the phenotype of a deletion of this 50-kb *MAT$\alpha$* region was analyzed revealed the presence of additional $\alpha$-specific

components (83). In this study, a complete deletion of the 50-kb *MATα* region was generated in **a**/α diploid cells (no deletions were recovered in α haploids due to the presence of one or more essential genes in the locus). Because the process of diploid filamentation requires information from both **a** and α cells, it was predicted that diploid filamentation would be abolished in an **a**/Δ strain. However, diploid filamentation was still intact, suggesting that an additional factor or mechanism was functioning to maintain the **a**/α cell identity. Further experiments showing that a 2n-1 diploid strain missing the *MATα*-containing chromosome was deficient in diploid filamentation (and mated like an **a** cell) suggested that the required component(s) is located on the *MATα*-containing chromosome.

Two models for the regulation of diploid filamentation were proposed and evaluated. In the first model, the ploidy of the *MAT* chromosome could be regulating filamentation analogous to the regulatory system found in other organisms in which ploidy and/or dosage establish mating type (9, 141) or to *S. cerevisiae* in which recent studies have shown that expression of a set of genes is ploidy dependent (64). This ploidy-dependent model was ruled out by experiments in which **a**/**a** and α/α diploid cells were created and tested for diploid filamentation. Homozygous diploid strains were created using a novel assisted mating or "ménage à trois" reaction in which strains of the same mating type were induced to fuse by the addition of an opposite mating partner to the mix (83). The resulting homozygous diploid strains were unable to undergo diploid filamentation (although α/α diploids can still form filaments in a fruiting assay). This result showed that ploidy alone does not control diploid filamentation and supported a second model in which an additional α-specific factor(s) on the *MAT* chromosome acts to induce filamentation. The ploidy result suggested that a second *MATα* region resided on the same chromosome or that the locus was larger than previously thought. Two approaches were taken to explore these possibilities.

In the first approach, Hull et al. hypothesized that a second *MATα* locus might contain homeodomain DNA-binding proteins. Basidiomycete *MAT* loci generally consist of two separate regions, one that contains pheromones and pheromone receptors, and one that contains homeodomain proteins (20). Because the previously defined locus contained pheromones and a pheromone receptor (91, 129, 154), Hull et al. hypothesized that a second locus could contain DNA-binding proteins. Using a bioinformatics approach, they identified putative homeodomain protein sequences from the *C. neoformans* Genome Project at Stanford University. One of the identified sequences hybridized in a Southern blot to DNA from only α cells and was located on the *MAT*-containing chromosome. Deletion of the gene (*SXI1α* for Sex Inducer 1α) resulted in a severe reduction in diploid filamentation and mating. In addition a double deletion strain in which the 50-kb *MATα* region and the *SXI1α* gene were both deleted (**a**/Δ *sxi1α*Δ) was no longer capable of diploid filamentation and mated like an **a** cell. This result indicates that Sxi1α is a key α cell-identity factor required for specifying cell fate, analogous to the cell-fate determinants encoded by the *MAT* loci of other fungi (e.g., **a**1 and α2 of *S. cerevisiae*) (90).

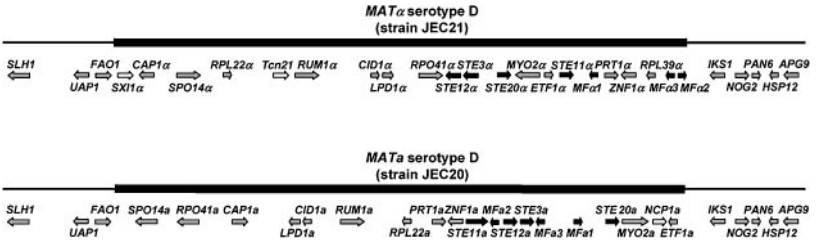



**Figure 3** The **a** and $\alpha$ alleles of the mating-type (*MAT*) locus. The *MAT*$\alpha$ and *MAT****a*** mating-type alleles and the adjacent genomic regions are depicted for serotype D. The mating-type specific regions are shown as bold lines and flanking regions as thinner lines. Sequences were analyzed using the BLASTX algorithm, and identified genes are shown as arrows in the direction of transcription. Genes encoding pheromone response pathway elements are shown as black arrows, and the remaining genes in the fragment are shown as gray arrows with the exception of the mating-type–specific genes *SXI1*$\alpha$, *Tcn21*, and *NCP1****a***, which are represented by open arrows.

In the second approach to understand the nature of the *MAT* locus, Lengeler et al. set out to elucidate the nucleotide sequence of the locus (110). Genomic BAC libraries were created for both the **a** and $\alpha$ mating types from the A and D serotypes (strains JEC20 and JEC21, H99 and 125.91). BAC clones containing *MAT* sequences were identified by hybridization, and overlapping clones were isolated and mapped in conjunction with BAC end sequences from Schein and colleagues (152). The BACs were sequenced and assembled to generate a *MAT* region for each strain (Figure 3). The sequences were aligned so that sequences identical between mating types flanked sequences that were different between mating types, thus defining a region of nonhomologous DNA sequence. The complete serotype D *MAT*$\alpha$ locus was found to contain the original 50-kb locus defined by Karos et al. as well as another 55 kb of *MAT* sequence. The sizes of all of the *MAT* alleles are in the range of 105 to 130 kb. The additional sequence contains apparent homologs for a mitochondrial RNA polymerase (*RPO41*$\alpha$), a dihydrolipoamide dehydrogenase (*LPD1*$\alpha$), a caffeine-induced death protein (*CID1*$\alpha$), an Rb binding protein 2 homolog similar to a regulatory protein in *U. maydis* that controls the expression of some sexual cycle genes (*RUM1*$\alpha$), a mariner-type transposon (*Tcn21*), a ribosomal protein (*RPL22*$\alpha$), a phospholipase D homolog (*SPO14*$\alpha$), a capsule-associated protein (*CAP1*$\alpha$), and the cell identity factor *SXI1*$\alpha$. Also identified within the original 50-kb locus was another ribosomal protein homolog (*RPL39*$\alpha$) and a putative zinc finger protein (*ZNF1*$\alpha$).

For almost every gene, there are similar, but not identical alleles in both *MAT*$\alpha$ and *MAT****a***. Exceptions include the $\alpha$-specific gene *SXI1*$\alpha$, the $\alpha$-only transposon *Tcn21*, and the **a**-specific gene *NCP1****a***. This is the only **a**-specific gene, and it shows sequence similarity to a single gene, an open reading frame identified by

the *N. crassa* sequencing project that shows no sequence similarity to any protein motifs and has no known function. Although *MAT**a*** is clearly allelic to *MATα*, the organization of *MAT**a*** reveals extensive rearrangement of the genes compared to *MATα* and may explain the absence of recombination in this *MAT* locus. Overall, minor differences occur between the two serotypes, but the final conclusion is that the *C. neoformans MAT* locus is >100-kb in length and encodes approximately 20 genes of diverse function (Figure 3). This substantial effort has resulted in a molecular definition of *MAT* in *C. neoformans*.

The *MAT* locus in *C. neoformans* is significantly larger than any other known fungal *MAT* locus, and its architecture differs from that of its basidiomycete relatives. Instead of containing two distinct loci, *C. neoformans* has one large locus encoding all of the traditional *MAT* components (pheromones, pheromone receptor, and DNA binding regulator) as well as a number of genes never seen in a *MAT* locus before. A few examples of *MAT* loci containing unusual genes exist (84, 175), but they are rare and involve only a few genes. *C. neoformans* is apparently the founding member of a new class of *MAT* loci in which genes not traditionally present in the *MAT* locus (such as signal transduction components) have mating type–specific alleles. A region of the genome of *Pneumocystis carinii* contains apparent signal transduction components as well, suggesting a strong similarity with the *C. neoformans MAT* locus (156). Perhaps these loci represent evolutionary intermediates between *MAT* loci in fungi and the sex chromosomes of multicellular eukaryotes. Because the regulation of the sexual cycle in *S. cerevisiae* has been studied extensively, and the molecular details of how cell fate is established and how sexual cycle progression occurs have been mapped in detail, we can look to this system for clues as to how the process might work in *C. neoformans*. In *S. cerevisiae*, **a** cells produce the homeodomain protein **a**1, and α cells produce the homeodomain protein α2. When **a** and α cells mate to form a diploid **a**/α cell, **a**1 and α2 interact to form a novel transcriptional regulatory activity that represses the expression of haploid-specific genes, thus establishing the diploid state (Figure 4) [reviewed in (90)]. The Sxi1α protein might interact with an **a**-specific partner protein to create a novel transcriptional regulatory complex in *C. neoformans* that would signify the dikaryotic state and promote progression through sporulation (Figure 4). This heteromeric regulatory complex model has been characterized in other basidiomycetes, including *U. maydis* where heterodimers composed of different forms of the bE and bW homeodomain proteins interact to induce sexual cycle progression [reviewed in (20)]. Although such a scheme for *C. neoformans* is appealing, no obvious partner proteins are apparent in the *MAT**a*** allele. The single **a**-specific gene, *NCP1**a***, shows no sequence similarity to any known protein motif, making it a poor candidate for a DNA-binding protein.

An alternative model is one in which one of the **a** alleles in *MAT**a*** has a disparate function from its partner allele in *MATα*. For example, the *ZNF1α/**a*** genes may encode proteins (predicted zinc-finger DNA-binding proteins) that interact with Sxi1α with different affinities to create complexes with different regulatory properties. Such complexes would be similar to the regulatory complex in *S. cerevisiae*

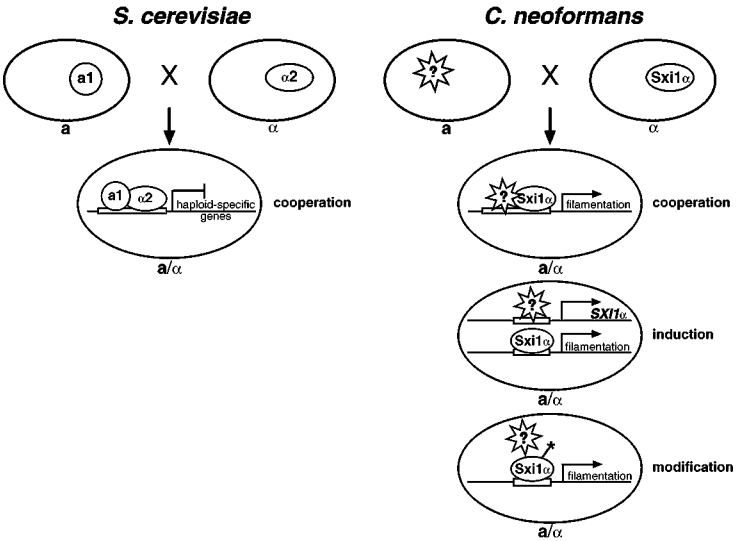



**Figure 4** Models of Sxi1$\alpha$ function. Ovals labeled **a** and $\alpha$ represent haploid cells. Larger ovals labeled **a**/$\alpha$ represent diploid cells formed after mating. Objects within the ovals represent proteins. *Left panel*: In *S. cerevisiae*, the homeodomain proteins **a**1 and $\alpha$2 are produced in **a** and $\alpha$ cells, respectively. Cell fusion produces an **a**/$\alpha$ diploid cell, and these proteins interact in a cooperative manner to create a novel transcriptional regulator. Through the repression of haploid-specific genes, this site-specific DNA binding activity specifies the diploid state. *Right panel*: Three models are proposed for how Sxi1$\alpha$ controls cell fate in *C. neoformans*. (i) In the Cooperation Model a factor from **a** cells (*star*) and a factor from $\alpha$ cells (Sxi1$\alpha$) interact to form a new activity that controls expression of genes important for development, similar to that seen in *S. cerevisiae* and other fungi. (ii) In the Induction Model *SXI1*$\alpha$ is controlled at the transcriptional level by an **a**-specific regulator that induces the expression (or stabilizes the transcript) of *SXI1*$\alpha$ upon cell fusion. Once Sxi1$\alpha$ is produced, it acts as a transcriptional regulator to control sexual development genes. (iii) In the Modification Model an **a**-specific modifier protein acts directly on Sxi1$\alpha$, modulates its activity, and leads to altered gene expression.

composed of the homeodomain protein Pho2 (a.k.a. Grf10) and the zinc-finger protein Swi5 (14). In $\alpha$ cells, a Sxi1$\alpha$-Znf1$\alpha$ complex could confer $\alpha$ cell identity, while after cell fusion, a Sxi1$\alpha$-Znf1**a** complex could form and specify the dikaryotic state. Alternatively, an **a**-specific allele could alter *SXI1*$\alpha$ transcript levels by inducing expression in the dikaryon, by regulating splicing of the pre-mRNA product, or by stabilizing the transcript (Figure 4). The regulation could also be a posttranslational event in which an **a**-specific kinase phosphorylates and activates the Sxi1$\alpha$ protein to allow it to function as a regulator of the dikaryotic state.

## MOLECULAR APPROACHES

The application of Koch's postulates revolutionized the study of microbial pathogenesis by providing a sound scientific basis to link pathogenic organisms to the diseases they cause. The four postulates are (*a*) all individuals with the disease harbor the presumptive causative agent; (*b*) the organism can be isolated from the infected host and propagated in the laboratory; (*c*) reintroduction of the organism results in the original disease in susceptible individuals; and (*d*) the organism can be reisolated from experimentally or accidentally infected individuals. Falkow and colleagues modified these postulates to formulate a molecular version of Koch's postulates: (*a*) The property studied should be associated with pathogenicity or infectivity; (*b*) mutation of genes involved in this property should lead to a loss of virulence; and (*c*) reintroduction of the gene should restore virulence of the organism. Thus, fulfilling Falkow's molecular postulates requires the identification of genes involved in virulence attributes, specific approaches to mutate these genes such as transformation and homologous recombination, methods to reintroduce the wild-type gene, and animal models to assess the impact of these molecular manipulations on virulence.

Recent genetic and molecular advances have allowed Falkow's molecular postulates to be fulfilled for a variety of different phenotypic traits and signaling cascades in *C. neoformans*. Early studies linking the polysaccharide capsule and production of the pigment melanin to virulence of *C. neoformans* utilized mutants isolated following chemical mutagenesis. Acapsular and albino strains were avirulent in animal models, and these mutant phenotypes cosegregated in genetic crosses, providing evidence that both properties are linked to virulence (87, 103).

### Gene Disruption

In the early 1990s, two different transformation systems were developed for *C. neoformans*. In the first, DNA is introduced by electroporation (54). In the second, DNA is delivered by biolistic delivery of gold microprojectiles decorated with DNA (170). This second approach was developed to circumvent difficulties in delivering exogenous DNA through the polysaccharide capsule. Both transformation systems relied on the use of genes involved in nucleotide biosynthesis, *URA5* or *ADE2*, as selectable markers (54, 164). Recipient strains for transformation were obtained either by the selection of *ura5* auxotrophic strains on medium containing 5-fluoro-orotic acid (which is converted to a toxin by the Ura5 enzyme) (104), or by the isolation of *ade2* mutants (which form pink or red colonies) following mutagenesis with $\gamma$-rays or UV light (143). DNA introduced into the cell by electroporation was found to frequently undergo the spontaneous addition of telomeres and to replicate in an extrachromosomal linear fashion (53). DNA delivered by biolistics was more frequently found to integrate into the chromosomal DNA, either ectopically by nonhomologous recombination or sequence specifically via

homologous recombination. These studies set the stage for an analysis of gene function by transformation and gene disruption by homologous integration.

An initial set of gene disruptions was conducted by biolistic transformation in the serotype A *ade2* recipient strains M001 and M049 (derived from strain H99) and by electroporation in *ura5* serotype D recipient strains of the JEC21/B3501 strain series (4, 21, 116, 136, 151). Two general principles emerged. First, biolistic transformation yields higher rates of integration and homologous recombination than electroporation in both serotype A and D strains (45). Second, it is essential that gene disruption alleles be constructed with DNA sequences that are isogenic to the recipient strain, because DNA polymorphisms result in abortion of homologous recombination events by the DNA mismatch repair system (45). In several recent studies, the frequency of homologous recombination that can be achieved with ~1000 bp of flanking sequence on each side of the selectable marker is in the range of 2% to 25%, with some exceptional cases at higher efficiency (42, 43, 154, 182, 183, 198). Most recent studies employ either the *URA5* marker and a spontaneous 5-FOA-resistant *ura5* mutant of the serotype A strain H99, or a dominant selectable marker that confers resistance to the aminoglycoside nourseothricin (123). The wild-type gene can be reintroduced ectopically by cotransformation with a linked selectable marker, either *URA5* or the hygromycin resistance gene (36, 82). Alternatively, the wild-type genetic locus can be reconstituted by starting with the *xyz1::URA5* disruption strain, transforming with the wild-type gene, and selecting for loss of the integrated *URA5* marker on 5-FOA medium to result in a wild-type *ura5* auxotrophic strain in which the wild-type gene is reconstituted at the endogenous locus (182). For virulence studies, the *URA5* gene is then reintroduced ectopically in a third transformation event. Although the resulting strains have been subjected to three independent transformation events, which some have argued might be deleterious if transformation itself is mutagenic, in several recent examples this approach has resulted in the full reconstitution of virulence to the wild-type level (182).

## Essential Genes

Three genes have been shown to be essential in *C. neoformans*. In the first example, the N-myristoyl transferase gene, *NMT1*, a temperature-sensitive allele was isolated based on a known, temperature-sensitive *nmt1* mutation in *S. cerevisiae* (116). The corresponding allele of the *C. neoformans NMT1* gene was found to be temperature sensitive in an *S. cerevisiae nmt1* mutant host. Next, the *nmt1-ts* allele was integrated into the *C. neoformans* genome linked to the *ADE2* selectable marker, and transformants in which the temperature-sensitive allele had replaced the wild-type locus were identified as temperature-sensitive transformants and confirmed by molecular analysis (116).

In the second example, the *TOP1* gene encoding topoisomerase I was shown to be essential in *C. neoformans* (47). In this case, no disruption mutants were identified from a screen of 8000 transformants obtained with a *top1::ADE2* deletion

allele, suggesting the gene was essential. When a second copy of the *TOP1* gene was introduced into the genome ectopically, the endogenous *TOP1* gene was readily disrupted by the same *top1::ADE2* disruption allele at 7% efficiency, demonstrating that the *TOP1* gene is essential (47).

In the third example, the *FKS1* gene encoding the unique $\beta$-1,3 glucan synthase gene was shown to be essential using a novel targeting strategy (169). In this case, the *ADE2* selectable marker was cloned between the 5′ flanking region of the *FKS1* gene and a 5′ truncated portion of the gene. Integration of the circular form of this disruption allele can occur in two ways. Integration into the upstream region on one side of the *ADE2* marker leaves the *FKS1* gene intact, whereas integration into the internal portion of the *FKS1* gene disrupts the gene. If the *FKS1* gene were nonessential, both types of integration events would be observed. In this case, homologous integration occurred at a frequency of 7.9% and 0/26 transformants analyzed contained a targeting event into the *FKS1* locus, indicating that the gene is essential. This finding is of particular interest given that the Fks1 glucan synthase is the target of the antifungal drug caspofungin, which exhibits poor activity against *C. neoformans* even though the *FKS1* gene is essential. Other factors involved in the action of this novel antifungal agent likely remain to be defined.

Given that only three genes have been documented to be essential in *C. neoformans* and that three different methods were used, more facile approaches would clearly be welcome. Stable congenic **a**/$\alpha$ diploid strains of *C. neoformans* were recently described (155). These strains grow as a budding yeast at 37°C, yet when grown at 24°C spontaneously filament, form basidia, and undergo meiosis and sporulation. These strains can be used to study an essential region of the *MAT*$\alpha$ allele of the mating-type locus (83). Deletion of an ~50-kb region of the *MAT*$\alpha$ allele resulted in a *MAT***a**/$\Delta$ diploid strain that sporulates to produce only *MAT***a** segregants that are all *ura5* and lack the *URA5* marker integrated at the site of deletion in the *MAT* locus. These studies reveal that one or more of the 12 genes contained in this interval is essential. Thus, these congenic diploid strains can be used to demonstrate that genes are essential, analogous to tetrad dissection in the model ascomycetous yeast *S. cerevisiae*.

Two recent reports suggest alternative approaches might also be applicable to study gene functions and essential proteins in *C. neoformans*. First, expression of two different antisense mRNAs was found to inhibit function of the *ADE2* gene or the gene encoding the calcineurin A catalytic subunit Cna1, which is required for both growth at 37°C and virulence (73). In a related study, expression of dsRNA was found to repress expression of the corresponding gene (114). In this case, the actin gene promoter was utilized to drive expression of a tandem gene in an inverted repeat orientation, to result in expression of a hairpin double-stranded RNA molecule. Hairpin structures were sufficient to repress expression of the *ADE2* gene, resulting in pink/red auxotrophic colonies. In a second example, dsRNA repression of the *CAP59* gene resulted in acapsular cells. Both approaches might find utility in further studies, but have thus far been applied to only three genes (*ADE2*, *CNA1*, *CAP59*) whose mutant phenotypes have been extensively characterized

by conventional mutational or gene disruption approaches. In addition, these novel approaches do not provide stable mutants for further genetic or phenotypic studies, such as virulence tests.

## Plasmids, Multicopy Libraries, and the Two-Hybrid System

Several different types of plasmids are available for molecular studies in *C. neoformans*, including both integrating and non-integrating vectors (Figure 5). First, a series of plasmids containing the *URA5* or $HYG^r$ selectable markers are available for integration of foreign DNA into the *C. neoformans* genome (36, 53, 54, 82). These plasmids have frequently been used to introduce dominant alleles for epistasis analysis, or to reintroduce wild-type genes into mutant strains to ensure that phenotypes are complemented and satisfy the third of Falkow's molecular postulates of virulence.

A second series of plasmids for *C. neoformans* are non-integrating plasmids that can be shuttled between *E. coli* and fungal cells. Two commonly used non-integrating plasmids are pCnTel1 and pPM8 (Figure 5) (53, 127). Both plasmids are maintained as circular plasmids in *E. coli* and contain both an origin of replication derived from pBR322 and an ampicillin resistance marker for selection in bacteria. These plasmids also contain two other special features. First, both contain the *C. neoformans URA5* gene as the marker for selection in *C. neoformans ura5* mutant cells. Importantly, the *URA5* gene can also be counterselected on medium containing 5-FOA, allowing tests of plasmid dependence to be readily performed (104).

A second important feature of both of these plasmids is that two telomeric sequences are present in an inverted orientation flanking the kanamycin resistance gene for selection in bacteria. Circular plasmid DNA from *E. coli* can be cleaved with a rare cutting restriction enzyme (Meganuclease I-SceI), which releases the Kan resistance gene cassette and reveals telomeric sequences that are converted into functional telomeres when introduced into *C. neoformans* (Figure 5). The plasmids are thus maintained as circular DNA molecules in bacteria and as linear, telomeric plasmids in fungal cells. The copy number of these plasmids has been determined to be $\sim$5 to 10 copies per cell, and studies are in progress to determine how these plasmids replicate in fungal cells (C. Newlon, personal communication). Both plasmids can be rescued from fungal cells by isolating DNA, cleaving with NotI (which removes the telomeric repeats), circularizing under dilute ligation conditions, and rescuing in *E. coli* by transformation and selection for ampicillin resistance (Figure 5). These approaches allow the introduction of genes into fungal cells, the identification of genes by complementation approaches, and the rescue of these genes in bacterial cells for molecular analysis.

The pPM8 plasmid has an additional feature that makes it exceptionally well suited for certain cloning procedures and the construction of genomic libraries (127). The pCnTel1 plasmid is somewhat unstable in *E. coli*, possibly due to the inverted telomeric repeats, and it has been difficult to construct stable genomic

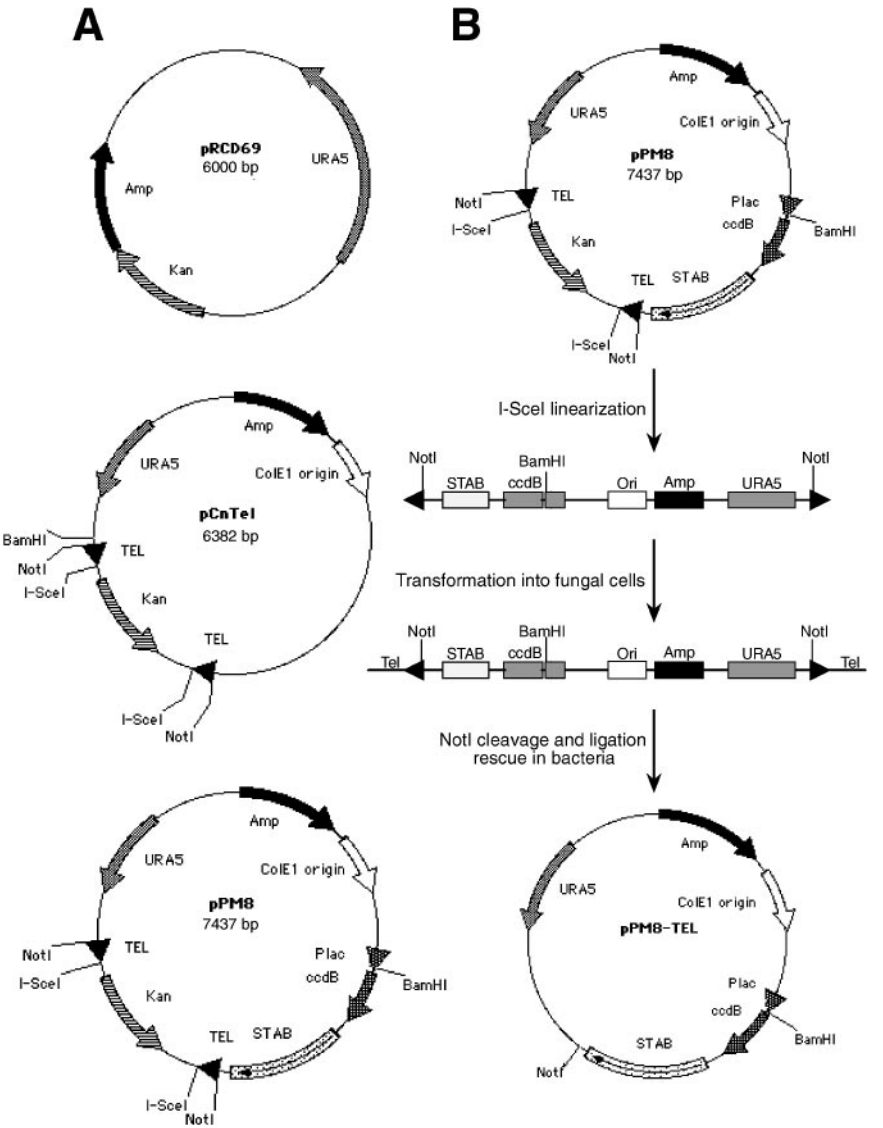



**Figure 5** Plasmids for gene identification and analysis. *A.* Plasmids for expression analysis in *C. neoformans*. pRCD69 (*top*) is an integrating vector. pCnTel (*middle*) is a telomeric vector that can be linearized prior to transformation and maintained as a stable linear fragment. pPM8 (*bottom*) is a telomeric plasmid derived from pCnTel that is optimized for use with genomic library fragments. All contain the *URA5* gene as a selectable marker. *B.* Schematic shows how pPM8 vector can be used to transform *C. neoformans* and then be recovered in *E. coli*, facilitating library screens.

libraries in this plasmid. To circumvent this issue, Mondon et al. constructed the pCnTel1 derivative pPM8 that allows plasmids containing inserts to be positively selected in bacterial host cells (127). The *ccdB* gene encoding a gyrase inhibitor was cloned under the control of the lac promoter in the pCnTel1 plasmid to create pPM8. Introduction of this plasmid into *E. coli* laci$^q$ cells is tolerated because *ccdB* expression is repressed, whereas addition of IPTG induces *ccdB* and kills the cells. Introduction of DNA inserts into the BamHI cloning site in the middle of the *ccdB* gene inactivates the toxin, allowing the positive selection of clones bearing inserts on medium containing IPTG.

Using this plasmid system, we have constructed a *C. neoformans* serotype D genomic library using the congenic $\alpha$ strain JEC21. Sau3A partial fragments in the size range of 6 to 11 kb were cloned in the vector. The library contains >90% inserts and represents ~6.7X coverage of the genome. This library has been successfully used in two independent screens to identify multicopy suppressors of two different mutants. In the first, a gene that restores growth at elevated temperature in a calcineurin B (*cnb1*) mutant strain was identified as a candidate component of the calcineurin signaling pathway required for growth at elevated temperature and virulence (39; D. Fox & J. Heitman, in preparation). In the second screen, clones that restore melanin production in a mutant strain lacking the Gpa1 G$\alpha$ protein involved in nutrient sensing were identified and may include novel components involved in melanin biosynthesis or its regulation (B. Allen & J.A. Alspaugh, personal communication).

The pPM8 plasmid also contains an additional unique sequence element known as the STAB sequence. The STAB region was thought to have been originally derived from a *C. neoformans* minichromosome and to confer increased stability to episomal plasmids (176). More recent studies have revealed that the STAB sequence is actually a region of the *E. coli* chromosome that has no effect on plasmid stability in *C. neoformans* (C. Newlon, personal communication).

A two-hybrid library has been constructed from cDNA grown under a variety of conditions and used to identify the novel calcineurin binding protein Cbp1, which is homologous to the calcineurin inhibitor Rcn1 in *S. cerevisiae* and DSCR1/calcipressin in humans (72).

## GENOME SEQUENCING PROJECTS AND GENETIC AND PHYSICAL MAPS

One of the most exciting recent developments in microbial genetics and pathogenesis is the application of genomic technologies and their integration with genetic and molecular biology approaches. In this regard, *C. neoformans* is no exception. Starting in 1999, an international consortium was formed to focus on the *C. neoformans* genome sequencing project whose goal is to provide the complete and annotated genome sequences, including bioinformatics tools and reagents, for at least two and possibly as many as four strains representing the major serotypes/varieties/species

for this organism (80). Several overlapping approaches will complement the direct sequencing. First, physical and genetic maps will ensure that the genome sequences faithfully represent the structure and architecture of the genome and provide tools to analyze the genome experimentally. Second, extensive EST analysis will be used to identify genes and assist in the annotation of this intron-rich genome. Finally, the genome information will be used to develop genome arrays for expression profiling and genotyping. The genome of this organism spans some 18 to 24 Mb and is divided into ~12 chromosomes. Thus, determining the genomic sequences of three related but diverged strains will require the acquisition of ~75 Mb of genomic sequence information.

The *C. neoformans* genome project has made considerable progress since its inception. BAC libraries were constructed for representative serotype A (H99/125.91), serotype D (JEC20/JEC21), and serotype B (WM276/E566) strains of both mating types. The serotype A, D, and B BAC libraries have been fingerprinted and end-sequenced at the Vancouver Genome Centre by Kronstad and colleagues, providing BAC maps for the genomes, which are serving as scaffolds for the genome wide assembly (152) (VGC website: http://rcweb.bcgsc.bc.ca/cgi-bin/cryptococcus/cn.pl). Shotgun sequencing for the serotype D strains JEC21 and the ancestral precursor strain B3501 have reached ~6X coverage for each strain at Stanford University and The Institute for Genome Research, and chromosome size assemblies have been achieved in large part based on paired plasmid reads and BAC end reads to scaffold the assembly (Stanford website: http://www-sequence.stanford.edu/group/C.neoformans/index.html, TIGR website: http://www.tigr.org/tdb/e2k1/cna1/index.shtml). It is anticipated that closure and annotation of the JEC21 reference serotype D genome will be achieved by the end of 2003. A comparative genomics project to determine the genome sequence for the serotype A strain H99 has begun at the Duke Center for Genome Technology (Duke CGT website: http://cneo.genetics.duke.edu). A BAC library has been fingerprinted and end-sequenced to serve as a scaffold for the sequencing project (152). ~61,000 sequence traces or 1.5X coverage has been determined by shotgun sequencing of paired plasmid reads. Initial comparisons reveal that global genome architecture may be conserved between the divergent serotype A and D strains (F. Dietrich, personal communication). Finally, studies on a representative serotype B variety *gattii* strain, WM276, have begun to extend the comparative aspects of the genome project to three divergent varieties or sibling species of this human fungal pathogen.

A challenging aspect of annotating the *C. neoformans* genome is the presence of multiple small introns in virtually every gene. This challenge is being met by two complementary approaches. First, a large-scale EST sequencing project at Oklahoma Health Science Center has provided cDNA sequence information for several thousand genes from both serotypes A and D, and the information revealed about intron-exon borders is being used to train gene finding software for annotation (OHSC website: http://www.genome.ou.edu/cneo.html). Second, comparative genomics approaches between serotype A and D genomic sequences provide a novel means to identify the most highly conserved regions of the genome,

which include exon sequences (but not intron sequences). Thus, the determination of two different but related genome sequences has the power to make the analysis of the genome much more tractable than analysis of a single reference genome.

In addition to these physical and sequence-based maps of the genome, a meiotic-based recombination map of the genome has recently been generated (60). This map was based on an analysis of meiotic segregants from the serotype D *MATα* strain B3501 and the *MAT**a*** strain B3502. 280 meiotic progeny were analyzed by AFLP analysis of polymorphic markers, and a map consisting of 14 major linkage groups was established. This map is currently being extended and refined using microsatellite loci. These studies provide a starting point for the definition of higher-resolution maps to be used as tools in the analysis of quantitative trait loci and their contribution to physiology and virulence of the organism. These types of analysis will also provide mapping information and resources that can be used to identify centromeres using an array of microsatellite markers and basidiospores analyzed from individual basidia in which a single meiotic event gives rise to parental ditype, nonparental ditype, or tetratype segregation patterns (98).

## ANIMAL MODELS OF VIRULENCE

One of the most attractive features of *C. neoformans* as an experimental system for studies of pathogenesis is that several robust animal model systems have been developed and widely implemented. These include models in several different animal species and which probe unique aspects of the infectious cycle. As described above, *C. neoformans* most commonly occurs in immunocompromised hosts but can also be a primary pathogen in individuals with no apparent immune system dysfunction. Second, *C. neoformans* is acquired from the environment by inhalation of spores or desiccated yeast cells, and thus initially infects the lung (Figure 1). While virtually any organ can be infected by *C. neoformans*, the most common clinical manifestation is hematogenous dissemination to the central nervous system where meningoencephalitis ensues. An important aspect is that latent infections can be established in the lung, which can then lead to dissemination years or decades later. The animal models in current use seek to emulate these features of the infectious cycle.

The animal models most commonly used are the murine tail vein injection model, the murine inhalation model, the rat inhalation model, and the rabbit intracerebral model. Each has strengths, and together these models provide a robust platform to examine virtually every aspect of *C. neoformans* pathogenesis. The murine models have distinct advantages in terms of the size and cost of animals and the large number of inbred and genetically altered lines that are available. The murine inhalation model recapitulates virtually all aspects of the natural course of infection in humans. Animals are anesthetized and infected with drops of fungal cells deposited on the nares, which are inhaled (35, 42, 182). The infection begins in the lung and spreads to the CNS. Virtually all infected animals die from

cryptococcal meningitis within 30 days of infection with the serotype A strain H99 delivered at an infecting inoculum of $5 \times 10^4$ cells. A/Jcr mice are commonly used in this model. These animals are immunocompetent but can be readily infected with pathogenic serotype A strains. For the congenic serotype D lab strains, which are significantly attenuated compared to serotype D or A clinical isolates, a tail vein injection model has been commonly employed. These studies are generally carried out using DBA mice lacking the C5 component of complement, although studies using BALB/c mice have also been reported (21, 23, 40, 154, 182). These two models allow study of the lung-blood-CNS virulence cycle following infection by inhalation, or to bypass the lung and study only the blood-CNS cycle following tail vein injection. Models employing direct intrathecal inoculation in either rabbits or mice allow one to bypass both the lung and the bloodstream to analyze only the CNS part of the infectious cycle.

The rabbit model has advantages in that the animals are immunosuppressed with steroids (mimicking a common human host state), and that larger quantities of CSF and fungal cells can be obtained for analysis (142). Disadvantages of the rabbit model are the large size and high cost of animals, that expertise is required to deliver the infecting inoculum and recover CSF fluid, and that the normal body temperature of the rabbit is 39°C (higher than normal human body temperature). Nevertheless, the rabbit model has been enormously useful for studies of antifungal drug action and mutants and will continue to play a valuable role in allowing access to large numbers of fungal cells and RNA for gene expression profiling.

Finally, the rat inhalation and intratracheal models provide a valuable animal model system in which to study latency and dissemination of infection (68, 69, 71). In contrast to the murine and rabbit models, in which infection progresses rapidly to symptomatic disease, infection in the rat leads to a latent infection that can persist for as long as 18 months. This provides a window of opportunity to study the latent phase of the infection in the lung and to analyze molecular determinants that could contribute to understanding how this important aspect of the natural history of infection might be controlled in the human host. In practice, for new investigators in this area, establishing the murine inhalation and tail vein injection models would provide a robust and flexible platform to conduct a variety of virulence studies.

## ROLE OF MAPK AND cAMP SIGNALING IN CELL DIFFERENTIATION

Two conserved signaling pathways, the pheromone-activated MAP kinase pathway and a nutrient-sensing cAMP-protein kinase A pathway, control differentiation of *C. neoformans* (Figure 6). Of particular importance is that several components of the MAP kinase pathway (Ste20, Ste11, and Ste12) are encoded by the mating-type locus, and thus $\alpha$ and **a** cells express distinct alleles of these signaling elements. While the role of the MAP kinase pathway in controlling mating and haploid fruiting has been definitively established, the role of the MAP kinase pathway in

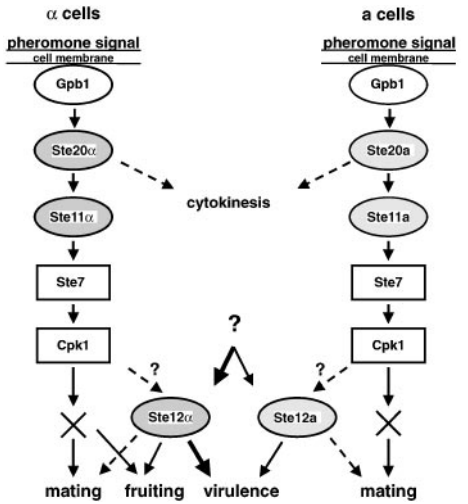



**Figure 6** MAP kinase signaling cascades. Some components of the signaling cascades leading to mating, fruiting, and virulence in *C. neoformans* are mating-type specific. In $\alpha$ cells, pheromone activates a MAP kinase cascade through a G-protein–coupled receptor and a heterotrimeric G-protein. The G$\beta$ subunit Gpb1 transduces the signal to the mating-type–specific PAK kinase Ste20$\alpha$, which relays the signal to the MAP kinase cassette consisting of the mating-type–specific MEK, Ste11$\alpha$ and the nonmating-type–specific kinases Ste7 and Cpk1. Signals from this cassette likely impinge on the $\alpha$-specific transcription factor Ste12$\alpha$ to control fruiting and virulence and an as-yet unknown factor to control mating. This signaling pathway is mirrored in **a** cells utilizing the cell type-specific factors Ste20**a**, Ste11**a**, and Ste12**a**.

controlling virulence remains less clear. Instead, the protein kinase A pathway plays a central role in controlling virulence, and mutations that disable signaling in this pathway attenuate virulence, whereas those that activate signaling enhance virulence.

A MAP kinase signaling cascade has been defined that functions in sensing pheromones during mating [reviewed in (109, 181)]. Three genes encoding the MF$\alpha$ pheromone have been identified in *MAT*$\alpha$ (46, 129, 154). These genes encode a precursor peptide that is modified by proteolysis and C-terminal farnesylation at a CAAX motif. The MF$\alpha$1 pheromone has been produced synthetically, and induced conjugation tube formation in **a** cells. By both reporter gene assays and northern blots, the *MF*$\alpha$*1*, *2*, and *3* genes are induced in response to nutritional limitation and co-culture with **a** cells. Deletion of the three genes results in an *mf*$\alpha$*1,2,3* pheromoneless triple mutant strain that is mating impaired but still capable of producing mating filaments and recombinant basidiospores at about 1% the efficiency of wild-type cells. This is in stark contrast to *S. cerevisiae*, in which mating pheromones are essential for mating, but similar to other basidiomycetes

in which cell-cell fusion events are more promiscuous. The MF$\alpha$ pheromone is required for $\alpha$ cells to induce the morphological changes associated with mating in confronting **a** cells. The *MF$\alpha$1* pheromone gene is induced in vivo in the central nervous system of infected animals (48), but the pheromoneless triple mutant strain exhibits only a very modest defect in virulence (154). Three related genes encoding the MF**a** pheromone produced by **a** cells have also recently been identified (121). The three genes are encoded by *MAT**a*** and encode lipid modified peptide pheromones that share some limited amino acid sequence similarity with the MF$\alpha$ pheromones.

Two G-protein–coupled receptor homologs that likely function in pheromone sensing are encoded by the *MAT$\alpha$* and *MAT**a*** alleles of the mating-type locus, and both share sequence identity with the *S. cerevisiae* **a**-factor receptor Ste3 (27a, 110). These receptors are coupled to a heterotrimeric G-protein whose G$\beta$ subunit is encoded by the *GPB1* gene (183). Mutants lacking Gpb1 are sterile, and this mating defect is suppressed by overexpression of the MAP kinase homolog Cpk1, implicating Gpb1 in control of the MAP kinase cascade. Overexpression of Gpb1 promotes conjugation tube formation in both $\alpha$ and **a** cells, supporting a model in which the $\beta\gamma$ subunit plays an active signaling role analogous to the $\beta\gamma$ heterodimer Ste4-Ste18 in *S. cerevisiae*. The $\alpha$ subunit coupled to Gpb1 has not been definitively identified but may be encoded by the *GPA3* gene. The $\gamma$ subunit remains to be identified.

Recent studies have implicated a second GTP binding protein, the Ras1 protein, in cellular responses to pheromone (2, 185). *ras1* mutant cells are sterile, defective in responses to pheromone in confrontation assays, and fail to express mating pheromones or pheromone-activated genes in co-culture. Overexpression of Gpb1, or of the MF$\alpha$1 pheromone, restores mating of *ras1* mutant cells. Finally, when the MF$\alpha$1 mating pheromone is expressed from a heterologous promoter in *$\alpha$ ras1* mutant cells, the ability to promote pheromone-induced morphology changes in confronting **a** cells is restored, but the *$\alpha$ ras1* mutant cells still fail to respond to MF**a** pheromone. These findings illustrate that Ras1 is required both for pheromone production and pheromone response and suggest that Ras1 functions early in the pheromone response pathway in *C. neoformans*. An analogous situation occurs in *S. pombe* in which the ras1 homolog functions with the G$\alpha$ subunit gpa1 to promote activation of the MAP kinase cascade by mating pheromones (132). In *S. cerevisiae*, the Ras2 protein plays a dual signaling role and activates both cAMP production by adenylyl cyclase and MAP kinase signaling during filamentous and invasive growth (130). The functions of Ras appear to be more restricted to MAP kinase signaling in fission yeast and in the basidiomycete *C. neoformans*.

The genes encoding several kinases that function in pheromone sensing and mating have been identified, including *STE20$\alpha$/**a***, *STE11$\alpha$/**a***, *STE7*, and *CPK1* (28, 44, 182). Three genes encoding homologs of the PAK kinases have been identified and studied in detail, including the mating type–specific Ste20$\alpha$ and Ste20**a** kinases encoded by the *MAT* locus and the nonmating type–specific Pak1 kinase (182). Mutants lacking the Ste20 kinases exhibit a bilateral mating defect, and

*ste20α* mutants fail to respond to MF**a** pheromone in confrontation assays. Mating is restored in *ste20* mutant strains by overexpression or mutational activation of the Ste11α or Cpk1 kinase, providing epistasis evidence that the PAK kinases function upstream in activating the MAP kinase signaling pathway.

Importantly, the genes encoding the principle components of the MAP kinase pathway, *STE11α/**a***, *STE7*, and *CPK1*, are both mating-type specific and nonspecific. Genetic evidence links these three kinases in a common pathway that senses pheromones and controls mating (44). Mutants lacking any of the three kinases exhibit a very similar severe sterile phenotype and fail to respond to mating pheromones in confrontation assays. The function of all three kinases is required for induction of the MFα mating pheromone genes in response to MF**a** pheromone secreted by **a** cells. Epistasis evidence supports a model in which components of the Ste11-Ste7-Cpk1 module function downstream of both the pheromone-activated heterotrimeric G-protein and the Ste20α PAK kinase. This pathway is thus composed of both mating-type–specific components (Ste20α/**a**, Ste11α/**a**) and nonmating-type–specific components (Ste7, Cpk1). The Ste7 and Cpk1 kinases therefore must have evolved to function with two different types of partner subunits depending on the cell type in which they are expressed. As a consequence of this unique specialization, α and **a** cells use two somewhat different signaling cascades to respond to pheromone during mating. This may in part give rise to some of the unique features exhibited by the two cell types during mating: namely, that α cells respond to **a** cells by haploid fruiting, while **a** cells respond to α cells by initially making short conjugation tubes and then dramatically enlarging, possibly to provide an easier target for cell fusion with an extending filament tip or basidiospore.

One candidate target of the MAP kinase pathway is Ste12, which is encoded by the mating-type locus and therefore exists as both Ste12α and Ste12**a** (25, 91, 189, 198). However, *ste12α* mutant cells exhibit only a modest defect in mating, respond normally to pheromones in cell-cell confrontation assays, and pheromone gene expression is inducible by co-culture with cells of the opposite mating type (26, 44, 198). Moreover, overexpression of Ste12α suppresses the mating defects of mutants lacking the Ste11α, Ste7, or Cpk1 kinases, indicating that simply increasing Ste12 levels in the cell can bypass the normal requirement for MAP kinase signaling (44). These findings could be accommodated by models in which Ste12 is one of two redundant downstream targets in which overexpression of Ste12 bypasses a requirement for MAPK action. Alternatively, Ste12 might be the target of a different signaling pathway, such as the PKA cascade (42). The *C. neoformans* Ste12 homologs contain an N-terminal homeodomain presumed to be involved in specific DNA binding, and a C-terminal zinc finger region of unknown function. The *C. neoformans* Ste12 homologs lack the peptide binding sites for the Dig1/2 repressor proteins found in *S. cerevisiae*, and thus are likely to be regulated in a distinct fashion from their ascomycete counterparts.

In *S. cerevisiae*, one component of the MAP kinase pathway has been functionally specialized into two divergent forms. The Fus3 MAP kinase promotes

mating, whereas the Kss1 MAP kinase promotes pseudohyphal growth in the diploid (33, 120). While Kss1 can in part support mating in haploid yeast cells, Fus3 is not expressed in diploids and plays no role in diploid pseudohyphal growth. This theme is revisited in *C. neoformans* but now three different signaling components, Ste20 and Ste11 (kinases), and Ste12 (a transcription factor) are all expressed from the mating-type locus in $\alpha$ and **a** cell type–specific forms.

Studies on the pheromones and pheromone-activated G-protein reveal an autocrine signaling loop that promotes haploid fruiting of $\alpha$ cells in the absence of **a** cells (154). Mutants lacking the MF$\alpha$ mating pheromones or the G-protein $\beta$ subunit, Gpb1, exhibit defects in haploid fruiting, providing evidence that an autocrine signaling loop involving MF$\alpha$ pheromone acting on $\alpha$ cells contributes to cellular differentiation (183). One analogy is to self-filamentous mutants of *S. commune* and *C. cinereus*. In these organisms, single amino acid changes allow a mutant pheromone to act on a normally nonresponsive receptor, or broaden the ligand-binding specificity of a pheromone receptor such that a cell responds to its own pheromone (61, 137). Similar mutations may have arisen in *C. neoformans* and could explain the specialized ability of $\alpha$ cells to differentiate in response to nutrient limitation. Haploid fruiting conditions result in the induction of *MF$\alpha$* expression, which could then act as a partial agonist on the MF**a** pheromone receptor or another as-yet undefined receptor.

The MAP kinase cascade that plays a role in pheromone sensing during mating also functions during haploid fruiting in response to nitrogen limitation and desiccation. Mutants lacking the Ste20$\alpha$, Ste11$\alpha$, Ste7, or Cpk1 kinases all exhibit profound defects in haploid fruiting (28, 44, 182). Although the Ste12$\alpha$ kinase plays only a very minor role in mating, *ste12$\alpha$* mutants exhibit a profound defect in haploid fruiting in response to nitrogen limitation and desiccation (26, 198). In epistasis experiments, the haploid fruiting defect of *ste12$\alpha$* mutant cells is suppressed by overexpression of the MF$\alpha$ pheromone, a dominant activated Ste11$\alpha$-1 kinase, or overexpression of Cpk1 (44). These findings do not support a model in which Ste12$\alpha$ functions downstream of the MAP kinase pathway in a strictly linear fashion, since activation of the upstream components bypasses the requirement for Ste12$\alpha$. In addition *ste12$\alpha$* mutant cells can haploid fruit in response to MF**a** pheromone produced by confronting **a** cells, whereas *ste20$\alpha$*, *ste11$\alpha$*, *ste7*, and *cpk1* mutants do not. These findings reveal that the MAP kinase cascade and Ste12$\alpha$ both control haploid fruiting, but again reveal that the functions of the upstream elements of the pathway are distinguished from the transcription factor that has been thought to be a possible target of the cascade. These findings underscore a molecular link between the signaling pathways that allow the cell to differentiate during haploid fruiting and mating. This may reflect a role for haploid fruiting in response to pheromone in the earliest steps of mating to assist cells in locating a mating partner. In turn, the ability to haploid fruit to a more limited extent in the absence of a mating partner may reflect evolution of an asexual pathway from a sexual one to promote survival under adverse conditions.

The finding that *MATα* is linked to virulence fueled early speculation that the pheromone-activated MAP kinase pathway might play a central role in virulence. Yet a different picture has emerged from recent studies of the pathogenic potential of mutant strains lacking defined elements of this signaling pathway. First, the Ste11α, Ste7, and Cpk1 kinases are completely dispensable for virulence of the serotype D strain JEC21, and a serotype A *cpk1* mutant strain is also fully virulent (44). Independent studies by Wickes and colleagues reveal that *ste11α* mutants are nearly fully virulent (28). These findings clearly indicate that the MAP kinase pathway does not play an important role in virulence.

Two other components have been linked to virulence but appear to play serotype-specific roles. When the Ste20α PAK kinase is mutated in the serotype A pathogenic isolate H99, virulence is attenuated in both rabbits and mice (182). In contrast, serotype D mutants lacking either Ste20α or Ste20**a** exhibit no defect in virulence compared to wild-type cells. The virulence defect of serotype A strains lacking the Ste20α kinase is likely attributable to several findings. First, these mutants exhibit a cytokinesis defect that becomes more severe at elevated temperature and results in a temperature-sensitive growth defect. Second, the *ste20α* mutants adopt a filamentous growth form in vivo in which elongated buds are produced and many of the mutant cells exhibit a defect in capsule production. In contrast, serotype D strains lacking Ste20α or Ste20**a** exhibit a cytokinesis defect but no temperature-sensitive growth defect, which may explain why they are dispensable for virulence in serotype D.

A similar scenario has emerged with respect to the Ste12 transcription factor homolog. In this case, serotype D mutants lacking Ste12α or Ste12**a** are attenuated for virulence, whereas serotype A *ste12α* mutants are fully virulent (25, 26, 198). *ste12α* mutant cells exhibit a modest defect in capsule production in vivo. These findings link a second component of the *MAT* locus to virulence and also underscore the divergence that has occurred between these two varieties or species.

During mating, the MAP kinase pathway functions in parallel with a nutrient-sensing Gα protein-cAMP-protein kinase A signaling cascade that also plays a central role in controlling virulence of this organism (4, 5, 42). The Gα protein Gpa1 was first identified by Courchesne and colleagues who hypothesized that it might play a role in mating, possibly as an element of the pheromone response pathway (171). Studies in *S. cerevisiae* on the homologous Gα protein Gpa2 revealed a distinct role in nutrient sensing during diploid pseudohyphal growth (117). Alspaugh et al. established that *C. neoformans gpa1* mutants are viable and exhibit a sterile phenotype in the serotype A pathogenic strain H99 (4). This initial finding could be explained by a role in nutrient sensing, pheromone sensing, or both, since both signals are required for mating. Alspaugh et al. went on to show that *gpa1* mutant cells exhibit defects in producing two inducible virulence factors: capsule in response to iron limitation and melanin in response to carbohydrate limitation. Both capsule and melanin had previously been linked to virulence in classic studies, and mutants lacking either capsule or melanin are avirulent or

severely attenuated for virulence. Thus, the *gpa1* mutant cells have two strikes against them, and in two different animal models (the rabbit CNS model and the murine inhalation model), *gpa1* mutant strains are dramatically attenuated (4, 42).

The *S. cerevisiae* Gpa2 G$\alpha$ protein has been implicated in activation of cAMP production and a protein kinase A signaling pathway that promotes filamentous growth (30, 117, 138, 139, 140, 148). Analogously, provision of exogenous cAMP restores mating, capsule production, and melanin production in *C. neoformans gpa1* mutant cells. Direct measurement of intracellular cAMP concentrations reveals that Gpa1 is required for cAMP production in response to glucose in *C. neoformans* (42). Mutants lacking the enzyme adenylyl cyclase (Cac1) exhibit phenotypes strikingly similar to mutants lacking the heterotrimeric G$\alpha$ protein Gpa1 (5). The *CAC1* gene encoding adenylyl cyclase was identified by low-stringency PCR with primers targeted to conserved regions of homologous fungal enzymes, and the gene was disrupted by transformation and homologous recombination. The resulting *cac1* mutant cells are viable, sterile, and defective in producing melanin and capsule and are avirulent in the murine inhalation model (5). These cells lack any detectable cAMP, illustrating that adenylyl cyclase is not essential for viability in this pathogenic basidiomycete, in contrast to the essential function of the homologous enzyme and pathway in *S. cerevisiae*. Provision of exogenous cAMP restores mating, capsule, and melanin production in cells lacking adenylyl cyclase, indicating that the functions of this large peripheral membrane protein are catalytic and not structural.

The target of cAMP, the cAMP-dependent protein kinase PKA, plays a central role in controlling the virulence of *C. neoformans* (42). PKA is highly conserved and exists as a tetramer of two regulatory subunits bound to two catalytic subunits. In cells with low cAMP concentrations, the enzyme is in an inactive complex; however, when cAMP concentrations increase, the binding of cAMP to the regulatory subunits induces conformational changes that release and activate the catalytic subunits. D'Souza et al. identified and characterized the genes encoding the catalytic and regulatory subunits of protein kinase A from *C. neoformans* (42). Two genes encode the catalytic subunits, *PKA1* and *PKA2*, whereas the regulatory subunit is encoded by a single unique gene, *PKR1* (42; C. D'Souza, J. Hicks, G.M. Cox & J. Heitman, in preparation). In the pathogenic serotype A strain H99, *pka1* mutants exhibited phenotypes strikingly similar to *gpa1* and *cac1* mutant cells lacking the G$\alpha$ protein Gpa1 or adenylyl cyclase (42). *pka1* mutant strains are sterile, fail to produce capsule or melanin in response to inducing conditions, and are avirulent in the murine inhalation model. *pka1* mutant strains generated in the congenic lab-adapted serotype D JEC20/JEC21 background exhibit no phenotype, are fertile, produce melanin and capsule, and are fully virulent (C. D'Souza, J. Hicks, G.M. Cox & J. Heitman, in preparation). Instead, serotype D *pka2* mutant cells exhibit phenotypes in common with serotype A *pka1* mutants and are sterile in bilateral crosses and fail to produce melanin or capsule in response to inducing conditions.

The two PKA catalytic subunits share limited sequence identity, and their functions have clearly diverged. This specialization of PKA catalytic subunitsunderscores the molecular divergence that has occurred in the ~20 million years that separate the serotype A var. *grubii* and the serotype D var. *neoformans* from a common ancestor (195) and is reminiscent of similar functional specialization that has occurred in *S. cerevisiae*, in which the three PKA catalytic subunits Tpk1, Tpk2, and Tpk3 play a redundant role in growth but unique roles to promote (Tpk2) or inhibit (Tpk1/3) filamentous growth (138, 148). Mutations altering the PKA regulatory subunit gene *PKR1* constitutively activate the PKA signaling cascade and result in increased capsule production and a hypervirulent phenotype in animal models. The *PKR1* gene encoding the protein kinase A regulatory subunit was identified by PCR with primer pools under low-stringency conditions (42). The gene is unique in *C. neoformans* and was disrupted by transformation and homologous recombination. In contrast to mutations in the catalytic subunit genes, *PKA1* and *PKA2*, which prevent signaling via the pathway, mutations in the regulatory subunit have two different physiological outcomes. First, *pkr1* mutations release the Pka1 and Pka2 catalytic subunits in an active form, and second, the pathway is severed from upstream regulatory signals that control cAMP levels.

*pkr1* mutant strains lacking the Pkr1 regulatory subunit are viable and produce enlarged capsules under normally inducing conditions, similar to wild-type cells exposed to exogenous cAMP (42). These observations provide further evidence that the PKA pathway controls capsule production, that signaling via the pathway is limiting for capsule production and can be increased, and that signaling via the cAMP-PKA pathway functions independently of the sensor for the iron-limitation signal that induces capsule production. The consequences of increasing capsule production on virulence were examined in animal models. In both the murine inhalation and tail vein injection models, *pkr1* mutant strains were hypervirulent in comparison with the congenic wild-type strain (42). The fungal burden in the central nervous systems of infected animals was increased four- to sixfold with the *pkr1* mutant compared to the wild-type strain. Importantly, when the fungal cells were examined directly in the CNS of infected animals, the *pkr1* mutant cells produced dramatically enlarged capsules whose volumes were increased 12-fold compared to those produced in response to host signals by the wild-type strain. The capsule of wild-type and *pkr1* mutant cells in the inoculum (grown in vitro in YPD-rich medium) did not differ, indicating that this dramatic induction is in response to host signals following infection. These findings suggest that the PKA pathway modulates the ability of another sensing mechanism to effect changes in virulence factor production. The finding that *pkr1* mutant strains are hypervirulent may be relevant in two clinical settings. First, although *C. neoformans* is classified as an opportunistic pathogen, infections can occur in hosts with no known immune dysfunction (1, 18, 135). These individuals may have subtle immune defects or, alternatively, may be infected with hypervirulent strains. In fact, two clinical case reports identified patients who were infected with atypical forms of

*C. neoformans* that produce dramatically enlarged capsules (37, 119). Whether these represent naturally occurring lesions in the *PKR1* gene or of other elements of the PKA-controlled pathway regulating capsule remains to be explored experimentally. A second potentially relevant clinical finding is that whereas serotype A and D strains infect predominantly immunocompromised hosts, strains of the serotype B and C *gattii* variety infect immunocompetent hosts and are rarely isolated from AIDS patients [reviewed in (157)]. Thus, the serotype B and C strains may be hypervirulent with respect to host immune function. Alternatively, it has been proposed recently that serotype B and C strains do not establish latent infections and thus give rise only to primary infections in immunocompetent hosts rather than to reactivation of latent infections in response to immunosuppression which occurs with serotype A and D strains (T. Sorrell, personal communication).

The hypervirulent *pkr1* mutant strain is one of the few mutants to be molecularly defined in any pathogenic system. Two other mutations that enhance virulence of *C. neoformans* have been identified recently. Janbon and colleagues identified the *CAS1* gene, which encodes an integral membrane protein involved in capsule acetylation that generates specific epitopes detectable by anticapsular monoclonal antibodies (89). *cas1* mutant serotype D strains have lost these capsular epitopes, and were hypervirulent in the murine tail vein injection model, leading to 100% lethal infections by day 20 postinfection compared to ~60 days with the congenic wild-type strain (89). A third hypervirulent mutant strain was recently identified in a signature-tagged mutagenesis approach (131). Following random insertion of the hygromycin resistance gene into the *C. neoformans* genome, mutants were identified that were altered in ability to survive in vivo in mice compared to the parental wild-type strain (H99, serotype A). One such mutant, the 2A2 strain, resulted in a tenfold greater burden of fungal cells in the CNS and animals infected with this strain exhibited frank signs of infection in as short as 10 days following infection, a time at which animals infected with the wild-type remained largely healthy. Identification of the insertional mutation in the 2A2 mutant strain, and demonstration that it causes the mutant phenotype, should provide significant insight into the nature of this intriguing hypervirulent isolate.

What remains to be learned about the G$\alpha$-cAMP-PKA pathway and its role in virulence? First, the molecular identity of the receptor that activates this pathway has not been established. One hypothesis is that the receptor will be a member of the G-protein–coupled receptor family, and in fact GPCRs that are linked to the homologous G$\alpha$ proteins in both *S. cerevisiae* and *S. pombe* have been identified. In these model yeasts, the Gpr1 and git3 receptors are GPCRs that span the membrane seven times, share homology with each other, and are involved in sensing glucose and activating cAMP production (93, 118, 186, 196). In contrast, although a homologous G$\alpha$ protein exists in the basidiomycetes *C. neoformans* and *U. maydis*, no receptor homolog has as yet been identified. A second interesting feature of the signaling pathway is that no G$\beta\gamma$ subunits linked to the G$\alpha$ protein Gpa1 have been identified. Gpa1 shares marked sequence identity with

known heterotrimeric G$\alpha$ protein subunits, but the only G$\beta$ subunit identified, Gpb1, is linked to pheromone sensing and plays no role in virulence factor production or virulence and does not function as a subunit with Gpa1 (183). A similar situation exists in *S. cerevisiae* in which the G$\alpha$ protein Gpa2 does not interact with the only known G$\beta\gamma$ subunits (Ste4-Ste18) that function in mating but not filamentous growth (115, 147). Very recent studies have identified several novel proteins that function as G$\beta$ subunit structural mimics in the Gpa2 signaling pathway in budding yeast (76). These proteins, Gpb1 and Gpb2, physically interact with Gpa2 and play an inhibitory signaling role. Remarkably, these proteins lack the signature seven WD-40 repeats of all known G$\beta$ subunits but contain instead seven copies of a different sequence, the kelch repeat, previously implicated in mediating protein-protein interactions. The x-ray structure of one enzyme (galactose oxidase) with seven kelch repeats is known, and the protein folds into a seven bladed $\beta$–propeller that is essentially superimposable on the known structure of the G$\beta\gamma$ complex (86). Finally, the targets of the PKA pathway that function to promote mating, capsule and melanin production, and virulence also remain to be identified. By analogy with previous studies on PKA signaling in *S. cerevisiae*, *S. pombe*, and *U. maydis*, it is anticipated that one or more transcription factors that control target gene expression may lie downstream of PKA [reviewed in (41, 109)]. For example, in *S. cerevisiae* the Tpk2 catalytic subunit of PKA is nuclear localized and functions to promote expression of the cell surface flocculin Flo11 that is necessary for filamentous and invasive growth (140). Tpk2 governs differentiation by a dual mechanism in which phosphorylation of the Sfl1 repressor promotes dissociation of the dimeric repressor, causing it to disengage from the *FLO11* promoter (31, 140, 148). Tpk2 also phosphorylates the activator Flo8, enabling it to bind to the *FLO11* gene promoter and activate transcription (140). In *U. maydis*, a key transcription factor target of the PKA pathway is the Prf1 pheromone response factor, a member of the HMG box transcription factor family (77, 78). PKA controls Prf1 by promoting expression of the *PRF1* gene, and it likely phosphorylates and activates Prf1 as well. A homolog of the Prf1 factor has recently been identified in *C. neoformans*, and studies are in progress to establish whether this is the target of the Pka1 catalytic subunit that controls virulence factor production and pathogenesis (C. D'Souza, J. Hicks & J. Heitman, unpublished results). Additional studies are in progress using genome microarray approaches to define the transcriptional profiles of cells with engaged or defective PKA signaling routes.

## GENETICS OF VIRULENCE

*C. neoformans* produces two specialized virulence factors that play a central role in its ability to survive in the harsh host environment and cause symptomatic infection. These specialized virulence factors are the polysaccharide capsule and the antioxidant pigment melanin (18). The production of a darkly pigmented cell

ensheathed in a thick capsule makes a stark contrast between this pathogenic budding yeast and the model yeast *S. cerevisiae*. Growth of *C. neoformans* cells under certain conditions results in the dramatic induction of a complex polysaccharide capsule. These inducing conditions include growth in limiting iron concentrations or in physiological concentrations of $CO_2$, both conditions found in the infected host (74, 177). The capsule can be readily detected microscopically by its ability to exclude India ink particles, resulting in the appearance of a halo surrounding the cell. The capsule can also be detected with polyclonal or monoclonal sera raised against carbohydrate epitopes, and this forms the basis of the common serotyping assay that detects differences in the capsule structure that exist in strains of different serotypes. In addition to cell-associated capsule, the growing fungal cells shed capsular material into the growth medium, both in vitro and in vivo in infected animals [reviewed in (50)]. The capsule plays myriad roles in virulence, including inhibiting phagocytosis in some settings and promoting intracellular survival in macrophages following phagocytosis (15). In addition, the shed capsular antigen wreaks havoc on the host immune system by multiple mechanisms, further enfeebling the already immunocompromised host. Of all the virulence traits associated with *C. neoformans*, the capsule most closely fulfills the criteria often associated with virulence factors of, for example, bacterial pathogens. All clinical isolates make capsule, mutants lacking capsule are avirulent, and restoration of capsule production restores virulence.

A panoply of genes involved in capsular biosynthesis have already been identified, and many more likely remain to be discovered. In early studies, both Bulmer et al. and Kozel & Cazin identified a limited number of acapsular mutant strains (16, 92). Subsequently, Jacobson and colleagues identified a series of acapsular mutant strains (*cap43*, *cap44*, *cap48*, *cap53*, *cap54*, *cap55*, *cap59*, and *cap64*) following mutagenesis with N-nitrosoguanidine or UV irradiation in a serotype D strain background (87). Acapsular and hypocapsular mutants were identified initially as nonglistening colonies and then screened by microscopic examination with India ink. These mutants were isolated in the serotype D background in the B3501 mating-type $\alpha$ and B3502 mating-type **a** strains. Importantly, these strains are the f1 progeny of the ancestral precursor strains NIH12 and NIH433 that gave rise to the congenic JEC20/JEC21 strain pair [reviewed in (79)]. B3501 and B3502 are not congenic with each other, with NIH12 or NIH433, or with JEC20 or JEC21. They do, however, share ~50% of their genomes with their parents and ~75% with their offspring, and the B3501 genome is one of the two serotype D strains being subjected to sequence analysis.

Genetic outcrosses were conducted with the isolated hypo- and acapsular mutant strains and the four spore chains were either isolated as a spore suspension, or else directly micromanipulated and dissected from individual basidia (162). The mutant phenotypes segregated in a ratio of 1 wild-type:1 mutant in meiotic progeny. Subsequent studies involving genetic crosses between two different acapsular mutant strains yielded evidence for independent segregation of alleles in a limited number of cases (~3:1 ratio of mutant to wild-type), but in most cases the

mutations appeared to be linked (all mutant progeny) (162). Subsequent molecular studies revealed in some cases that *CAP* genes thought to be linked by genetic analysis were in fact located on different chromosomes (24). These studies were among the first to indicate that standard yeast genetic approaches could be applied to this pathogenic basidiomycete. They also suggest caution in isolating recombinants by the wire loop en masse approach, which is not a suitable substitute for careful basidiospore dissection.

Subsequently, these acapsular mutant strains served as a valuable resource to clone the corresponding genes. In an elegant series of molecular biological studies, Chang & Kwon-Chung cloned and characterized the *CAP10*, *CAP59*, *CAP60*, and *CAP64* genes (21–24). These genes were isolated by introducing a genomic library into the respective acapsular mutant strains, and complementing clones were isolated by the alteration in density and charge of capsular vs. acapsular mutant strains in a polyethylene glycol partitioning gradient. The cloned genes were sequenced and then used to produce gene disruption alleles to mutate the corresponding genes. Each gene was disrupted with the *ADE2* selectable marker for positive selection, and the transforming DNA was introduced by electroporation. Homologous recombination was achieved with a selection method in which nonhomologous recombination events were counterselected using a flanking *URA5* marker distal to the region of homology and selection of the desired *ura5* transformants on 5-FOA medium. These approaches are similar to those originally developed for gene disruption in murine embryonic stem (ES) cells. Although homologous recombination was achieved, the frequency was extremely low in several cases ($\sim$1/10,000). Two factors likely contributed to these low frequencies. First, electroporation is less efficient than biolistic methods for transformation and gene disruption in this organism (45). Second, in several cases the gene disruption alleles were likely constructed from DNA sequences that were not strictly isogenic with the transformation recipient strain, and DNA polymorphisms inhibit targeted integration of homologous sequences in bacteria, yeasts, and murine ES cells.

In these examples, targeted disruption of the *CAP10*, *CAP54*, *CAP59*, and *CAP64* genes each resulted in acapsular mutant strains that were avirulent in the murine tail-vein injection model, and reintroduction of the wild-type gene restored virulence. Several of these genes that were thought to be linked based on earlier genetic studies (162) were found to be encoded by different chromosomes (24). Finally, the sequences of this series of *CAP* genes provide little or no clue as to their functions in capsule biosynthesis.

More recent studies have begun to attack the construction of the capsule by biochemical, immunological, and cell biological approaches wedded to molecular biology and genetics. For example, Janbon et al. identified the *CAS1* gene, which encodes a membrane protein that is essential for O-acetylation of sugar hydroxyl groups on the GXM polysaccharide (89). The *cas1* mutant strain was isolated in the serotype D JEC21 strain background and was identified following UV irradiation as nonreactive in ELISA assays with a monoclonal antibody raised against capsular antigen. The gene was cloned by complementation with a genomic library

constructed in the pCnTel1 telomeric shuttle vector by colony hybridization for restoration of antibody cross-reactivity. The gene was cloned and sequenced, and shown to be the correct gene by identification of a mutation in the *CAS1* gene in the original *cas1* mutant strain. The gene was disrupted by electroporation and positive-negative selection. Importantly, the *cas1* disruption mutant failed to react with the original monoclonal, and most interestingly, this mutant was hypervirulent in mice. These studies illustrate the power of combining immunological reagents against the capsule with genetic and molecular biology approaches to dissect the characteristics of this virulence trait.

Doering and colleagues have pioneered the use of cell biological and biochemical approaches to analyze capsule structure and construction (50). In an elegant series of studies, novel imaging techniques were employed to examine how and where new capsule material is formed (144). Newly synthesized capsular material is deposited near the cell wall, displacing outward older capsular antigen. In addition, as the outer capsule is displaced and therefore occupies a greater volume, the original capsular material becomes diluted and appears to acquire additional newly synthesized material. Finally, the capsule surrounding the bud is newly synthesized and originally has a looser configuration that can give rise to an unusual India ink staining pattern in which grains of the ink penetrate the capsule of the bud but not the mother cell. This finding may be analogous to the recent finding in budding yeast that the septins form a diffusion barrier between the plasma membranes of mother and daughter cells (168).

In addition to these cell biological studies, Doering and colleagues have begun to apply biochemical approaches to identify enzymes involved in capsule biosynthesis. These studies have identified a unique $\alpha$-1,3 mannosyltransferase that transfers mannose from a donor molecule to a dimannoside receptor to establish the critical $\alpha$-1,3 linkages that buttress the capsule (49). Doering and colleagues also recently identified the enzyme that produces the UDP-xylose donor for capsule synthesis and the *USX1* gene encoding it (8).

The second specialized virulence factor elaborated by *C. neoformans* in response to nutritional and host signals is the pigment melanin [reviewed in (19)]. This dark polymer is produced from precursors such as dopa or caffeic acid following their oxidation by the enzyme laccase, and expression of the laccase enzyme is induced by carbohydrate limitation. The production of melanin serves to identify *C. neoformans*, which produces darkly pigmented colonies on dopa or bird seed agar (which contains diphenolic precursors) (158, 163). Melanin is deposited in the cell wall, and protects the cell from oxidative and nitrosative challenge by the host immune system and likely also plays a role in defense against other stresses. Melanized cells are extremely durable, and following an overnight exposure to boiling acid, the shells of these cells (so called melanin ghosts) remain (133, 149). Melanin ghosts can be recovered by this procedure from the organs of infected mice and rats, and from the brain tissue of human patients with cryptococcal meningitis examined at autopsy, proving that melanin is produced during infection (133, 134). Virtually all clinical isolates of *C. neoformans* make both capsule and melanin,

although a few rare nonpigmented variants that retain virulence in animal models have been reported (103, 174).

Mutants that fail to produce melanin are significantly attenuated in animal models. In early studies, Mel$^-$ mutant strains were isolated following mutagenesis and found to have defects in the enzyme required for melanin production, and an apparent defect in active transport of precursors required for melanin biosynthesis (102). These Mel$^-$ mutant strains were significantly attenuated in animal models, and those fungal cells that could be isolated from the CNS had reverted to a Mel$^+$ phenotype (145). In other cases, strains that exhibit a temperature-sensitive phenotype and produce melanin at 24°C or 30°C but not at 37°C have been reported (103). Even wild-type strains produce significantly less melanin at 37°C than at lower growth temperatures in vitro, which introduces a paradox with respect to this virulence factor unless other conditions mitigate this effect in vivo (88). Importantly, mutations in the Gpa1-cAMP-PKA pathway confer a defect in melanin production that is most apparent at 37°C and less so at 30°C (4), suggesting that earlier temperature-sensitive Mel$^-$ mutants might have altered elements of this signaling cascade that controls virulence factor production.

The *LAC1* gene encoding the melanin biosynthetic enzyme laccase has been disrupted by transformation and homologous recombination (151, 191). Following a series of backcrosses, the *lac1* mutant strain was found to be attenuated compared to the congenic wild-type in a murine tail vein injection model. However, in contrast to acapsular mutants, which are completely avirulent, the *lac1* mutant strains were significantly attenuated but not avirulent and some lethal infections were observed. Thus, in rare apigmented clinical isolates, other mutations may mitigate the loss of melanin and thereby restore virulence.

Genetic approaches analogous to those applied to capsule have also been applied to melanin biosynthesis. Torres-Guererro & Edman subjected strains of the congenic serotype D JEC21/JEC20 strain series to mutagenesis with the alkylating agent EMS (172). A series of mutants that produce no melanin or reduced levels of melanin was isolated as those that produced white or light tan colonies on birdseed or dopamine medium. Crosses between wild-type and Mel$^-$ mutant strains yielded wild-type and mutant progeny in a ratio of 1:1, indicating single nuclear mutations confer the observed phenotypes. Genes were identified as allelic by segregation tests in which crosses between Mel$^-$ strains yielded either 100% mutant progeny (allelic) or 75% mutant/25% wild-type (nonallelic). This approach assigned 18 mutants to seven different genes defined as segregation groups (by analogy to complementation groups). Importantly, the *mel2* mutant strain was found to be complemented by the cloned *LAC1* gene encoding laccase and shown to harbor a mutation in a conserved histidine residue (H164Y) in a putative copper-binding site of the laccase enzyme (151).

Several of the other Mel$^-$ mutants exhibited additional interesting phenotypes (172). First, melanin production was restored by the addition of exogenous copper ions to the *mel1*, *mel3*, *mel5*, and *mel7* mutant strains. Many laccase enzymes are copper-zinc enzymes, and thus mutations affecting proteins involved in metal

ion transport and delivery to charge the active site of the cryptococcal laccase enzyme might result in melanin defects. The second intriguing phenotype is that the *mel1*, *mel5*, and *mel7* mutants also exhibited a recessive sterile phenotype. These mutants mate with wild-type strains, but $\alpha$ *mel1* strains fail to mate with **a** *mel1* strains and no filaments, basidia, or spores were produced. These mutants are therefore bilaterally sterile. This sterile phenotype cosegregated in genetic crosses to wild-type with the Mel$^-$ phenotype, indicating a single mutation confers both phenotypes. Copper not only suppresses the melanin defect of *mel1* mutant cells but also restores mating in a *mel1* by *mel1* cross. Finally, two other mutants, *mel3* and *mel6*, exhibited an unusual bilateral mating defect and produced filaments but no basidiospores. These studies reveal an unexpected link between mating and laccase production. Thus far, only the *mel2* mutant has been characterized at the molecular level, and the remaining Mel$^-$ mutants represent a unique vantage point to begin a molecular dissection of melanogenesis and its contribution to virulence.

All pathogenic organisms must survive within the infected host, and thus an important property essential for virulence is the ability to grow at 37°C. Of the many thousands of fungal species, only a few dozen cause human infection. The only common trait that links those fungi capable of causing systemic disease is their shared ability to proliferate at 37°C. Other fungi that colonize and infect humans but are not capable of growth above ~35°C are relegated to causing dermatophytic infections of the skin, hair, and nails. Although the model yeast *S. cerevisiae* is an uncommon human pathogen, more than 50 clinical isolates have been reported, and their one common feature is an ability to grow at temperatures up to 42°C, whereas laboratory strains of yeast do not grow above 39°C (122). Novel quantitative trait-mapping approaches have begun to pinpoint the molecular basis of this virulence trait in yeast (161).

Early studies in *C. neoformans* defined temperature-sensitive, avirulent mutants (102). More recent molecular studies have revealed important roles for calcineurin, Ras1, and the Ste20$\alpha$ kinase in growth at elevated temperature and virulence (2, 40, 62, 136, 182, 184). Calcineurin is a serine-threonine specific protein phosphatase that consists of two subunits, the calcineurin A catalytic subunit and the calcineurin B regulatory subunit. In addition the calcineurin AB heterodimer is bound and activated by calmodulin in response to intracellular calcium increases. Calcineurin is the molecular target of the immunosuppressive drugs cyclosporin A (CsA) and FK506, and both drugs were found to inhibit growth of *C. neoformans* at 37°C but not at 24°C (136). The isolation of FK506-resistant mutants that retained sensitivity to CsA identified two genes involved in FK506 antifungal action (136). Three mutations (*frr1-1*, *frr1-2*, *frr1-3*) conferred resistance to both FK506 and another related drug, rapamycin, and were recessive in genetic crosses. All three harbor mutations within the *FRR1* gene encoding the FK506/rapamycin binding protein FKBP12 (38). The remaining mutation, *FKR1-1*, conferred resistance to FK506 but not to rapamycin, was dominant in heterokaryon analysis, and segregated independently from the *FRR1* gene encoding FKBP12. Subsequent genetic and molecular analysis revealed the *FKR1-1* mutation results from a tandem

duplication of 6 bp that inserts two amino acids into the calcineurin B subunit that prevents FKBP12-FK506 binding (62).

The finding that calcineurin inhibitors prevented growth at 37°C but not at 24°C suggested that calcineurin might be essential for growth at elevated temperature and for virulence. The genes encoding the calcineurin A and B subunits were cloned and disrupted by transformation and homologous recombination (40, 62, 136). The resulting *cna1* and *cnb1* mutant strains are viable at 24°C, but inviable at 37°C and completely avirulent in animal models. These studies were the first to define a molecular determinant of growth at elevated temperature necessary for infection. Importantly, this phenotype could not have been predicted from studies in model yeasts or even other pathogenic yeasts. Calcineurin is not essential for growth at elevated temperature in either lab strains or pathogenic isolates of *S. cerevisiae*. Calcineurin is also dispensable for growth of *C. albicans* at 37°C or 39°C, but interestingly does play a role in virulence (J. Blankenship & J. Heitman, in preparation). Thus, the functions of calcineurin are linked to virulence in two different fungal pathogens but via distinct mechanisms.

Several other genes have recently been linked to growth at elevated temperature and virulence, including those encoding the small GTP binding protein Ras1, the protein kinase Ste20$\alpha$ encoded by the mating-type locus (discussed above), the cyclophilin A protein Cpa1 (180), and a component of the vacuolar proton ATPase Vph1 (59). Mutations in all of these genes impair growth at elevated temperature and compromise the ability of the organism to establish infection and cause disease.

Finally, recent studies have revealed that two secreted enzyme activities, phospholipase B and urease encoded by the *PLB1* and *URE1* genes, respectively, also contribute to the full virulence composite but are not strictly essential for virulence. Serotype A *plb1* mutant strains were found to be significantly attenuated for virulence in both the murine inhalation model and the rabbit meningitis model but remained capable of causing 100% lethal infections in mice (34). Serotype A *ure1* mutant strains lacking urease showed no defect in fungal cell survival in the rabbit CNS, but in the murine inhalation and tail vein injection model the mutant was attenuated compared to wild type (35). Mice infected with the wild-type or the *ure1* reconstituted strain in which the wild-type gene was introduced exhibited signs of pulmonary distress and hydrocephalus at late stages of infection, whereas animals infected with the urease mutant strain showed only hydrocephalus. Thus, the ability of urease to produce ammonia and regulate local pH might play a role in promoting fungal cell survival in the lung.

## EVOLUTION OF A FUNGAL PATHOGEN

Serotype A variety *grubii* and serotype D variety *neoformans* strains are both found in association with pigeon droppings worldwide. Virtually all humans are exposed to *C. neoformans*, and in most individuals the immune system rapidly clears an asymptomatic infection. There is recent evidence, however, that the majority of

children in the Bronx, NY, are infected early in life, and some of these infections are symptomatic (70). In other individuals, the initial infection in the lung enters a pulmonary lymph node complex and establishes a dormant phase in which the organism may replicate and survive inside macrophages (6, 65).

This macrophage survival is thought to enable *C. neoformans* to establish latent infections. Studies in France reveal that Asians and Africans who emigrated to France many years previously present with *C. neoformans* infections with strains endemic from Asia or Africa, rather than those present in France (52, 65). A series of autopsy studies in the 1950s (7), and studies repeated recently in Japan (85), reveal that 0.5–1% of normal individuals exhibit evidence of cryptococcal infection in hilar lymph nodes. These findings suggest that in the 2–3% of transplant recipients in which *C. neoformans* develops many of these infections may represent reactivation of latent infections acquired much earlier in life.

Although many bacterial and fungal pathogens have adopted strategies to survive inside macrophages, it is curious when these pathogens are organisms generally found in the soil. How have saprophytes developed mechanisms to survive in response to the mammalian immune system? One possibility is that the defense mechanisms developed by organisms like *C. neoformans* to survive in the presence of other microorganisms can be put to use in the presence of the host immune response. It has been proposed that the interaction of *C. neoformans* with soil amoebae as competitors in the environment has allowed *C. neoformans* to develop strategies to survive within macrophages (160). Macrophages and amoebae are similar in many respects, including the abilities to phagocytose particles into vacuoles and digest them with secreted enzymes. Steenbergen et al. found that *C. neoformans* is in fact phagocytosed by the amoeba *Acanthamoeba castellanii*, and that once inside the amoeba, divides, and leads to amoeba killing (160). This apparent survival strategy is very similar to that seen in macrophages where *C. neoformans* can be phagocytosed and then persist for years (71). Using the amoeba as a comparative model for macrophage survival may provide valuable insights into the success of *C. neoformans* as a human pathogen, and allow a better understanding of the latency phase of infection.

Fully understanding the epidemiology of infection and latent disease depends on knowing the environmental reservoirs for *C. neoformans*. *C. neoformans* var. *neoformans* (serotype D) and var. *grubii* (serotype A) are clearly associated with birds, particularly pigeons (57, 66, 166), but the environmental reservoir for var. *gattii* (serotype B) has been much more elusive. The *gattii* variety is found primarily in subtropical and tropical regions. In Australia, Ellis & Pfeiffer found this variety to be associated with *Eucalyptus* trees (primarily the red gum *E. camaldulensis*) (55). They conducted large-scale sampling of air, soil, and vegetation on a weekly basis over an 8-month period and found var. *gattii* in vegetation only from *E. camaldulensis* (56). Var. *gattii* can be cultured out of tree hollows and the koalas that inhabit these trees (32), and it appears to be cultured most easily from flowering trees (56). The authors suggest that this discovery explains the unexpected epidemiology of var. *gattii*: It is not common among AIDS patients in Australia.

Because the majority of the AIDS population in Australia lives where there are no *E. camaldulensis* trees, the opportunity for exposure is low. As a result, most of the cases of cryptococcosis in Australia are found in individuals who are immunocompetent and likely to be outdoors in areas of the country where eucalypts grow.

Finding var. *gattii* in eucalypts may have revealed the environmental reservoir in Australia, but it does not explain the distribution of var. *gattii* worldwide. For example, almost all cases of cryptococcosis in Papua New Guinea (PNG) are caused by var. *gattii*, yet there are almost no *Eucalyptus* trees in PNG. This finding suggests that other natural habitats must exist for var. *gattii*, and thus far no one has succeeded in isolating var. *gattii* from the environment in PNG (105). On the other hand, efforts to culture *Cryptococcus* from the environment in South America have resulted in the discovery of both var. *gattii* and var. *neoformans* on trees other than eucalypts. (106, 107, 128). Clearly, there is great variation in environmental habitats among the *C. neoformans* varieties around the world.

Another difference between var. *gattii* and the other varieties is that most *gattii* infections appear to represent primary infections, and susceptibility is not limited to immunocompromised individuals (157). In fact most infections are in apparently healthy people. Interestingly, an outbreak of cryptococcosis caused by var. *gattii* in Vancouver, B.C. beginning in 1999 has affected more than 40 individuals, 27 of them exhibiting no apparent immune deficiencies (63). Investigations are under way to identify an environmental source.

Given the striking differences in epidemiology, distribution, environmental habitats, and molecular and biochemical characteristics between var. *gattii* and vars. *grubii* and *neoformans*, Boekhout et al. have proposed that the *gattii* variety be reclassified as a different *Cryptococcus* species (11). This idea is supported by their thorough investigation of the AFLPs of 153 var. *neoformans* and *grubii* isolates and 54 var. *gattii* isolates from AIDS and non-AIDS patients, the environment and animals from all over the world. Boekhout et al. showed that the AFLP patterns for the two varieties cluster into two main branches, which seem to correspond with reproductively isolated populations. Based on their data and the accumulated evidence of differences between the varieties, they recommend designating var. *neoformans* and var. *grubii* (serotypes A, D, and AD) as *Cryptococcus neoformans* and var. *gattii* (serotypes B and C) as *Cryptococcus bacillisporus*. The *bacillisporus* designation was first used by Kwon-Chung in 1976 to distinguish the sexual states of serotypes A/D (*Filobasidiella neoformans*) from serotypes B/C (*Filobasidiella bacillisporus*) on the basis of observations of the spores resulting from intraserotype crosses (96). Subsequent mating experiments suggesting successful interserotype mating led to the designation of varieties instead of separate species for the two classes of serotypes (100, 153). Now, however, the overwhelming phenotypic and molecular data indicate separating the serotypic groups into species. These conclusions support the idea that the species concept, both biologically and phylogenetically, is a continuum, and as such, speciation determinations need to be made on the basis of many criteria.

## CONCLUSION

Genetic analysis of *C. neoformans* over the past two decades has revolutionized what we know about its life cycle and virulence properties. As we proceed, the value of having defined the sexual cycle will continue to be high. Although scientists have found the *C. neoformans* sexual cycle to be invaluable, it is much less clear what its value is to *C. neoformans*. Even though it can be seduced to mate in the laboratory, there is limited evidence that *C. neoformans* undergoes sexual reproduction in nature.

The absence of active sexual reproduction in *C. neoformans* is curious given that the sexual cycle is an important part of the life cycles of most eukaryotes. The purpose of sexual reproduction is a matter of debate, but what is known is that sexual reproduction affords a fitness advantage to most organisms, and those that lose or never develop this ability are likely to face extinction more rapidly than those that do [reviewed in (17)]. The simple explanation for the advantage that sexual reproduction affords is that it allows recombination between different genetic backgrounds to occur, facilitating the propagation of beneficial mutations into a population. Alternatively, recombination could afford the opportunity to purge deleterious mutations from the population (199). Either way, it appears that recombination is important enough to drive many organisms to invest energy in the processes of mating and meiosis.

Even with the strong selection for sexuality, it appears that *C. neoformans* is not actively engaging in sexual reproduction. Sporulation structures have not been observed in patients or the environment, and the populations that have been studied show linkage disequilibrium consistent with clonal growth (10, 13, 27). Clonal growth could, in part, be explained by the finding that almost all clinical and environmental isolates are of a single mating type ($\alpha$) providing little opportunity for sex. Of ~1500 serotype A strains screened, only two were of the **a** mating type, suggesting that very little opportunity exists for natural crossing to occur (112, 178). On the other hand, there is very little evidence of recombination even when strains of different mating types are found in the same environmental location and, presumably, have every opportunity to mate. Serotype B *MAT**a*** and *MAT*$\alpha$ isolates from *Eucalyptus* trees in Australia have been found on the same tree, but show no crossing, and all attempts to induce mating between these strains in the laboratory have failed (75; W. Meyer, personal communication).

It seems that a piece of the lifestyle puzzle for *C. neoformans* is still missing. Perhaps extremely infrequent mating is all that is required for healthy *C. neoformans* populations, so the sexual cycle is maintained, but used very rarely in an as-yet unidentified environmental niche. Maybe **a** and $\alpha$ strains rarely engage in sexual reproduction but still enhance fruiting to promote spore formation and thus increase survival in response to harsh conditions. Alternatively, perhaps *C. neoformans* is evolving to be an asexual organism. Maybe the ability of $\alpha$ strains to form spores in the absence of **a** cells has led to their dispersal and prevalence in the environment, while **a** strains are disappearing. Whatever

the intricacies of the *C. neoformans* life cycle, there is still much to be learned about how this unusual pathogen survives in both the environment and human beings. Understanding the complexities of when, where, and how *C. neoformans* reproduces will provide insights not only into how *C. neoformans* infects humans but also into how all fungal pathogens live, reproduce, and evolve strategies to survive.



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