MOLECULAR AND CELLULAR BIOLOGY, Mar. 1993, p. 1962–1970
0270-7306/93/031962-09$02.00/0
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Vol. 13, No. 3

The α-Mating Type Locus of *Cryptococcus neoformans* Contains a Peptide Pheromone Gene

TRACEY D. E. MOORE[1] AND JEFFREY C. EDMAN[2]*

Hormone Research Institute[1,2] and Departments of Pharmaceutical Chemistry[1] and Laboratory Medicine,[2] University of California, San Francisco, California 94143-0626

Received 25 August 1992/Accepted 18 December 1992

The opportunistic fungal pathogen *Cryptococcus neoformans* has two mating types, *MAT*a and *MAT*α. The *MAT*α strains are more virulent. Mating of opposite mating type haploid yeast cells results in the production of a filamentous hyphal phase. The *MAT*α locus has been isolated in this study in order to identify the genetic differences between mating types and their contribution to virulence. A 138-bp fragment of *MAT*α-specific DNA which cosegregates with α-mating type was isolated by using a difference cloning method. Overlapping phage and cosmid clones spanning the entire *MAT*α locus were isolated by using this *MAT*α-specific fragment as a probe. Mapping of these clones physically defined the *MAT*α locus to a 35- to 45-kb region which is present only in *MAT*α strains. Transformation studies with fragments of the *MAT*α locus identified a 2.1-kb *Xba*I-*Hin*dIII fragment that directs starvation-induced filament formation in *MAT*a cells but not in *MAT*α cells. This 2.1-kb fragment contains a gene, *MF*α, with a small open reading frame encoding a pheromone precursor similar to the lipoprotein mating factors found in *Saccharomyces cerevisiae*, *Ustilago maydis*, and *Schizosaccharomyces pombe*. The ability of the *MAT*a cells to express, process, and secrete the *MAT*α pheromone in response to starvation suggests similar mechanisms for these processes in both cell types. These results also suggest that the production of pheromone is under a type of nutritional control shared by the two cell types.

Cryptococcus neoformans causes a serious and often fatal meningitis in patients with AIDS and in other immunocompromised patients (45). This pathogen is a heterothallic basidiomycetous yeast with two mating types, *MAT*a and *MAT*α, which are determined by a single-locus, two-allele system (34). *C. neoformans* has a well-defined life cycle in which mating occurs only between opposite mating types. The haploid *MAT*a or *MAT*α yeast cells divide vegetatively by budding and are induced to mate by starvation. Haploid yeast cells of opposite mating types fuse during mating and differentiate into the dikaryotic hyphal phase with clamp connections. As in most fungi, cell fusion does not lead directly to nuclear fusion but rather results in a dikaryon in which the separate haploid nuclei are maintained within a common cytoplasm and divide in synchrony. Basidia form at the hyphal tips, inside which nuclear fusion, meiosis, and spore formation occur (34). Dimorphism (yeast to hyphal form) in *C. neoformans* is sexual and occurs in response to mating, not to temperature and/or environmental conditions as is common in other fungal pathogens. The primary infection usually occurs in the lungs following inhalation of the organism, most likely the spore form. *C. neoformans* has a worldwide distribution and can be isolated from pigeon nests, roosts, and droppings.

The mechanisms of **a**-α cell recognition and starvation-induced mating in *C. neoformans* have not been identified. Genetic studies in other fungi have demonstrated the role of mating type loci genes in cell type control, cell-cell signaling, yeast-hypha differentiation, and maintenance of the filamentous state (7, 20, 23, 24, 32, 49, 51). The components involved in these processes (homeodomain proteins, receptors, and heterotrimeric G proteins) have been found to be similar between fungi and higher eukaryotes (6, 16, 24, 25, 31). The roles of the genes within the *C. neoformans* mating type loci and their functions have yet to be identified.

Mating type loci genes and their roles in mating and subsequent developmental changes have been successfully explored in other fungi by using mating type loci mutants. Mating type genes have also been isolated by chromosome walking from genes which are known to be linked to mating type. To date, no *C. neoformans* genes that are linked to mating type have been identified, and no mutants that map to the *MAT*a or *MAT*α locus have been described.

Genetic analysis of *C. neoformans* has identified four factors associated with virulence: growth at 37°C, a polysaccharide capsule, production of melanin, and α-mating type. Thermotolerance is an obvious prerequisite for the infection of mammalian hosts, and the capsule and melanin appear to offer some protection from host defense mechanisms (17, 27, 36). However, the increase in virulence associated with α-mating type is not understood (35). No morphological or biochemical differences between *MAT*a and *MAT*α congenic strains which could account for the difference in virulence have been identified. Therefore, the *MAT* loci must be isolated and analyzed to understand the mating process in *C. neoformans* and the role of α-mating type in virulence at the molecular level.

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

MATERIALS AND METHODS

***C. neoformans* strains and media.** The strains used are described in Table 1. The initial *MAT*a auxotrophic mutants were isolated from UV-mutagenized cells. Subsequent

* Corresponding author.

TABLE 1. Strains

Strain	Genotype	Reference
JEC20	*MAT***a**	35
JEC21	*MAT*α	35
JEC34	*MAT***a** *ura5*	This work
JEC50	*MAT*α *ade2*	This work
JEC157	*MAT*α *ade2 ura5 lys1*	This work
JEC168	*MAT***a** *lys2 ade2 ura5*	This work
JEC169	*MAT***a** *ade2 ura5 lys1*	This work
JEC170	*MAT*α *lys2 ade2*	This work
JEC171	*MAT***a** *lys2 ade2*	This work

*MAT***a** and *MAT*α strains were constructed by crossing auxotrophic strains and dissecting spore progeny with a micromanipulator. YEPD (1% yeast extract, 2% Bacto Peptone, 2% dextrose) was used as the maintenance medium. Synthetic dextrose (SD) contained, per liter, 6.7 g of yeast nitrogen base without amino acids, 20 g of glucose, and appropriate amino acid or nucleotide supplements. V8 starvation crossing medium contained 5% V8 vegetable juice (Campbell Soup Co.), 0.5 g of KH_2PO_4 per liter, and 4% agar adjusted to pH 7.0 with KOH.

Difference cloning of *MAT*α-specific DNA. Difference cloning was performed by using the phenol-enhanced reassociation technique (PERT) and molecular cloning of specifically reassociated molecules (33). Genomic DNA was isolated from congenic wild-type strains JEC20 (*MAT***a**) and JEC21 (*MAT*α). The *MAT***a** DNA was sheared to an average size of 1,000 bp by sonication for 15 s of a solution containing 100 μg of genomic DNA in 500 μl. *MAT*α DNA (5 μg) was digested to completion with *Taq*I restriction endonuclease at 65°C for 1 h. After digestion, 2 μl of 0.5 M EDTA and 1 μl of 10-mg/ml proteinase K were added to the 20-μl digestion mixture, and the mixture was incubated at 65°C for 30 min. For the reassociation reaction, 25 μg of sonicated *MAT***a** DNA was mixed with 0.125 μg of *MAT*α DNA, and the mixture was heated to 100°C for 5 min, cooled on ice, and added to a final reaction volume of 250 μl in 7% phenol–1.25 M $NaClO_4$–0.12 M $NaPO_4$. The reassociation mixture was vortexed for 37 h. After reassociation, the mixture was extracted twice with chloroform, dialyzed against TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), ethanol precipitated, and resuspended in 10 μl of TE.

Ligations were carried out in a 10-μl reaction volume containing 2 μl of the PERT-reassociated DNA, 25 ng of Bluescript digested with *Cla*I, and 1 U of T4 DNA ligase (BM) at 25°C for 30 min and 4°C for 15 h. *Escherichia coli* JM101 electroporation-competent cells were prepared and transformed according to published procedures (12). The cells were found to have a transformation efficiency of 10^9 transformants per μg of DNA.

Preparation of *C. neoformans* DNA. *C. neoformans* genomic DNA was isolated from 100-ml cultures grown in SD plus adenine, uracil, or both. The cells were collected by centrifugation and resuspended in 20 ml of 1 M sorbitol–1% β-mercaptoethanol–1-mg/ml lytic enzymes (Sigma) and incubated for 30 to 60 min. The spheroplasts were collected by centrifugation and lysed by incubation at 65°C in 10 ml of 50 mM EDTA–1% sodium dodecyl sulfate (SDS)–100-μg/ml RNase A for 30 min. Proteinase K was added to 167 μg/ml, and the incubation continued for another 30 min. One-third volume of 5 M potassium acetate was added, the mixture was kept on ice for 30 min and clarified by centrifugation, and the supernatant was precipitated with isopropanol. The DNA was resuspended in TE, heated to 65°C for 15 min, and precipitated with ethanol and 1/10 volume of 7.5 M ammonium acetate. Approximately 10 μg of genomic DNA was recovered per ml of culture (14).

Southern analysis. For Southern analysis, genomic DNA (5 μg) was digested to completion with restriction enzymes (Boehringer Mannheim and New England Biolabs), run on a 0.8% agarose gel, transferred to a nylon membrane, and fixed by UV cross-linking (Stratalinker; Stratagene). All membranes were hybridized with digoxigenin-labeled probes labeled according to the manufacturer's instructions (Genius; Boehringer Mannheim). Blots were prehybridized at 42°C for 2 h in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide–0.2% SDS–5% Genius blocking reagent–0.1% *N*-lauroylsarcosine. Blots were hybridized overnight at 42°C in the same mixture with the addition of the probe. All blots were washed according to the manufacturer's instructions and visualized by using the chemiluminescent substrate AMPPD (Tropix).

Screening of phage and cosmid libraries. A *C. neoformans* *MAT*α genomic library with 5- to 10-kb inserts prepared in lambda ZAP II (15) was screened by using digoxigenin-labeled *MAT*α-specific probes. Phagemid were rescued from all libraries by coinfection of *E. coli* XL-1B with 10^7 PFU of lambda phage and 10^{10} PFU of R408 helper phage in 10 ml of LB. After 6 h of shaking at 37°C, the culture was heated to 70°C for 30 min and clarified by centrifugation, and the supernatant containing rescued phagemid was saved. One milliliter of XL-1B cells was infected with 2 μl of the rescue supernatant. Plasmid DNA was harvested from the resulting colonies by boiling 5-h cultures, and the resulting supernatant was used to transform competent *E. coli* HB101 cells.

A cosmid library was prepared by partial digestion of genomic *MAT*α DNA with *Xba*I, *Spe*I, *Nhe*I, and *Avr*II, selection for 30-kb fragments on a sucrose gradient, and insertion into the *Xba*I site of pAUcos11, which contains sequences from Bluescript, the *C. neoformans ADE2* and *URA5* genes, and a cosmid fragment. Approximately 15,000 colonies were grown on 100-mm-diameter LB-ampicillin (100 μg/ml) plates at 37°C until the colony diameter was 0.2 mm. Replica filters were prepared by pressing the lifted filter to a clean filter between filter paper and glass plates. The filters were placed colony side up onto LB-ampicillin (100 μg/ml) plates and grown until the colonies were approximately 0.5 mm in diameter. The two replica filters were then placed colony side up onto chloramphenicol (250 μg/ml) plates for 20 h to amplify the DNA. The filters were denatured, neutralized, washed in 2× SSC, and UV cross-linked (Stratalinker; Stratagene). The fixed filters were washed at 65°C in 0.1× SSC–0.1% SDS for 30 min and then probed and developed as described for the Southern blots.

Electroporation of *C. neoformans*. Strains JEC157 and JEC169 were grown overnight in YEPD at 30°C with shaking at 300 rpm. The cells were diluted 1:50 in fresh YEPD and then grown for an additional 5 h at 30°C. The cells were washed in EB (10 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$, 270 mM sucrose) plus 1 mM dithiothreitol and resuspended in 0.5 ml of EB. DNA constructs were linearized with *Rsr*I or *Pac*I prior to transformation. In the linear construct, the insert DNA is flanked by the markers *ADE2* and *URA5*. Prior to electroporation, 50 μl of cells was mixed with 100 to 500 ng of sample DNA and 660 ng of salmon sperm DNA. The mixture was transferred to a 0.2-mm electroporation cuvette and electroporated at 470 V and 25 μF. Cells were plated immediately to SD plus lysine. Transformants were

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

Filament formation assay. Constructs containing *MATα* DNA and vector alone as a negative control were transformed into *MAT***a** *ade2 ura5 lys1* strains, streaked onto V8 medium, and starved for 24 to 48 h. These cells were then examined with a Zeiss microscope for the production of filamentous projections. All constructs were also transformed into *MATα ade2 ura5 lys1* strains as a negative control but did not induce filament formation in these strains.

DNA sequencing. Subclones used for sequencing were created as nested deletions or restriction fragments. Nested deletions were made by digestion with exonuclease III followed by the mung bean nuclease treatment system. Deletion clones were constructed in both directions (Stratagene). DNA sequencing was performed on double-stranded templates on an Applied Biosystems model 470 DNA sequencer or with [^{35}S]dATP, using a Sequenase kit.

RESULTS

Isolation of *MATα*-specific DNA. *C. neoformans* does not switch mating type, suggesting a lack of silent copies of the mating type loci. Each mating type should therefore contain unique DNA sequences at the mating type locus responsible for the determination of mating type. This difference could be exploited to isolate the mating type loci by difference cloning (33), which allows the isolation of DNA sequences which are present in one DNA preparation but not in another. DNA from wild-type *MATα* cells (JEC21) was digested with *Taq*I and hybridized to an excess of randomly sheared DNA from a congenic *MAT***a** strain (JEC20) by using PERT. During reassociation, DNA fragments unique to *MATα* will reanneal to form the original *Taq*I fragments with two cohesive ends. The hybridization mixture was ligated to Bluescript which had been linearized with *Cla*I. Transformation of *E. coli* with the ligation mixture selected for fragments with two cohesive ends, as only these would allow the formation of transformation-competent circles. Sixteen plasmid clones were recovered in this way and used to probe genomic Southern blots of *MAT***a** and *MATα* DNA. One clone, α24, contained a 138-bp insert that hybridized solely to *MATα* DNA and was chosen for further analysis. The remaining plasmid clones did not hybridize to *C. neoformans* DNA or hybridized to repetitive sequences found in both mating types.

Linkage analysis of α24, the *MATα*-specific DNA fragment. To determine the linkage of clone α24 with the *MATα* locus, *MAT***a** and *MATα* strains were crossed and 10 progeny were analyzed for cosegregation of hybridization of clone α24 with mating type. *C. neoformans* JEC34 (*MAT***a** *ura5*) and JEC50 (*MATα ade2*) were crossed, and single basidiospores were isolated. Genomic DNA was isolated from both parents and 10 progeny (five **a** and five α). A Southern blot of genomic DNA from the parents and progeny was probed with α24 DNA (Fig. 1). The probe hybridized to a single 12-kb *Pst*I fragment in genomic DNA from the *MATα* parent and all *MATα* progeny, but no fragment was detected in DNA from the *MAT***a** strains. Cosegregation of mating type and the α24 probe confirmed that α24 is linked to the α-mating type locus.

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

The *MATα* locus was found within five genomic *Kpn*I fragments encompassing a region of 64 kb (Fig. 2). The *MATα* locus itself is 35 to 45 kb in size. The exact size of the locus depends on the precise location of the junction between *MATα*-specific and *MAT*-nonspecific flanking regions within the 6-kb *Not*I-*Kpn*I fragment from the left side and the 4-kb *Kpn*I fragment from the right side. The locus contains *MATα*-specific DNA and repetitive DNA which hybridizes to numerous fragments in both *MAT***a** and *MATα* genomic DNA. The repetitive DNA sequences are dispersed throughout the *MATα* locus (data not shown). The Southern blots probed with two internal nonrepetitive DNA fragments (Fig. 2b and c) demonstrate mating-type-specific hybridization, since both fragments hybridized to a single fragment in *MATα* genomic DNA. Fragments from regions flanking the locus showed hybridization which was nonspecific to mating type. For example, in Fig. 2a, the probe used was from a region outside the locus and hybridized to *Eco*RI and *Kpn*I fragments of the same size in *MAT***a** and *MATα* DNA. In Fig. 2d, however, the probe hybridized to both α-specific and nonspecific fragments in a genomic Southern blot. The probe hybridizes to a 5.0-kb *Kpn*I fragment present in both mating types. *Eco*RI-digested DNA shows a mixture of mating-type-specific fragments (1.5 kb in *MAT***a** and 9.3 kb in *MATα*) and nonspecific fragments (1.0 and 3.0 kb). This restriction fragment length polymorphism suggests that the probe spans the junction region between the *MATα* locus and the flanking DNA outside the locus. These data demonstrated that an extensive *MATα*-specific region and homologous flanking regions had been isolated.

Transformation of *C. neoformans* with *MATα* DNA. To identify regions with mating type activity, a series of constructs containing various fragments of the *MATα* locus was introduced into *MAT***a** and *MATα* strains (Fig. 3). Strains transformed with vector only served as negative controls for the behavior of strains transformed with various fragments. Transformants were mated with the *MAT***a** and *MATα* strains JEC170 and JEC171. Mating can be assayed by the production of the hyphal phase. By this assay, no fragments resulted in transformants which have shown altered mating activity such as dual-mating ability (mate with both *MAT***a** and *MATα* strains) or sterility (will not mate). All show mating behavior identical to that of their corresponding untransformed haploids. However, when isolated transformants were placed on V8 starvation medium alone, one segment of the locus was found to induce filaments (Fig. 3). The active region was localized to a 2.1-kb *Xba*I-*Hin*dIII fragment which caused *MAT***a**, but not *MATα*, transformants to form hyphal filaments which were visible within 24 to 48 h after starvation (Fig. 3 and 4). Filaments were formed only when transformants were transferred from SD medium onto V8 starvation plates. Southern analysis of the filament-forming transformants showed that the DNA was present in multiple copies as autonomously replicating extrachromosomal fragments (data not shown). Southern analysis demonstrated that both *MAT***a** and *MATα* transformed strains contained the plasmid (data not shown).

Molecular analysis of the filament-inducing fragment. The 2.1-kb fragment which was able to direct filament formation was subcloned and sequenced (Fig. 5). The nucleotide sequence contains a gene with a 114-bp open reading frame (ORF), which encodes a 38-amino-acid peptide with similar-

ity to the fungal mating factor precursors found in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Ustilago maydis*, which we call *MF*α (Fig. 6) (7, 8, 11). All are similar in size, with small ORFs encoding peptides between 36 and 44 amino acids in length. An asparagine residue is present at amino acid 24 of *C. neoformans* propheromone and is in a similar position to asparagine residues found to precede the amino-terminal cleavage site in *S. pombe* **M**-factor and *S. cerevisiae* **a**-factor. The predicted polypeptide contains the prenylation motif CAAX (where C is cysteine, A is aliphatic, and X is any amino acid) at the carboxy terminus (9). This motif has been found in *S. cerevisiae* **a**-factors (MF**a**1 and MF**a**2), in *S. pombe* **M**-factors (MFm1 and MFm2), and in the rhodotorucine A precursors, encoding the mating pheromone of *Rhodosporidium toruloides*, all of which are modified by the addition of a farnesyl group on the cysteine residue. The CAAX in the **a**-factor precursor of *S. cerevisiae* is required for farnesylation, which is necessary for biological activity. The *C. neoformans* and *U. maydis* pheromone genes also contain this motif; however, the peptide pheromones themselves have yet to be isolated. Processing of the *C. neoformans* propheromone can be predicted by analogy with *S. cerevisiae* and *S. pombe* (Fig. 6). Addition of a farnesyl group occurs in mating pheromones from a variety of fungi, including *S. cerevisiae*, *R. toruloides*, *Tremella mesenterica*, *Tremella brasiliensis*, and *S. pombe* (2, 22, 26, 28, 46).

Putative promoter elements TATAAA and CAAAT in the nucleotide sequence of the pheromone gene are shown in Fig. 5. The region upstream of the ORF contains a pair of large (150-bp) inverted repeats and three shorter (16-bp) repeats. The shorter repeats in *C. neoformans* contain sequences which are similar to those of the repeats found in the pheromone genes recently isolated from *U. maydis* and may be involved in transcriptional regulation of the pheromone genes in response to opposite mating type pheromone or nutritional factors (7). Analysis of the entire 2.1-kb fragment for other gene sequences revealed no other ORFs with significant similarity to sequences in the Protein Identification Resource.

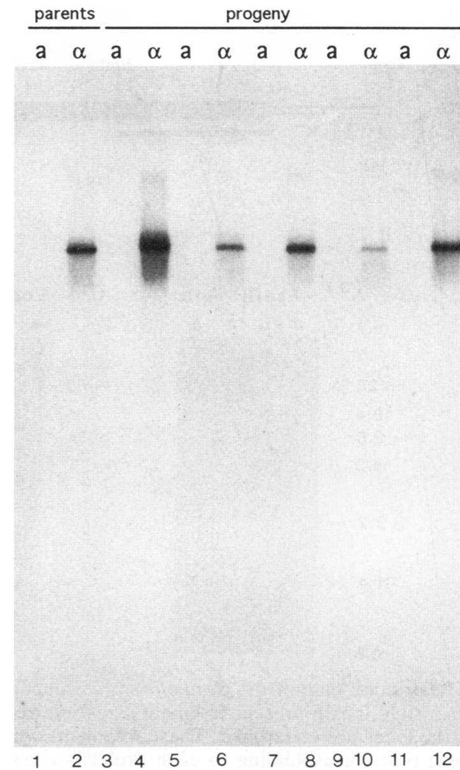

FIG. 1. Evidence that *MAT*α-specific DNA segregates with mating type. Southern blot analysis was performed on *C. neoformans* genomic DNA isolated from the parents and 10 progeny of a *MAT***a** *ura5* × *MAT*α *ade2* cross. DNA was digested with *Pst*I and hybridized to digoxigenin-labeled α24. Lanes: 1 and 2, parent strains *MAT***a** *ura5* and *MAT***a** *ade2*, respectively; 3 to 12, progeny strains *MAT***a** wild type, *MAT*α wild type, *MAT***a** *ade2*, *MAT***a** *ade2*, *MAT***a** *ura5*, *MAT***a** *ura5*, *MAT***a** wild type, *MAT*α wild type, *MAT***a** *ura5 ade2*, and *MAT***a** *ade2*, respectively.

DISCUSSION

Several observations confirm that the *MAT*α locus of *C. neoformans* has been isolated. The DNA is present in a single copy in *MAT*α but not *MAT***a** strains and cosegregates with α phenotype in a cross (Fig. 1). Southern analysis demonstrates that silent copies of alternate mating type alleles are not present within the genome. This can explain the failure to observe mating type switching as is seen in *S. cerevisiae*. Subfragments of the region induce filaments only in *MAT***a** cells, consistent with the introduction of *MAT*α-specific information (Fig. 3 and 4). The *C. neoformans* *MAT*α allele is much larger, 35 to 45 kb (Fig. 2), than previously isolated mating type alleles from other fungi, such as *S. cerevisiae* (**a**, 640 bp; α, 750 bp [43]), *U. maydis* (**a1**, 4.5 kb; **a2**, 8 kb [7]), *Neurospora crassa* (**A**, 5.3 kb; **a**, 3.2 kb [21, 50]), and *S. pombe* (**P** and **M**, 1.1 kb [29]).

The mating type loci of fungi are complexes of genes with related functions. The alternative alleles or complexes are not homologous in sequence or physical size between mating types, and these limited genomic differences have been compared with the more expansive genomic differences of sex chromosomes (16). The ascomycete *S. cerevisiae* has the best characterized of the fungal mating type loci. *S. cerevisiae* has a single locus with two alleles, **a** and α, which are codominant (24). The **a** and α alleles code for the transcription factors at the top of the regulatory cascades which control cell type and mating functions. Fungal mating type loci commonly encode regulatory proteins (31). In *S. cerevisiae*, *S. pombe*, and *N. crassa*, genes involved in mating are controlled by transcription factors encoded within their mating type loci. Because the mating type loci of both *S. cerevisiae* and *S. pombe* encode regulatory proteins, the relatively small mating type loci are able to control cell type and mating functions through the transcriptional regulation of a large set of genes dispersed throughout the genome.

Basidiomycetes have multiple mating type loci each containing more than one gene, each of which may have several alleles. In general, at least one allele at each locus must be nonidentical between the mating pair to maintain the dikaryon and progress through the sexual cycle. Each locus controls a specific set of developmental changes associated with mating or the dimorphic change (6, 40). For example, *Schizophyllum commune* has two loci, **A** and **B**, each containing two genes with multiple alleles. The **A** locus is responsible for nuclear pairing, conjugate nuclear and cell division, clamp connections, and cell septation, whereas the **B** locus controls nuclear migration and septal dissolution (19). The A locus of *Coprinus cinereus* has recently been shown to be composed of two subcomplexes which contain a total of seven genes, four of which contain homeodomain-

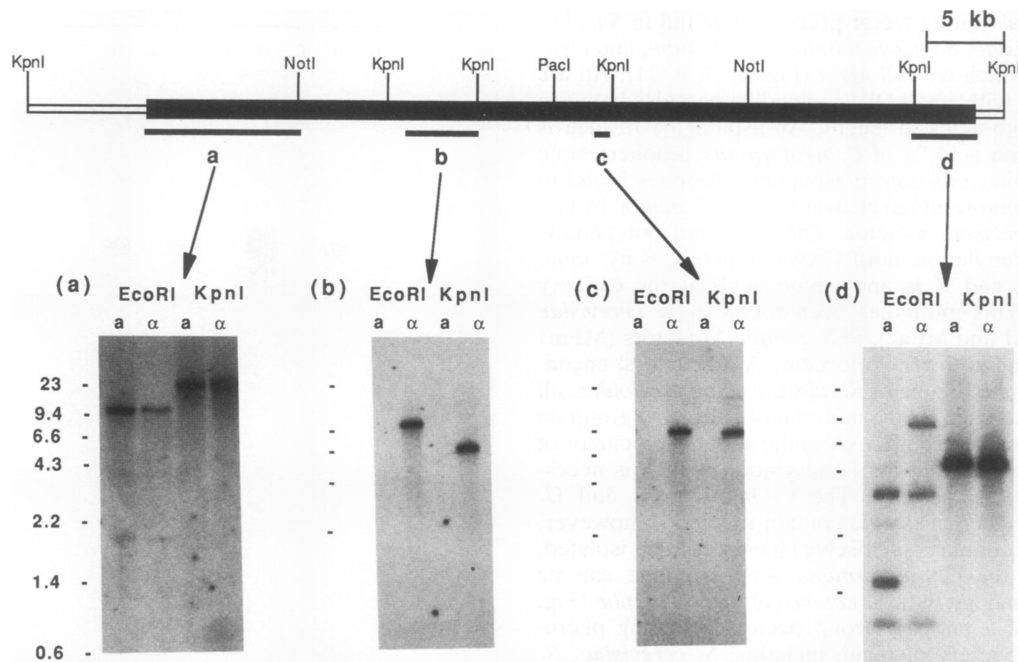

FIG. 2. Restriction map of the *C. neoformans* α-mating type locus. Thick lines represent the region present in overlapping cosmid and phage clones; thin lines represent fragment sizes inferred from genomic Southern analysis. DNA fragments used as probes from regions throughout the locus are underlined. These fragments were used to probe *MAT***a** and *MAT*α genomic DNA digested with *Eco*RI and *Kpn*I. The Southern blot corresponding to each probe is shown. (a) 10-kb fragment; (b) 4.5-kb fragment; (c) 138-bp α24 fragment; (d) 4.0-kb fragment.

related motifs and are known to regulate A-mating type sexual development (32). Another basidiomycete, the corn pathogen *U. maydis*, has two loci, **a** and **b**. The **a** locus encodes cell signaling components proposed to control cell fusion; the **b** locus contains genes that regulate self/non-self recognition and pathogenicity. Products from both loci are required for development and maintenance of the dikaryon (3, 7, 20). The **b** locus contains two genes, each of which encodes a protein with a variable N-terminal domain, a constant C-terminal domain, and a homeodomain-related motif (20).

Models have been proposed for both *U. maydis* and *S. commune* in which a homeodomain protein encoded by the mating type locus of each partner is required to form a heteromultimeric regulatory species which regulates the developmental events associated with mating. This would be analogous to the **a**1-α2 heterodimer which is formed after mating in *S. cerevisiae* and regulates diploid cell type. The situation is different at the **a** locus in *U. maydis*, where the two alleles contain genes for mating pheromones and the receptor for the opposite mating type pheromone (7). For example, the **a1** allele codes for the **a1** pheromone and the receptor for the **a2** pheromone. Sex-specific transcription of these genes which are directly involved in mating behavior depends solely on the presence or absence of the gene within each cell type. This is in contrast to *S. cerevisiae* in that although similar peptide pheromones and receptors are used to identify partners of the opposite mating type, the genes for the pheromones and receptors are present in the genomes of both mating types. In the *S. cerevisiae* system, mating-type-specific expression of these genes is controlled by the transcription factors encoded by the mating type alleles.

C. neoformans appears to have a mating type system genetically less complex than those of the basidiomycetes discussed above, with a single mating type locus (34). The *C. neoformans MAT*α allele encodes at least one gene whose product is directly involved in filament formation, a pheromone. The pheromone genes in *S. cerevisiae* and *S. pombe* are not present within the mating type loci. Each cell of either mating type contains the genes for both sex-specific pheromones; however, the synthesis of these pheromones is under mating type control. Since the *C. neoformans* pheromone gene is located in the mating type locus and therefore present only in a single cell type, it could be under mating type and/or nutritional control. This type of organization, wherein the sex-specific genes are found within the mating type locus, is similar to that of the **a** locus of *U. maydis*. *C. neoformans*, however, has only one locus controlling mating type. It is possible that the single large locus of *C. neoformans* is functionally analogous to a fusion of both the **a** and **b** loci of *U. maydis*. In any event, it is likely that the *C. neoformans MAT*α locus contains more than the pheromone precursor gene identified here. The unusually large size of the *C. neoformans* α-mating type allele suggests that additional genes required for determining cell type and mating behavior may be encoded directly in the mating type locus, possibly a pheromone receptor, transcription factor, or other genes.

Binding of mating factors to their receptors on cells of the opposite mating type in *S. cerevisiae* causes the cells to shmoo, agglutinate, undergo G_1 arrest, and induce genes required for cell and nuclear fusion (39). Upon exposure to its mating factor, the cells of *R. toruloides* form conjugation tubes toward the opposite mating type cell (28). Tremerogens specific for *T. mesenterica* or *T. brasiliensis* and for **A** or **a** strains induce mating tube formation in the opposite mating type strain of the same species. *C. neoformans* has

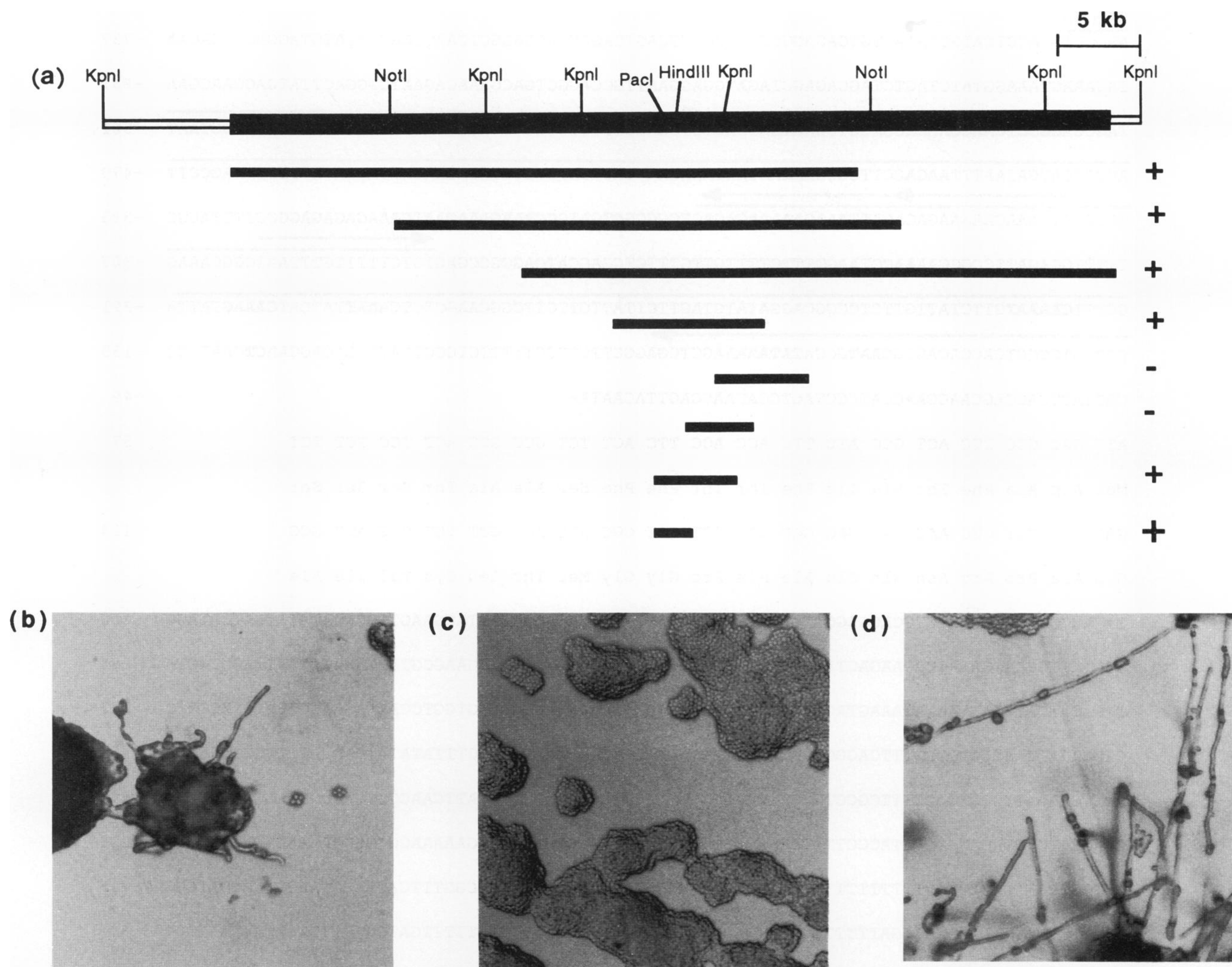

FIG. 3. Localization of the filament-forming activity by transformation of *C. neoformans* with fragments from the *MAT*α locus. *C. neoformans* JEC157 (*MAT*α *ade2 ura5 lys1*) and JEC169 (*MAT***a** *ade2 ura5 lys1*) were transformed by electroporation with various fragments of the *MAT*α locus and evaluated for filament-forming activity on V8 starvation medium. (a) +, fragments that enabled the *MAT***a** recipients to form filaments; −, those unable to confer filament-forming ability. All restriction sites of *Pac*I, *Not*I, and *Kpn*I are shown. One *Hin*dIII site is included for orientation with the sequenced DNA shown in Fig. 4. (b) *MAT***a** strain transformed with *MAT*α DNA fragment; (c) *MAT***a** cells transformed with pAUcos11 (vector) only; (d) a normal mating (cross of *MAT***a** *ade2 ura5 lys1* and *MAT*α *lys2*).

not been observed to undergo any of these responses as part of its normal mating process.

To begin to elucidate the functions of specific genes within the *MAT*α locus of *C. neoformans* and their roles in mating behavior and filament formation, DNA fragments from the *MAT*α locus were used to transform haploid cells of the opposite mating type. A 2.1-kb fragment from the *C. neoformans MAT*α locus, after transformation into *MAT***a** cells, caused the transformants to produce filaments. This activity seems to be under appropriate control, as filament production occurs only when these transformants are starved. The 2.1-kb fragment that induces filament formation was found to contain a small ORF (114 bp) encoding a peptide with similarity to other fungal mating factors (Fig. 5 and 6).

Pheromones have been described in a variety of fungi, including *S. cerevisiae*, *S. pombe*, *U. maydis*, *R. toruloides*, and *Tremella* species (1, 7, 11, 13, 26, 28, 46). The two types of pheromone found in *S. cerevisiae*, **a**-factor and α-factor, are quite different in gene structure, processing, and secretion. The mature *S. cerevisiae* **a**-factor is a farnesylated dodecapeptide which is required for mating (42). This peptide is thought to be transported out of the cell via a peptide export protein (STE6) with similarity to bacterial permeases, the mammalian multidrug resistance protein, and the cystic fibrosis gene product (41, 44). The peptide encoded by the ORF found in the *C. neoformans MAT*α locus is similar to the *S. cerevisiae* **a**-factor and other fungal mating factors (Fig. 6). The similarity includes the small size of the propheromone (all between 36 and 44 amino acids), the abundance of threonine, alanine, and serine in the presumptive propheromone coding region, and a CAAX motif at the carboxyl terminus (9). This motif is the prenylation signal for a variety of proteins (Ras oncoproteins and the γ subunit of trimeric G proteins) and peptides (lipoprotein pheromones) (10, 48). Farnesylation, as directed by the CAAX motif in the *S. cerevisiae* **a**-factor precursor, is important for membrane

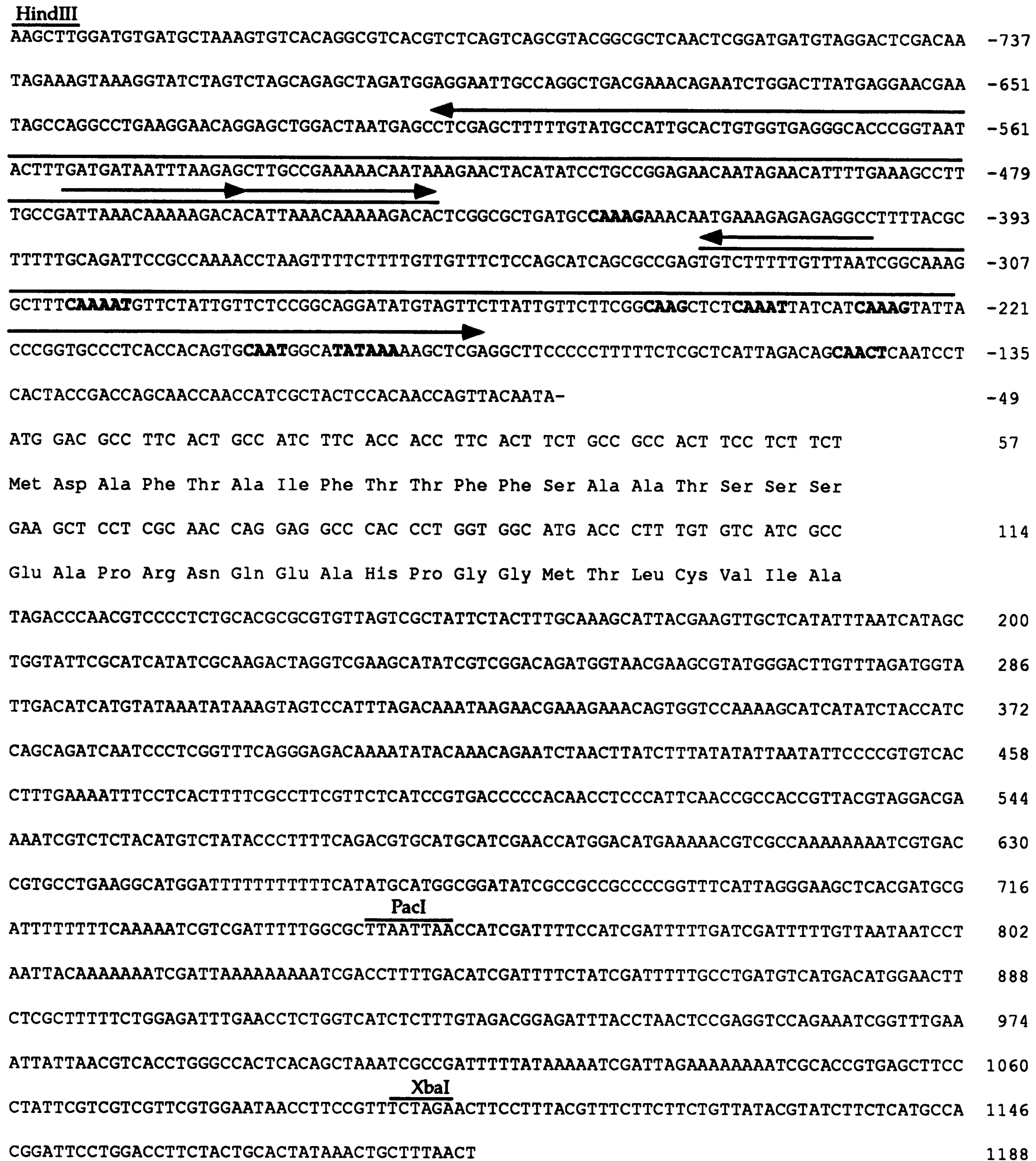

FIG. 4. Nucleotide sequence of the 2.1-kb fragment from the *MAT*α locus which induces filament formation in *MAT***a** cells. The sequence was found to contain an ORF which codes for a 38-amino-acid peptide with homology to peptide mating factors in other fungi, the sequence of which is printed below the nucleotide sequence. Long arrows show the 150-bp inverted repeats; short arrows show 16-bp repeat sequences. Bolding alone highlights the smaller repeat sequences, the consensus filamentous fungi core promoter, and the CAAT motifs. The −3 position is occupied by an adenine, as is the case in 90% of filamentous fungal genes (30).

localization, further propheromone processing, biological activity, and export (42, 48). *S. cerevisiae* **a**-factor, *S. pombe* M-factor, and rhodotorucine A have been shown to be modified by farnesylation, carboxymethylation, and N-terminal truncation. The tremerogens of *T. mesenterica* and *T. brasiliensis* also undergo these hydrophobic modifications (26, 46). Processing and the structure of the mature pheromone can be predicted by analogy with the *S. cerevisiae* gene (Fig. 6). Isolation and characterization of the pheromone will enable detailed study of its role in mating and the dimorphic change. Initially in *S. cerevisiae*, exogenously added **a**-factor was shown to be unable to complement the mating defect of a pheromone mutant (42). Recently, very high concentrations of exogenously added **a**-factor were found to be able to complement some mutations; however, the unmodified peptide is virtually inactive (40,000-fold less active) (38).

It is possible that the filaments seen in *MAT***a** *C. neoformans* cells transformed with the *MF*α gene are conjugation tubes, since closely related fungi such as *R. toruloides*, *T. mesenterica*, *T. brasiliensis*, and *Ustilago violaia* form conjugation tubes in response to pheromone production by opposite mating type cells (17). Peptide pheromone genes have been isolated in both *U. maydis* and *C. neoformans*,

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

FIG. 5. Amino acid alignment of the *C. neoformans* putative pheromone precursor with the precursor peptides of *U. maydis*, *S. pombe*, and *S. cerevisiae*. *S. pombe* **M**-factor and *S. cerevisiae* **a**-factor each have two genes encoding the pheromone and are shown. The single-copy pheromone genes from both the **a1** and **a2** mating types of *U. maydis* are shown. The CAAX prenylation motif and the conserved asparagine are shown in bold.

but no morphology changes have been observed in response to opposite mating type cells in either organism (7). Another possibility is that the production of the α-mating factor by transformed *MAT***a** cells allows the cells to mate and fuse with each other but that the subsequent steps in filament production are blocked. In this case, it may be that heterozygosity of some other mating type gene(s) is required for further filament production. Presumably, other regions of the *MAT*α locus or two entire nuclei of opposite mating types are required to complete the entire sexual cycle, as the filaments do not grow to normal lengths, produce basidia, or form spores. In contrast to *S. cerevisiae*, the *U. maydis* pheromone continues to be expressed in the diploid cell, and its production is required for filamentous growth to occur. A pheromone receptor mutant in *U. maydis* cannot mate if it is a haploid and cannot make filaments as a diploid (7). The exact role that the pheromone plays in mating and/or filament formation in *C. neoformans* is not understood. *MAT***a** cells can transcribe, process, and secrete the *MAT*α pheromone in response to starvation, which suggests that gene products which are common to both mating types are involved in these processes. These results also indicate that the production of pheromone is under a type of nutritional control which is common to both cell types.

*C. neoformans MAT*α strains are more virulent than *MAT***a** strains. The *MAT*α locus was isolated to begin to study mating in *C. neoformans* and the contribution of the locus to virulence. The unexpectedly large locus was found to contain a putative pheromone gene. This discovery provides new information about the organization of the mating type locus and cell-cell signaling in *C. neoformans*. The locus is expected to contain more genes, which as they are

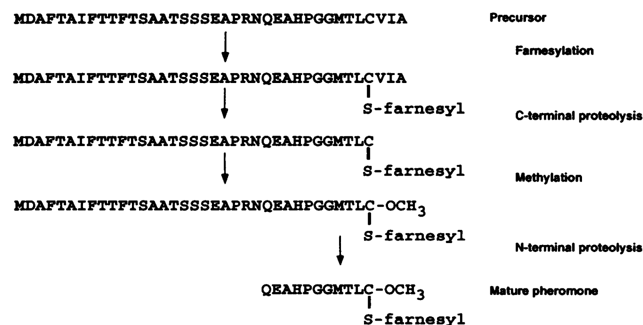

FIG. 6. Predicted processing of the *C. neoformans* mating factor. The processing of the *C. neoformans* pheromone precursor gene is predicted by comparison with the genes and mature peptide pheromones of *S. cerevisiae* **a**-factor (two genes) and *S. pombe* **M**-factor (two genes). The carboxy-terminal processing shown also occurs in *R. toruloides*.

identified will provide more insight into both of these processes. The homologous flanking regions can be used to isolate the analogous locus from *C. neoformans MAT***a** cells. This would enable a comparison to be made between the mating types. It is too early to tell whether mating type genes will form a focus for therapeutic research, but the identification and study of the genes within the *MAT*α locus should allow analysis of the mechanisms of cell-type-specific expression in *C. neoformans* as well as insight into mechanisms of virulence in cryptococcal meningitis.

ACKNOWLEDGMENTS

We thank Haydee Torres-Guerrero for the *MAT*α cosmid library, Bob Chadwick of the HRI sequencing facility for sequencing, K. J. Kwon-Chung for continuing advice and insight, and Flora Baunett, Jay Cross, Liz Hedstrom, and Ira Herskowitz for stimulating discussions and critical reading of the manuscript.

This work was supported by NIH grant A129312 to J.C.E.

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