A Homolog of Ste6, the a-Factor Transporter in Saccharomyces cerevisiae, Is Required for Mating but Not for Monokaryotic Fruiting in Cryptococcus neoformans

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Fungal pheromones function during the initial recognition stage of the mating process. One type of peptide pheromone identified in ascomycetes and basidiomycetes terminates in a conserved CAAX motif and requires extensive posttranslational modifications to become mature and active. A well-studied representative is the a-factor of Saccharomyces cerevisiae. Unlike the typical secretory pathway utilized by most peptides, an alternative mechanism involving the ATP-binding cassette transporter Ste6 is used for the export of mature a-factor. Cryptococcus neoformans, a bipolar human pathogenic basidiomycete, produces CAAX motif-containing lipopeptide pheromones in both MATα and MATα cells. Virulence studies with a congenic pair of C. neoformans serotype D strains have shown that MATα cells are more virulent than MATα cells. Characterization of the MATα pheromones indicated that an autocrine signaling loop may contribute to the differentiation and virulence of MATα cells. To further address the role of pheromones in the signaling loop, we identified a STE6 homolog in the C. neoformans genome and determined its function by gene disruption. The ste6 mutants in either mating-type background showed partially impaired mating functions, and mating was completely abolished in a bilateral mutant cross. Surprisingly, the MATα ste6 mutant does not exhibit a defect in monokaryotic fruiting, suggesting that the activation of the autocrine signaling loop by the pheromone is via a Ste6-independent mechanism. MFα pheromone itself is essential for this process and could induce the signaling response intracellularly in MATα cells. Our data demonstrate that Ste6 is evolutionarily conserved for mating and is not required for monokaryotic fruiting in C. neoformans.

Cryptococcus neoformans is a human fungal pathogen which primarily infects individuals with compromised immune functions. Unlike most of the frequently encountered human fungal pathogens, which are ascomycetes, C. neoformans is a basidiomycete. As a consequence of the increasing prevalence of immunosuppression caused by AIDS, chemotherapy, and high-dose steroid treatment, C. neoformans has emerged as the leading cause of fungal meningitis in the past 2 decades (3). The bipolar sexual cycle of C. neoformans was first identified in 1975 (21). The mating process is initiated by the fusion of haploid yeast cells of opposite mating types (α and α) and leads to the production of heterokaryotic hyphae with fused clamp connections. A specialized sporulation structure called the basidium forms at the tip of the hypha, where karyogamy and meiosis occur to produce sessile basidiospores terminally in basipetal chains by repetitious budding. An analysis of single basidiospore isolates revealed a 1:1 segregation of the two mating types, which indicated that a bipolar mating system existed (20). Upon germination, the basidiospores form yeast cells to regenerate the haploid yeast phase.

An alternative route for the vegetative cells to produce fruiting-body-like structures with spores is called monokaryotic, or haploid, fruiting. Upon desiccation and nitrogen starvation, MATα haploid yeast cells can differentiate, in the absence of a mating partner, into monokaryotic filaments with unfused clamp connections, producing four chains of basidiospores on the basidia. This haploid hyphal phase was initially reported to be associated exclusively with the α mating type (38), and it has been thought to be one of the factors contributing to the predominance of MATα cells over MATα cells in the environment. A recent study, however, reported the discovery of haploid fruiting in some MATα strains (36); as a consequence, whether haploid fruiting accounts for the α mating type predominance is still unclear.

Mating specificity in fungi is controlled by the mating-type (MAT) locus. The MAT locus was first characterized, and has been extensively studied, in the budding yeast Saccharomyces cerevisiae (16). In this region, homologous chromosomes contain nonhomologous sequences. The term idiomorph has been introduced to specify this variation and to distinguish the MAT loci from classical alleles. Information encoded by the MAT loci determines sexuality. Studies on the well-characterized mating systems of ascomycetous and basidiomycetous fungi present a conserved mechanism utilized by fungal cells (18). S. cerevisiae, which is an ascomycete, harbors a single MAT locus which differs between α and α cells. MATα encodes the transcriptional regulator α1, and MATα encodes the transcriptional regulators α1 and α2. Basidiomycetous fungi typically have a tetrapolar mating system with two unlinked MAT loci in the genome; one locus encodes homeodomain proteins, and the other encodes the pheromones and pheromone receptors. Unlike most of the basidiomycetes, C. neoformans does not employ a tetrapolar mating system. Instead, it has a bipolar mat-
ing system. The \textit{C. neoforms} MAT locus is larger than any other known fungal MAT loci (>100 kb) and contains multiple genes which have never been previously observed in a mating-type locus. The unusual size and structure of the \textit{C. neoforms} MAT locus may therefore indicate that it is an evolutionary intermediate between the MAT loci of fungi and the sex chromosomes of higher multicellular eukaryotic organisms, such as humans (14, 23).

An intriguing correlation between virulence and the MAT\textalpha locus has been noticed. Over 95\% of all \textit{C. neoforms} isolates are MAT\textalpha (3, 22). Congenic serotype D \textalpha cells have been shown to be more virulent than \textalpha cells in a murine model of systemic cryptococcosis (22). Additionally, previous studies showed that a MAT\textalpha pheromone triple-deletion mutant was greatly impaired for monokaryotic fruiting, and overexpression of the \textalpha pheromone enhanced this process. Pheromoneless MAT\textalpha mutants were modestly attenuated for virulence. All the data suggested that an autocrine signaling loop may function and contribute to the differentiation and virulence of MAT\textalpha cells (33). To further address how this signaling loop is activated by the pheromone, we have identified a homolog of the \textit{S. cerevisiae} \textit{ste6} gene in the \textit{C. neoforms} genome and determined its function.

It has been shown that fungal pheromones function in the initial recognition stage of the mating process (2, 28). Fungal pheromones can be divided into two broad categories based on their hydrophobicities. First, pheromones can be hydrophobic, like \textalpha-factor in \textit{S. cerevisiae}. Second, pheromones can be hydrophobic, like \alpha-factor in \textit{S. cerevisiae}. The mating pheromone \textalpha-factor is a specific, diffusible signaling molecule expressed only by \textalpha cells and is similar to peptide hormones secreted by higher eukaryotes. It is initially synthesized as a larger precursor that undergoes posttranslational modification. Pheromone maturation occurs by sequential events involving a carboxy-terminal CAAX modification (in which C is cysteine, A is an aliphatic amino acid, and X is cysteine, serine, methionine, glutamine, or alanine) and amino-terminal processing. The mature pheromone is finally exported from the cell via an alternative (nonclassical) mechanism that involves the ATP-binding cassette (ABC) transporter Ste6 (5, 27).

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\textit{STE6} encodes the \alpha-factor transporter, which is essential for mating in \textit{S. cerevisiae} (19, 26). Ste6 is a member of the ABC transporter superfamily composed of two homologous halves, each with six membrane-spanning segments and an ATP nucleotide binding domain (NBD). The only observable phenotype of the \textit{ste6} null mutant in \textit{S. cerevisiae} is the inability to export \alpha-factor and, consequently, to mate; thus, it appears that Ste6 has a defined role in \textit{S. cerevisiae} as a transporter dedicated to \alpha-factor (11). In addition, biochemical evidence has shown that Ste6 couples ATP hydrolysis with pheromone export, so Ste6 is one of a very few ABC transporters in which the presumed ATPase activity has been proven (17).

In this study, we report the identification of a \textit{C. neoforms} \textit{ste6} homolog that is not linked to the mating-type locus. Mutants lacking \textit{STE6} exhibit a bilateral mating defect, although discrepancies are noticed in different mating-type backgrounds. Monokaryotic fruiting in the MAT\textalpha \textit{ste6} mutants is surprisingly unaffected. Our results indicate that \textit{STE6} functions in both mating types and is required for mating but not for haploid fruiting in \textit{C. neoforms}.

\section*{MATERIALS AND METHODS}

\textbf{Strains and media.} The \textit{C. neoforms} strains used in this study are listed in Table 1. Congenic \textalpha strains JEC20 (MAT\textalpha) and JEC21 (MAT\textalpha) and their auxotrophic derivatives were used throughout the study (10, 29). All strains were handled by use of standard techniques and media as previously described (1, 12). Yeast extract-peptone-dextrose (YPD), yeast nitrogen base (YNB), V8 mating, and synthetic low ammonia dextrose (SLAD) media and filament agar were prepared as previously described (13, 38).

\textbf{Identification of a \textit{STE6} homolog in \textit{C. neoforms}.} \textit{C. neoforms} primers WC6 (5'-GTCAGGAGAATCTATAGAAA-3') and WC7 (5'-CTCCTACCTCTCTGTGCA-3') were designed based on the regions homologous to the \textit{S. cerevisiae} \textit{STE6} gene and were used for PCR amplification of a partial sequence of the \textit{C. neoforms} \textit{STE6} gene. This 1.8-kb PCR product was then used as a probe for subsequent identification of a \textit{C. neoforms} genomic clone from a JEC21 bacterial artificial chromosome library (Research Genetics). A 7.9-kb ClaI fragment from this clone was subcloned into pBluescript SK(+) (Stratagene) to generate plasmid pYPE3. Reverse transcription-PCR and sequencing with primers WC156 (5'-GCCCACGTCCGACGGCTCGCCTTTCCA-3'), WC185 (5'-CTTCGAGAAAGCTCTGTAAGGTCG-3'), WC179 (5'-CG TGGTAGACCTTGGCCAGCCGCTTAT-3'), and WC180 (5'-GTACACATTC ACCGAAGAGCAAC-3') allowed the coding regions to be recognized.

\textbf{Disruption and reintroduction of the \textit{STE6} gene.} The \textit{C. neoforms} \textit{STE6} gene was disrupted by replacing a 3.7-kb region within the open reading frame with a 1.9-kb fragment containing the \textit{URA5} gene. Primer pair WC46 (5'-GGACCGGAAATCTGTGATCTCTGTTAGGCGCGTTGA-3') and WC47 (5'-CAC ATCCGGGATCCGTGGGCTGTTAGCAGCG-3') and primer pair WC48 (5'-CGCATATTGATCCTGCCAGGACGCTAGGGCTGTA-3') and WC49 (5'-GGTGGATCTGCTATGTTAGCTGGGCTGAC-3') were used to amplify the 5'- and 3'-end homologous flanking regions of the \textit{STE6} open reading frame, respectively. The \textit{URA5} selectable marker (10) was released from pRCD69 (8) and cloned into the BamHI site to generate a \textit{ste6::URA5} deletion construct containing the left and right portions of \textit{STE6}. The resulting \textit{ste6::URA5} deletion construct, pYPE1, was introduced into the \textit{MAT\textalpha} strain JEC43 by biolistic transformation as described previously (35). Uracil prototrophic transformants were picked and screened by PCR and Southern analyses. To isolate \textit{MAT\alpha} \textit{ste6} strains, the \textit{MAT\alpha} \textit{ste6} strain YPC4 was crossed with JEC34 (MAT\textalpha ura5), the progeny were isolated on synthetic dextrose medium lacking uracil, and \textit{MAT\alpha}

\begin{table}[ht]
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\begin{tabular}{l|l|l}
\hline
\textbf{Strain} & \textbf{Description} & \textbf{Reference or source} \\
\hline
JEC20 & MAT\textalpha & \\
JEC21 & MAT\alpha & \\
JEC34 & MAT\alpha ura5 & \\
JEC43 & MAT\alpha ura5 & \\
JEC170 & MAT\textalpha ade2 his2 & J. Edman \\
JEC171 & MAT\textalpha ade2 his2 & J. Edman \\
WSC18 & MAT\textalpha ade2::ADE2 myo2::URA5 ade2 ura5 & 32 \\
YP4C & MAT\alpha ste6::URA5 & This study \\
YP7C & MAT\alpha ste6::URA5 & This study \\
YP13 & MAT\alpha ura5 ste6 (5-FOA') & This study \\
YP15 & MAT\alpha ura5 ste6 (5-FOA') & This study \\
YP17 & MAT\alpha ura5 ste6 STE6::URA5 & This study \\
YP18 & MAT\alpha ura5 ste6 STE6::URA5 & This study \\
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\end{tabular}
\caption{Strains used in this study}
\end{table}
strains containing the disruption construct were identified by mating and PCR and Southern analyses. The *ste6* versions of *ste6* mutant strains used for reconstitution were generated by selecting the original mutants on 5-fluoroorotic acid (5-FOA) medium, which is toxic to *URA5* cells. The *STE6* reconstitution clone pYPE4 was constructed by introducing the 1.9-kb *URA5* gene fragment into pYPE3 by blunt-end ligation and transformed into *ura5 ste6* mutant strains.

**Southern blot analysis.** Genomic DNA used for Southern analysis was prepared by a large-scale genomic DNA isolation method described previously (30). DNA was digested with ClaI and electrophoresed in a 0.8% × Tris-acetate-EDTA agarose gel. A 0.5-kb probe of the *STE6* flanking region was generated by PCR amplification with primers WC48 (5′-CCGATTTGAGATCCGACACGC-3′) and WC49 (5′-GTTGATCTGATGTGATCGGTGGCA-3′) and labeled by use of a Prime-IT II random primer labeling kit (Stratagene) with [γ-32P]ATP (NEN Life Science Products). Blotting and autoradiography were carried out by standard procedures.

**Northern blot analysis.** RNA used in the Northern analysis was isolated from yeast cells by using Trizol total RNA isolation reagent according to the manufacturer’s instructions (Invitrogen). Twenty micrograms of total RNA from each sample was separated by electrophoresis in a 1.2% agarose-formaldehyde gel. RNA was transferred by capillary action to a nylon membrane (Immobilon-Ny; Millipore) and was hybridized in hybridization buffer (0.12 M Na2HPO4, pH 7.2, 0.25 M NaCl, 1 mM EDTA [pH 8], 7% sodium dodecyl sulfate, 50% formamide). A 0.5-kb *STE6* probe was amplified with *WC9* (5′-TCTGGTCATCCTTCTTCCA-3′) and *WC7* (5′-TCTCCACCTCCTTCCTTGCA-3′). Probes were used for the detection of *MFA1* and *MFα1* were amplified with primer pair WC121 (5′-CCGGAATCCGACGACTTTGCAATGCTATC-3′) and WC7 (5′-GTTGATCTGATGTGATCGGTGGCA-3′) and primer pair WC83 (5′-CTCGAGGCTTTCCCCCTTTTT-3′) and WC84 (5′-ATTTGGAAAAAGATCAATGTG-3′), respectively. All probes were labeled as described for Southern blot analysis.

**Mating, haploid fruiting, and confrontation assays.** Strains for mating, haploid fruiting, and confrontation assays were first grown on YPD at 30°C for 2 days. Mating reactions were performed by co-cultivating the cells with desired partners on V8 or SLAD medium at 20°C in the dark for 1 to 5 days. The mating tester strains used were JEC20 (MATα), JEC21 (MATa), and JEC43 (MATa), and the mating strains used were JEC170 (MATα/α) and JEC171 (MATa/α). The cell suspensions were spotted onto V8 medium and incubated for more than 1 month until substantial filaments and basidiospores had formed. Strains for mating, the *ste6* mutant, the *ste6*::URA5 mutant, the *ste6*::URA5 disruption strains and *ste6*::URA5 reconstitution clone were also confirmed by Southern blot analysis.

**RESULTS**

**Identification and characterization of the *C. neoformans* STE6 homolog.** Based on the typical CAAX motif in the carboxyl terminus of *C. neoformans* MFAα and MFAα, we hypothesized that a homolog of the *S. cerevisiae* Ste6 pheromone transporter may exist and be responsible for pheromone secretion in *C. neoformans*. Therefore, we used a reverse genetics approach to identify a *STE6* homolog candidate in the *C. neoformans* genome. BLAST searches with *S. cerevisiae* Ste6 were performed with the Stanford *C. neoformans* genome database (see http://www-sequence.stanford.edu/group/C.neoformans/index.html). A partial sequence which was found to have similarity with *S. cerevisiae* Ste6 (29% identity and 52% similarity) has since been designated CNBA7570 in the database and has been submitted to GenBank under accession number AY587551. BLAST searches of the putative *C. neoformans* *STE6* homolog in GenBank revealed several members of the ABC transporter superfamily in various organisms. Genomic sequence analysis revealed a predicted coding region of 5.3 kb, and the gene has the expected modular architecture with two homologous halves. Each half contains one membrane-spanning domain (MSD) and one NBD that are distinct among the ABC transporter superfamily. The four core domains are in a single polypeptide with a forward order (MSD1-NBD1-MSD2-NBD2). Analysis of the cDNA sequence presented six introns within the coding sequence, and the predicted number of amino acid residues is 1,656. PCR and Southern blot analyses revealed that this gene is in strains with either mating type (see Fig. 2). The NBDs are the most conserved regions in ABC transporters, and several conserved motifs are located within this region. The results of the amino acid sequence alignment of Ste6 fungal homologs for *NBDB1* and *NBDB2* are shown in Fig. 1. Protein alignment showed that *C. neoformans* Ste6 shares 45% similarity with *Candida albicans* Hst6 (31) and 50% similarity with *Schizosaccharomyces pombe* Mam1 (6).

**Disruption of the *C. neoformans* STE6 gene.** To determine the function of the putative *STE6* homolog, the *C. neoformans* *STE6* gene was disrupted by homologous recombination. The *ste6::URA5* disruption allele (Fig. 2A) was introduced by boliotic transformation into the *ura5* strain JEC43 (MATα). Transformants were selected on synthetic medium lacking uracil and containing 1 M sorbitol and were then screened by the presumptive, impaired mating phenotype on V8 medium in crosses with the *MATa* tester strain JEC20. Four isolates with apparent reductions of filamentation were obtained among the 64 transformants selected. Genomic DNA from the four putative deletion strains was extracted, and a PCR analysis was conducted to confirm that the isolates impaired in mating all lacked wild-type *STE6* and contained only the *ste6::URA5* disrupted allele. Southern blot analysis with a flanking 1.5-kb fragment of the *ste6::URA5* construct confirmed the gene replacement by the reduction of the hybridization signal from a 7.9- to a 6.1-kb fragment among all four isolates upon Clal digestion (Fig. 2B and data not shown). To generate a *MATa* *ste6* mutant, the *MATα* *ste6* strain was crossed with the *ura5* strain JEC34 (MATα) on V8 medium and incubated for more than 1 month until substantial filaments and basidiospores had formed. Progeny were isolated by sectioning the agar block with filaments into sterile water, and the suspension was spread onto synthetic medium lacking uracil. Single colonies were picked and screened for mating type to identify *MATα* strains and analyzed by PCR to identify the *ste6::URA5* disrupted allele. Approximately half of the progeny were *MATα* strains with the *ste6::URA5* allele. Two strains were confirmed by Southern blot analysis to be *ste6::URA5* disruption strains and were selected for subsequent analysis. The *MATα* *ste6* and *MATα* *ste6* strains with the reconstitution of the wild-type fragment of the *STE6* gene were also confirmed by Southern blot analysis (Fig. 2B).

**ste6 mutant strains are impaired in mating.** For *C. neoformans*, mating occurs when *MATα* and *MATα* strains are cocultured on V8 or SLAD medium and is characterized morphologically by cell fusion, filamentation, basidium formation, nuclear fusion, meiosis, and sporulation. To determine the role of the *C. neoformans* *STE6* gene in mating, the *ste6* mutant
strains were crossed with the wild-type tester strains JEC20 (MATa) and JEC21 (MATα) on V8 or SLAD medium (Fig. 3 and data not shown). As predicted, on the basis of the sterile phenotype of the S. cerevisiae MATa ste6 deletion mutant, the MATα/ste6 and MATa ste6 mutant strains displayed a dramatic reduction in filament formation when crossed with a tester strain of the opposite mating type. Interestingly, different degrees of filamentation were observed in the mutants with different mating-type backgrounds. Filamentation in MATα/ste6 mutants was better than that in MATa ste6 mutants. Reconstitution with the wild-type STE6 gene in the mutant strains restored the mating efficiency to the wild-type level (Fig. 3). These findings show that the STE6 gene plays an important role in mating for both α and a cells. It functions bilaterally and is required but not essential for mating in C. neoformans. Furthermore, since we think that ste6 mutants might be impaired in the courtship stage of the mating process, fusion assays were performed to test this hypothesis. Compared to that of the wild-type cells, the fusion efficiency of the MATα ste6 or MATα/ste6 mutant was reduced to less than 1% after 24 h of incubation. The MATα ste6 mutant exhibited decreased fusion efficiency, to about 1.5%, after a 48-h period, and the MATα/ste6 mutant retained about 40.7% of the fusion efficiency (data not shown). This result is consistent with the filamentation phenotypes observed with V8 mating medium and suggests that ste6 mutants are partially impaired in the fusion step.

FIG. 1. Amino acid sequence alignment of NBD1 (A) and NBD2 (B) of Ste6 fungal homologs. Amino acid sequences from C. neoformans (Cn) Ste6 (accession number AY587551), S. cerevisiae (Sc) Ste6 (accession number NP_012713), C. albicans (Ca) Hst6p (accession number P53708), and S. pombe (Sp) Mam1 (accession number P78966) are compared. Walker A, Signature, and Walker B are conserved motifs in the NBDs of ABC transporters. Amino acids identical among all four proteins are shaded black, and amino acids identical among two or three proteins are shaded light grey or dark grey, respectively.
**STE6 is dispensable in haploid fruiting.** The MFα pheromone has been shown to regulate haploid fruiting of MATα cells, and the pheromone mfaI mfa2 mfa3 triple deletion mutant was found to have a significant defect in haploid fruiting when grown on a nitrogen-limiting medium. Overexpression of the MFα1 pheromone gene enhanced haploid fruiting in the wild-type cells (33). To our surprise, the MATα ste6 mutant was fully capable of undergoing haploid filamentation (Fig. 5). Under nitrogen limitation and desiccation conditions, the MATα ste6 mutant produced monokaryotic filaments and blastospores to at least the same degree as, if not more prolifically than, the wild-type MATα cells. Similar observations were also obtained with the confrontation assay, in which the MATα ste6 mutant cells produced filaments to an extent similar to that of the wild-type MATα cells while confronting the MATα or MATα wild-type or MATα ste6 or MATα ste6 mutant cells (Fig. 5). These results indicated that STE6 is not required for haploid filamentation in the MATα C. neoformans cells and suggested that the autocrine signaling loop may be triggered intracellularly.

**STE6 expression is induced during the mating process.** To examine the expression pattern of STE6, 6 × 10⁷ cells of the MATα JEC20 or MATα JEC21 overnightYPD culture/ml were inoculated onto solid V8 medium or mixed in a 1:1 ratio, respectively. Cells were harvested at 2, 6, 12, 24, and 48 h confronted with the MATa wild-type strain, the MATa wild-type cells did not respond to the opposite mating-type cells, suggesting that the MATα ste6 cells failed to secrete MFα pheromone. Similar results were observed when the MATα ste6 strain was confronted with the MATα wild-type strain. On the other hand, both MATα STE6 and MATα STE6 reconstitution strains can fully restore the ability to trigger morphogenesis in response to cells of the opposite mating type. These results indicated that the ste6 mutants are unable to secrete pheromones to induce morphological changes in opposite-mating-type cells.

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**ste6 mutant strains fail to secrete pheromone molecules in confrontation assays.** Confrontation assays have been established to examine the capabilities of the cells to secrete and sense pheromone (33, 37). MATα and MATα cells cultured in close proximity but without contact on filament agar induce morphological changes in response to the opposite mating type. Filamentation at the edges of MATα cells and the presence of swollen MATα cells are typically observed. Cells respond to pheromones secreted from cells with the opposite mating type by undergoing these morphological changes. Therefore, this assay not only determines the ability to respond to pheromones but also analyzes the pheromone secretion of the cells. As shown in Fig. 4, filamentation and swollen cell production of the MATα and MATα cells were observed in the confronting wild-type pairs. When a MATα ste6 strain was confronted with the MATa wild-type strain, the MATa wild-type cells did not respond to the opposite mating-type cells, suggesting that the MATα ste6 cells failed to secrete MFα pheromone. Similar results were observed when the MATα ste6 strain was confronted with the MATα wild-type strain. On the other hand, both MATα STE6 and MATα STE6 reconstitution strains can fully restore the ability to trigger morphogenesis in response to cells of the opposite mating type. These results indicated that the ste6 mutants are unable to secrete pheromones to induce morphological changes in opposite-mating-type cells.

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**FIG. 3. STE6 is required but not essential for mating in both mating types of C. neoformans.** Wild-type (WT) and ste6 mutant strains were co-incubated with mating partners on SLAD plates in the dark for 30 h at 26°C. The edges of the mating mixtures were photographed at a magnification of ×100.

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**FIG. 2. Construction of the ste6:URA5 allele and Southern hybridization analysis of wild-type, ste6, and STE6 reconstitution strains.** (A) The ste6 deletion allele was created by replacing STE6 with the URA5 selectable marker. (B) The genomic DNA of each strain was digested with Clal, electrophoresed, blotted, and hybridized with the ³²P-labeled STE6 fragment indicated. Lane 1, MATα wild type; lane 2, MATα wild type; lane 3, MATα ste6 mutant; lane 4, MATα ste6 mutant; lane 5, MATα ste6 mutant plus STE6; lane 6, MATα ste6 mutant plus STE6.
postincubation. RNA was extracted, and transcription of STE6, MFA, and MFα was examined by Northern blot analysis. Hybridization results revealed that STE6 was expressed at a basal level in response to nutrient limitation in both MATα and MATa cells (Fig. 6 and data not shown). Previous studies showed that a coculture of cells of opposite mating types dramatically induces the transcription of MFA and MFα genes (9, 33); therefore, we further examined the transcription of STE6 during mating. As shown in Fig. 6, a coculture of cells of opposite mating types significantly induced STE6 transcription at 6 h postincubation, and the expression of STE6 returned to the basal level at later time points. Hybridizations with probes for the MFA and MFα transcripts similarly demonstrated that the highest expression level of the pheromone also occurred at 6 h postinoculation (Fig. 6). Thus, the hybridization results suggest that the transcription of pheromones and pheromone transporter genes is highly coordinated and that the expression of STE6 might also be under the control of the pheromone response pathway in C. neoformans.

**DISCUSSION**

*STE6 functions bilaterally in C. neoformans.* By disrupting the gene and analyzing the phenotypes of mutants in different mating-type backgrounds, we found that Ste6 is responsible for pheromone secretion in both MATα and MATa cells and is the
first pheromone transporter to be characterized in a basidiomycete. In *S. cerevisiae*, *STE6* is expressed only in a cells, in which it functions as a transporter to secrete the mating pheromone a-factor. Studies of *S. cerevisiae* have shown that the C-terminal methyl moiety of a-factor is critical for recognition by Ste6 and secretion (32). Contributions of particular amino acids in this dodecapeptide have been assessed. Interestingly, most of the mutations on the a-factor do not affect the export of a-factor but impede interaction with the pheromone receptor (26). It seems that the interaction of a-factor with its transporter is more permissive than the interaction of a-factor with its receptor, for which high specificity is required (26). Studies of the *C. neoformans* MF/H9251 and MFa pheromone genes have shown that the structure of these pheromones is conserved and that 3 out of 10 or 13 amino acids are identical in the mature MF/H9251 and MFa1 peptides, respectively (9, 25). Therefore, it is plausible that in *C. neoformans*, the same transporter functions bilaterally in MATα and MATa cells through different substrate affinities.

**STE6 is involved in, but not essential for, mating in *C. neoformans***. The mating ability of the MFα pheromone triple deletion mutant is not completely abolished (33). As predicted, similar results have been observed for the pheromone transporter mutant. However, this mutant is in stark contrast to the ste6 deletion mutant in *S. cerevisiae*, which exhibits a sterile phenotype. The early cell-cell interaction during mating in *S. cerevisiae* has been inspected extensively. High levels of pheromone molecules have been proven to be required as signals for prezygotes to initiate cell fusion (2, 15). However, unlike the case with ascomycetes, such as *S. cerevisiae*, in which cell fusion is strictly controlled, cell fusion in basidiomycetes is more promiscous. This fact may account for the leaky, non-sterile phenotypes observed in the pheromoneless, pheromone receptor, and pheromone transporter mutant strains of *C. neoformans* (7, 33).

**MATα ste6 cells have a higher mating efficiency.** Interestingly, the mating efficiency of the ste6 mutant in the MATα background is higher than the mating efficiency of the ste6 mutant in the MATa background. One possible explanation may be that there is a second nonspecific pheromone transporter in the *C. neoformans* genome and that it is capable of secreting the MFα pheromone but does not have affinity for the MFa pheromone. However, when we examined the MATα ste6 and wild-type MATa cells, no morphological changes in the MATa cells were discerned. This result poses a problem for the aforementioned hypothesis. However, studies of other basidiomycetous systems may provide some hints. In *Schizophyllum commune*, a homobasidiomycete, numerous pheromone genes encoding the lipopeptide pheromones have been identified in the B mating-type locus. In a study, Fowler et al. (12) reported that heterologous expression of the *Schizophyllum* sex pheromones and receptors in *S. cerevisiae* can substitute for the original yeast pheromones and receptors to induce the pheromone response pathway and lead to cell cycle arrest. Because the *S. commune* lipopeptide pheromones are predicted to have structural similarity to the *S. cerevisiae* a-factor precursor, it was hypothesized that the same machinery used for the processing and secretion of the a-factor in *S. cerevisiae* is also used.

**FIG. 6.** *STE6* expression is induced during mating. Total RNA was prepared from cells grown on V8 plates for 0, 2, 6, 12, 24, and 48 h. A Northern blot was hybridized in succession with probes for *STE6*, MFα, and MFa. RNA loading is demonstrated by the ethidium bromide-stained RNA gel.
for the Schizopyllum pheromones. Interestingly, research has shown that one of the pheromones is secreted in a Ste6-independent manner (12).

In *S. cerevisiae*, the secretion of an AFRP (a-factor-related peptide) has also been found to be Ste6 independent. AFRP corresponds to the C-terminal 7 amino acids of mature a-factor, including both farnesyl- and carboxymethylcysteine. The AFRP does not have pheromone activity, and its biological function is still unknown (4). There are over 30 ABC transporters with similarity to Ste6 in *S. cerevisiae*, and it is possible that the secretion of the AFRP is through one of these transporters (34). In order to determine whether residual amounts of pheromone could still be secreted in the *C. neoformans* MATα or MATa ste6 mutants, an immunohistochemistry assay, such as immunoprecipitation of the pheromones, is required.

Another explanation for why the MATα ste6 mutant mates better is the intrinsic nature of filamentation of the *C. neoformans* MATα cells. It is known that some of the wild-type MATα strains undergo monokaryotic filamentation, while very few wild-type MATα strains demonstrate this capability (36). Therefore, it is possible that this intrinsic nature of filamentation contributes to the better mating efficiency of the MATα ste6 mutant.

The expression of STE6 is coordinated with nutritional status and pheromone sensing. The expression pattern of *C. neoformans* STE6 is somewhat different from the pattern of the homolog in *S. cerevisiae*. First, *C. neoformans* STE6 expression is not mating type specific; cells of both mating types express the gene at comparable levels (Fig. 6 and data not shown), in contrast to *S. cerevisiae*, in which the expression of STE6 is restricted to a cells. Second, the *C. neoformans* STE6 gene is not transcribed at a detectable level under nutrient-rich conditions, and the expression is induced by nutrient limitation. Despite these differences, the expression levels of both the *C. neoformans* and *S. cerevisiae* STE6 genes are elevated in response to pheromone signaling. The STE6 homolog from another pathogenic yeast, *C. albicans* (HST6), has a very different expression pattern. The HST6 gene is constitutively expressed in different cell types of diploid strains at similar levels. HST6 was originally isolated by complementation of the *S. cerevisiae* ste6 mutant (31). In a recent study, it was demonstrated that the HST6 gene is required for mating in MTLa but not in MTLα cells in *C. albicans* (24), indicating that the mating processes in *S. cerevisiae* and *C. albicans* are highly conserved. Evolutionarily, *C. albicans* is much closer to *S. cerevisiae* than *C. neoformans*, and currently we are addressing the question of whether *C. neoformans* STE6 can also functionally complement the mating defect of an *S. cerevisiae* ste6 mutant.

The autocrine signaling response is regulated via a Ste6-independent manner. The finding that the MATα ste6 mutant does not exhibit a defect in monokaryotic fruiting is intriguing. The MATα ste6 mutant appears to undergo haploid fruiting to a greater extent than the wild-type MATα cells do. Additionally, the MATα ste6 mutant strain reconstituted with the wild-type copy of STE6 produces fewer haploid filaments (Fig. 5). This observation suggests that the reconstitution strain may have an elevated level of STE6 expression and this, in turn, results in the hypersecretion of the pheromone molecules. Using real-time PCR analysis, we confirmed that the expression level of STE6 in the reconstitution strain is 1.5-fold higher than that of the wild-type MATα cell (data not shown). Taken together, these results imply that monokaryotic fruiting is regulated by the intracellular level of MFα pheromone and that Ste6 pheromone transporter is not required for the MFα pheromone-mediated autocrine signaling response.

To further address whether the autocrine signaling loop operates intracellularly or extracellually in MATα cells, we also created and analyzed mutants defective in the pheromone receptor gene. The capacity for monokaryotic filamentation in the pheromone receptor *cpra* mutant has been shown to be largely intact (7), and we have confirmed this finding in our experiment. Additionally, we have found that the MATα CPRα ste6 double mutant is also capable of undergoing haploid filamentation and that no significant difference in filamentation was discernible when it was compared to the CPRα single mutant (data not shown). Based on all these results, our present hypothesis is that the autocrine signaling loop is triggered intracellularly by the MFα pheromone. If this is the case, it is a novel phenomenon that has never been reported. Research to identify the corresponding intracellular targets is now under way to further characterize MFα pheromone signaling in *C. neoformans* MATα cells.

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