Identification of the MATa mating-type locus of *Cryptococcus neoformans* reveals a serotype A MATa strain thought to have been extinct

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Cryptococcus neoformans **is an opportunistic fungal pathogen with a defined sexual cycle involving mating between haploid MATa and MATα cells. Here we describe the isolation of part of the MATa mating-type locus encoding a Ste20 kinase homolog, Ste20a. We show that the *STE20a* gene cosegregates with the MATa mating type in genetic crosses, maps within the mating-type locus on a 1.8-Mb chromosome, and is allelic with the MATα locus. We identify the first MATa isolate of the most common pathogenic variety of *C. neoformans* (serotype A, variety *grubii*) which had been thought to be extinct. This serotype A MATa strain is sterile, fails to produce mating pheromone, and is less virulent than a serotype A MATα strain in an animal model. Our studies illustrate an association of mating type with virulence and suggest that, like *Candida albicans*, pathogenic isolates of *C. neoformans* may be largely asexual.**

The basidiomycetous yeast *Cryptococcus neoformans* was first described by San Felice in 1894 and as a fungal pathogen by Busse and Buschke in 1895 (reviewed in ref. 1). *C. neoformans* has been known to be a human fungal pathogen for over a century and has flourished as a pathogen in immunocompromised hosts over the last two decades. Although *C. neoformans* can infect apparently normal hosts, it primarily causes clinical infections in hosts immunosuppressed by HIV, cancer, and immunosuppressive therapies (2, 3). A better understanding of the ecology, epidemiology, and molecular biology of this fungal pathogen is therefore of significant clinical importance.

The sexual cycle of this heterothallic haploid yeast has been defined and is based on a bipolar mating-type system (4–6). In response to environmental conditions, such as nitrogen limitation, cells of opposite mating type (MATa and MATα) fuse to form a dikaryotic mycelium with fused clamp connections. The tips of these filaments differentiate into rounded structures, called basidia, where nuclear fusion, meiosis, and sporulation occur.

In addition to its role in sexual development of the organism, mating type has been linked to prevalence and virulence of *C. neoformans* (7, 8). MATα strains are much more common than MATa strains in clinical and environmental isolates (7). In addition, *C. neoformans* var. *neoformans* MATα strains are more virulent than congenic MATa strains in a murine tail-vein injection model (8). Furthermore, the basidiospores may be the infectious propagule because their size is ideal for deposition into host airways. On the basis of these findings, the molecular structures of the *C. neoformans* mating-type loci are of significant interest. The MATα mating-type locus has been identified by a difference cloning approach (9). Further analysis has revealed that the MATα mating-type locus spans an ≈55-kb region and contains several genes involved in mating and virulence (9–12). In contrast, much less is known about the MATa mating-type locus.

C. neoformans occurs in three varieties: variety *neoformans* (serotype D), variety *grubii* (serotype A), and variety *gattii* (serotypes B and C). These varieties show antigenic differences in the chemical structure of the capsular polysaccharide (13, 14) and have distinct ecological niches and unique features of disease. DNA sequence analysis revealed that the varieties *neoformans* and *grubii* are related but are estimated to have diverged from each other ≈18 million years ago (15, 16). The variety *gattii* diverged from the others even earlier, ≈40 million years ago, and the differences in ecology and molecular biology of this variety suggest that it may represent a distinct species. The classification of these varieties as one species is mainly based on a report of successful mating between variety *gattii* and variety *neoformans*/*grubii* strains (17).

Interestingly, in *C. neoformans* variety *grubii*, the most prevalent clinical isolate worldwide, no strains of the MATa mating type have been described. First, J. Kwon-Chung and colleagues have tested >600 serotype A strains of *C. neoformans* and found all to be MATα mating type (ref. 7; J. Kwon-Chung, personal communication). Second, in several other studies, no MATa serotype A isolates were identified (18, 19). Third, the few MATa serotype A strains that have been reported were all subsequently found to be MATα strains that undergo robust haploid fruiting (refs. 7, 10, 20; J. Kwon-Chung, personal communication, and E. Jacobsen, personal communication). These observations may indicate that, like *Candida albicans*, pathogenic isolates of *C. neoformans* var. *grubii* are largely asexual.

Here we describe the identification of the MATa locus from *C. neoformans* var. *neoformans* (serotype D). We isolated and characterized a MATa mating-type-specific gene encoding a homolog of the *Saccharomyces cerevisiae* Ste20 protein kinase that is involved in pheromone response and mating. In addition, by using primers derived from the serotype D *STE20a* sequence, we identified a MATa serotype A strain of *C. neoformans* var. *grubii*; thus, contrary to the prevailing dogma, the MATa locus has not become extinct in this variety.

Materials and Methods

Strains. Strains used in this study were the serotype A strain H99 (MATα), its auxotrophic derivatives M049 and M001 (both MATα *ade2*), the congenic pair of serotype D strains JEC20 (MATa) and JEC21 (MATα), and auxotrophic serotype D strains JEC53 (MATa *ura5 lys1*) and JEC34 (MATa *ura5*). Tanzanian clinical isolates 123.91, 124.91, and 125.91 were from the permanent strain collection of the Duke University Mycol-

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Abbreviations: 5-FOA, 5-fluoroorotic acid; YPD, yeast extract/peptone/dextrose.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF162330 (*STE20*α A), AF315635 (*STE20*α D), AF315636 (*STE20a* A), and AF315637 and AF315638 (*STE20a* D)].

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ogy Research Unit; 5-fluoroorotic acid (5-FOA)-resistant derivatives of strain 125.91 were obtained as follows. Cells of strain 125.91 were grown overnight in liquid yeast extract/peptone/dextrose (YPD) medium at 30°C, washed twice with sterile water, spread on synthetic dextrose solid medium containing 50 μg/ml uracil and 1 mg/ml 5-FOA, and incubated at 30°C until colonies appeared (21).

Serotyping. Serotype analysis was performed by using the Crypto Check serotyping kit from Iatron Laboratories (Tokyo). Strains were grown at 24°C on solid YPD medium or in liquid capsule-inducing cell culture medium [Dulbecco-modified Eagle's medium; Mediatech (Washington, DC) Cellgro] supplemented with 25 mM Na HCO_3. Cells were harvested and washed several times with normal saline (0.9%). Antibody reactions were performed as described by the kit provider, and strains were tested three independent times.

Mating and Confrontation Assays. Strains were grown on solid YPD medium for 2 days, and a small amount of the cells was removed, washed twice with sterile water, and diluted to 10^8 cells/ml. Equal volumes of strains were mixed, and 5 μl of the cell suspension were spotted on V8 solid medium and incubated at 24°C for several days. Filament and basidiospore formation were assessed by light microscopy every other day. In confrontation assays, strains were streaked onto filament agar medium in thin lines $\approx$2 mm apart without touching. Conjugation tube formation was examined after 24 and 48 h at 30°C. All of these experiments were repeated in triplicate.

PCR Analysis. The *STE20a* allele was isolated by using a touchdown PCR approach (22–24) with degenerate primers JOHE2090 (5′-GTNGCNATIAARCARATG) and JOHE2092 (5′-YTCNGGNGCCATCCARTA; I = inosine, R = A/G, Y = T/C, and N = A/T/C/G), which amplify a region within the highly homologous kinase domain of the p21-activated protein kinase family. PCR reactions (50 μl) were run by using 50–80 ng of genomic DNA and 50 pmol of each primer. PCR conditions were as follows: after an initial denaturing period of 5 min at 94°C, PCR cycles were 94°C denaturing for 30 s, 1 min annealing at 60°C with a temperature increment of -1°C at every cycle, and 45-s synthesis time at 72°C. After 20 cycles, an additional 20–25 cycles with the lowest annealing temperature were performed. PCR was finished by a 10-min synthesis period at 72°C. Primers used for serotype- and mating-type analysis were JOHE1909/JOHE1910 (*MFα2* D), JOHE3067/JOHE3068 (*STE20a* D), JOHE3069/JOHE3070 (*STE20α* D), JOHE1895/JOHE1896 (*STE11α*), JOHE1671/JOHE1672 (*STE12α* A + D), JOHE1671/JOHE2189 (*STE12α* A), JOHE3065/JOHE3066 (*CLA4* D), JOHE3066/JOHE3236 (*CLA4* A), JOHE2926/JOHE3238 (*CNA1* D), JOHE2926/JOHE3239 (*CNA1* A), JOHE2596/JOHE3240 (*GPA1* D), and JOHE2596/JOHE3241 (*GPA1* A). Primer sequences are listed in Lengeler *et al.* (25). PCR for mating and serotype analysis were repeated at least twice.

Pulsed-Field Gel Electrophoresis of *C. neoformans*. Cells were grown overnight in 5 ml of YPD medium at 30°C; 100 μl of the culture were added to 50 ml of yeast nitrogen base minimal medium and grown to OD_{600} 0.5–0.6 at 30°C at 225 rpm. The medium was supplemented with 1 M NaCl to repress capsule formation. Cells were pelleted, washed three times in 0.5 M NaCl/50 mM EDTA (pH 8.0), resuspended in 9.5 ml of water and 0.5 ml of β-mercaptoethanol, and incubated for 30–60 min at 37°C with gentle mixing. Cells were again pelleted, resuspended in 4 ml of spheroplasting solution [(1 M sorbitol/10 mM EDTA/100 mM sodium citrate (pH 5.8)], 100 μl of lysing enzyme solution was added (Sigma; $\approx$80–100 mg/ml spheroplasting solution), and cells were incubated for 1–1.5 h at 37°C with gentle mixing. Complete spheroplasting was checked by mixing $\approx$10 μl of cell suspension with the same volume of 10% (vol/vol) SDS and gently pipetting to monitor the increase in viscosity that results from the release of high molecular weight DNA (26, 27). Spheroplasts were pelleted at 1,800 rpm for 10 min (4°C; Sorvall RT7), washed twice with ice-cold spheroplasting solution, resuspended in the remaining spheroplasting liquid, and diluted to a concentration of 10^9–10^{10} cells/ml. The spheroplasts were then mixed with three volumes of low-melting agarose [1% low-melting agarose in 0.125 M EDTA (pH 8.0); 50°C] and poured into molds. Agarose was allowed to solidify for 30 min at 24°C and for an additional hour at 4°C. Agarose plugs containing spheroplasts were removed from the molds and spheroplasts were lysed for at least 24 h at 55°C in lysing solution [0.5 M EDTA/10 mM Tris·HCl (pH 10)/1% Sarcosyl]. Plugs were washed three times for 15 min with 0.5 M EDTA (pH 8.0) and stored in 0.5 M EDTA at 4°C.

A standard 1% gel [1% pulse field-certified agarose in 0.5$\times$ 90 mM Tris·HCl/64.6 mM boric acid/2.5 mM EDTA (pH 8.3)] was run in a Bio-Rad CHEF DR II apparatus. Plugs were placed into the wells of a standard 13 $\times$ 14 cm gel, sealed with low-melting agarose, and allowed to solidify for 30 min at 4°C. The gel was run in 0.5$\times$ 90 mM Tris·HCl/64.6 mM boric acid/2.5 mM EDTA (pH 8.3) buffer at 12°C with the following settings: initial A-time 75 s, final A-time 150 s, start ratio 1.0, run time $\approx$30 h, mode to 10; initial B-time 150 s, final B-time 300 s, start ratio 1.0, run time $\approx$54 h, mode to 11. The voltage was set to 125 V. The running buffer was changed every 24–48 h.

Results

Identification of the *STE20a* Gene. We recently identified a gene in the *C. neoformans* MATα mating-type locus that encodes a homolog of the *S. cerevisiae* Ste20 kinase (P. Wang, C. S. Breeding, K.B.L., M. E. Cardenas, G.M.C., J.R.P., and J.H., unpublished results). Here we tested the hypothesis that *C. neoformans* cells of the opposite mating type contain a divergent, MATa-specific allele of the same gene. PCR primers that were used to identify the *STE20α* gene from the serotype D MATα strain JEC21 were used in a touchdown PCR approach (22–24) to amplify a region of a *STE20* gene from genomic DNA of the serotype D MATa strain JEC20 under low-stringency conditions. Protein sequence analysis revealed that the serotype D Ste20a kinase shares 70% overall identity with the *C. neoformans* Ste20α kinases from serotype A or D strains, and 60% identity with Ste20 from *S. cerevisiae*. The regions with the highest level of identity were the putative pleckstrin homology domain, the Cdc42-binding domain, and the kinase domain (Fig. 1). In general, serotype A- and D-specific alleles of the same gene exhibit 5–7% DNA sequence divergence (28, 29); our findings show that this is also the case for genes encoded in the mating-type loci of a specific idiomorph (MATa or MATα) in *C. neoformans*. The higher level of divergence between the *STE20a* gene and both the serotype A and D *STE20α* genes supports the conclusion that *STE20a* is specific for the MATa mating type. Consistent with this prediction is the finding that *STE20α*-specific DNA does not hybridize with DNA from MATa strains in Southern blot analysis (data not shown).

The *STE20a* Gene Is Linked to MATa Mating Type and Maps to the MATa Locus. To establish that the serotype D *STE20a* gene is linked to the mating-type locus, the serotype D MATα strain JEC170 (*lys2 ade2*) was crossed with the serotype D MATa strain MCC3 (*cna1*::*ADE2 ade2 ura5*), basidiospores were dissected, mating type and auxotrophic mutations were scored, and genomic DNA was isolated. PCR analysis with primers specific to the serotype D *STE20α* and *STE20a* genes revealed that the *STE20a* gene is linked to the MATa mating type, and the *STE20α* gene is linked

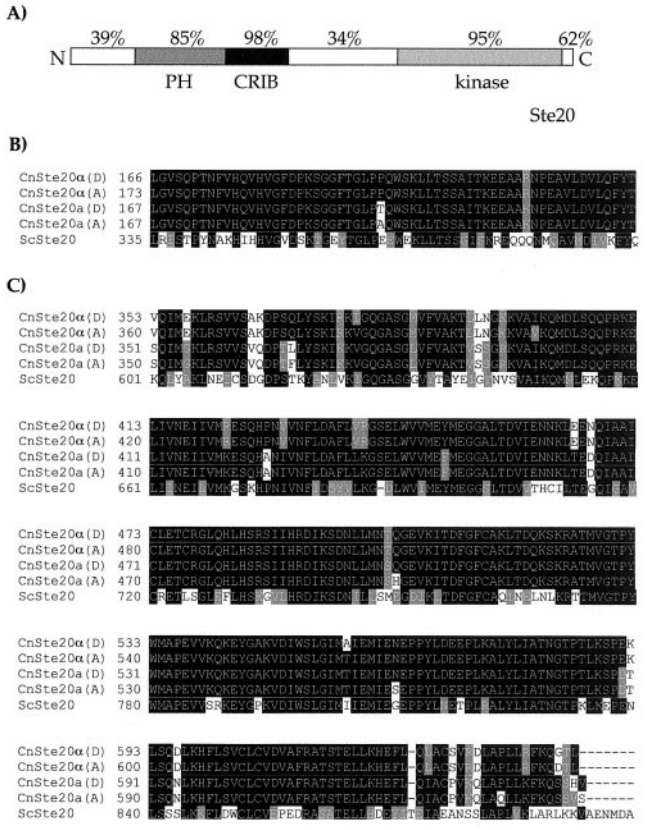

Fig. 1. Protein sequence alignment of Ste20 kinases of *C. neoformans*. The amino acid sequences of four Ste20 kinases from *C. neoformans* and their *S. cerevisiae* homolog were aligned by using the CLUSTALW algorithm. (*A*) Schematic drawing of the Ste20 protein of *C. neoformans* depicting a putative (pleckstrin homology) domain, the Cdc42-binding domain (CRIB), and the carboxyl-terminal kinase domain. Percent values indicate the similarity between the serotype D Ste20a and Ste20α proteins in the conserved domains and the linker regions. Similar results were obtained comparing the serotype A-specific proteins. (*B*) Alignment of the CRIB domains. (*C*) Alignment of the carboxyl-terminal kinase domains. Amino acids present in at least two of five proteins were shaded. Black shading indicates identical amino acids, and gray shading indicates conserved residues. (See also Figs. 7–11, which are published as supplementary material at http://www.duke.edu/~lengeler/PNAS.html.)

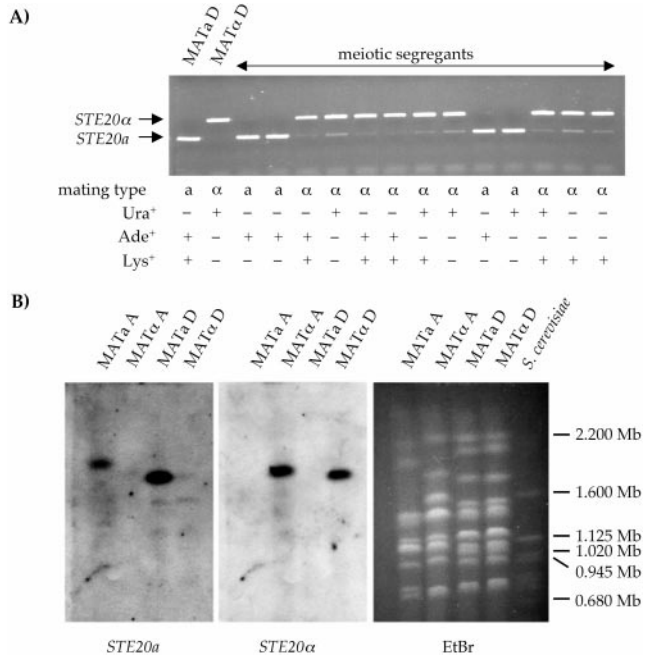

Fig. 2. The *STE20a* gene is MATa mating-type specific. (*A*) Progeny of a cross between the serotype D MATa strain MCC3 (*cna1*::*ADE2 ade2 ura5*) and the MATα strain JEC170 (*ade2 lys2*) were isolated and mating type was determined by PCR analysis and genetic backcrosses. The molecular analysis of mating type was performed by PCR amplification of the MATa or MATα-specific allele of the *STE20* gene. The MATa-specific PCR product was loaded on a standard agarose gel and run for ≈15 min before the MATα-specific reactions were loaded into corresponding wells. Under these conditions, some weak crossreactivity of the *STE20a*-specific primers was observed with DNA from MATα strains. Mating type of the meiotic segregants was determined by genetic backcrosses and is indicated below the gel. In addition, progeny were tested for recombination by scoring auxotrophic markers inherited from the parental strains. (*B*) Chromosomes from the serotype A strains 125.91 (MATa) and H99 (MATα), the serotype D congenic pair of strains JEC20 (MATa) and JEC21 (MATα), and *S. cerevisiae* (size marker) were separated by pulsed-field electrophoresis, and the gel was stained with ethidium bromide (*Right*). The chromosomes were then blotted onto a nylon membrane, and Southern hybridizations were performed by using probes specific to the serotype D *STE20a* or *STE20*α genes (*Left* and *Center*). Identical results were obtained by using probes derived from serotype A-specific DNA (data not shown).

to the MATα mating type. This finding also demonstrates that the *STE20a* and *STE20*α genes are allelic (Fig. 2*A*). In genomic Southern blots, the *STE20a* gene hybridized to genomic DNA isolated from MATa strains but not from MATα strains (data not shown). By pulsed-field gel analysis and Southern blot, the *STE20a* gene was mapped to a 1.8-Mb chromosome that comigrates with the chromosome containing the MATα locus in the congenic serotype D strain JEC21 and that hybridized to the *STE20*α gene (Fig. 2*B*; ref. 27). These findings demonstrate that the *STE20a* gene is a component of the MATa mating-type locus.

Identification of a Serotype A MATa Strain. Both MATα and MATa strains have been isolated in the serotype D, var. *neoformans* lineage of *C. neoformans*. In fact, the ability to use these strains in genetic crosses was critical in deciding to sequence the genome of this variety (30). In contrast, despite some effort (>600 strains tested by J. Kwon-Chung, personal communication), only MATα isolates have ever been reported in the serotype A *C. neoformans* var. *grubii* strains, despite their common occurrence as clinical and environmental isolates (7). Thus, the MATa locus has been thought to have been lost in this variety. However, within a group of clinical isolates, we discovered an unusual serotype A strain, 125.91, which was isolated from the cerebrospinal fluid of a patient with cryptococcal meningitis in Tanzania and clearly serotyped to be serotype A. By PCR (Fig. 3) and Southern analysis (data not shown), this strain lacked several of the genes encoded by the MATα mating type locus, including *STE12*α, *MF*α*2*, *STE20*α, and *STE11*α. This strain was also sterile in genetic crosses.

We considered two possible explanations: either strain 125.91 has a large deletion of the MATα locus or strain 125.91 is MATa. By low-stringency PCR using serotype D *STE20a*-specific primers, we were able to demonstrate that the *STE20a* gene was present in strain 125.91 (Fig. 3 *Bottom*; see arrow). This finding was further confirmed by Southern analysis of pulsed-field gels (Fig. 2*B*; see MATa A strain). In contrast to the congenic pair of serotype D strains JEC20 and JEC21 (MATa D and MATα D), the chromosomal banding pattern of strain 125.91 (MATa A) and the well-characterized strain H99 (MATα A) differed, confirming the variability in karyotypes of nonisogenic *C. neoformans* strains that has been described (26, 27). The *STE20a* gene from strain 125.91 was cloned and characterized. Sequence analysis revealed that the gene shares 95% identity with the

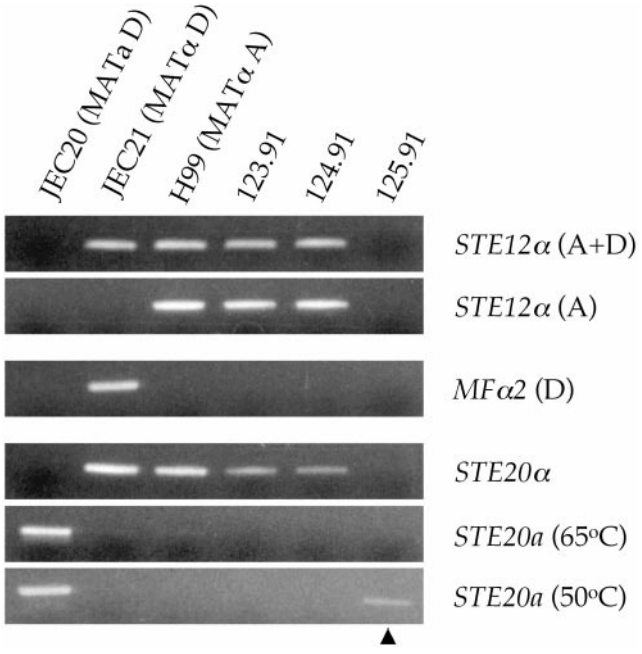

Fig. 3. Serotype A strain 125.91 contains a novel MATa-specific *STE20* allele. Genomic DNA was isolated from three control strains JEC20 (MATa serotype D), JEC21 (MATα serotype D), and H99 (MATα serotype A), and from three clinical isolates from Tanzania (123.91, 124.91, and 125.91). PCR analysis was conducted with primers specific to mating-type- and/or serotype-specific alleles of the *STE12*α, *MF*α*2*, *STE20*α, and *STE20a* genes. Under low-stringency conditions, a novel *STE20a* allele from strain 125.91 was identified and is indicated by an arrow.

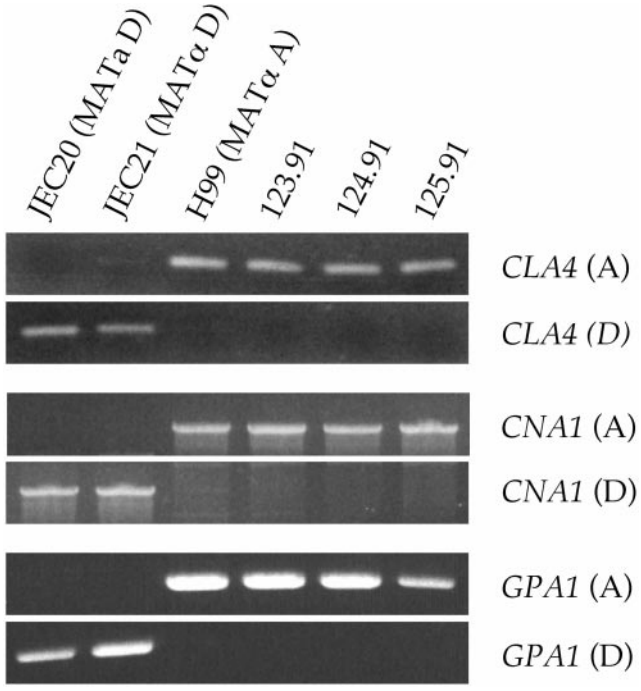

Fig. 4. MATa strain 125.91 is serotype A. Genomic DNA was isolated from strains JEC20 (MATa, serotype D), JEC21 (MATα, serotype D), and H99 (MATα, serotype A), and three clinical isolates from Tanzania (123.91, 124.91, and 125.91) and tested for the absence or presence of serotype-specific alleles of the *CLA4*, *CNA1*, and *GPA1* genes by PCR analysis.

serotype D *STE20a* gene and is much more divergent from the serotype A or D *STE20*α genes (67% identity). Because serotype A- and D-specific alleles normally differ by $\approx$5% at the nucleotide level, we conclude that this newly identified *STE20a* allele is serotype A specific. This is the first description of a MATa mating-type strain of *C. neoformans* var. *grubii*.

Recent studies have also revealed that unusual serotype AD strains are either diploid or aneuploid and often heterozygous for the mating-type locus (25). We demonstrated by two approaches that the serotype A MATa strain 125.91 is not a serotype AD isolate. First, by antibody agglutination tests, we confirmed that this strain is in fact serotype A and not serotype AD (data not shown). Second, by PCR analysis with primers that are specific for serotype A or D genes, we found that the serotype A MATa strain 125.91 contained only the serotype A-specific alleles of the *GPA1*, *CLA4*, and *CNA1* genes (Fig. 4).

We have recently discovered two transposable elements that are common in the genomes of serotype D strains, but absent or only rarely present in serotype A strains (M. C. Cruz and J.H., unpublished results). When genomic southern blots and chromosome hybridizations were performed with probes specific for these transposable elements, strain 125.91 exhibited a hybridization pattern typical for serotype A strains and largely or completely lacked these elements that are common in the genome of serotype D strains (see Figs. 7–11). These findings provide additional stringent evidence that the MATa strain 125.91 is indeed a serotype A *C. neoformans* var. *grubii* isolate.

Serotype A MATa Strain 125.91 Is Mating Defective. Previous studies have established the mating type of *C. neoformans* isolates by genetic crosses with known MATα and MATa strains. By a similar approach, we found that the serotype A MATa strain 125.91 is sterile, and fails to form filaments, basidia, or basidiospores when cocultured on a variety of different media (V8, synthetic low ammonium dextrose, filament agar, yeast nitrogen base, and YPD) with mating-type tester strains. Mating is often enhanced by auxotrophic mutations in *C. neoformans*, but there was still no mating observed when auxotrophic mating-type tester strains were crossed with a *ura5* auxotrophic (5-FOA-resistant) derivative of the serotype A MATa strain 125.91. In confrontation assays, the serotype A MATa strain 125.91 failed to stimulate conjugation tube formation in the serotype D MATα strain JEC21 or the serotype A MATα strain H99, suggesting it does not produce MFa pheromone. Strain 125.91 also failed to respond to MATα strains and produced no conjugation tubes or enlarged cells. As expected, strain 125.91 showed no reaction in mating or confrontation assays with MATa tester strains.

Several genes have been identified in *C. neoformans* that encode components of a pheromone response pathway involved in mating and virulence of this fungal pathogen (10, 11, 31, 32). When the G-protein β-subunit Gpb1 or the transcription factor Ste12α were overexpressed from the *GAL7* galactose-inducible promotor in strain 125.91, Ste12α induced filament formation but Gpb1 did not (Fig. 5), indicating that this strain might be defective in signal transduction pathways necessary for mating in *C. neoformans.*

Serotype A MATa Strain 125.91 Is Virulent in an Animal Model. The serotype A MATa strain 125.91 is a clinical isolate from a Tanzanian AIDS patient who died of cryptococcal meningitis, indicating that this strain is virulent in a human host. Therefore, the relative pathogenicity of this MATa strain was compared with the well-characterized serotype A MATα strain H99 in a murine tail vein injection model. Groups of 10 BALB/c mice were infected with 10^6 fungal cells of either strain and survival was monitored over the course of 2 months. Whereas 100% mortality occurred by day 32 with the serotype A MATα strain

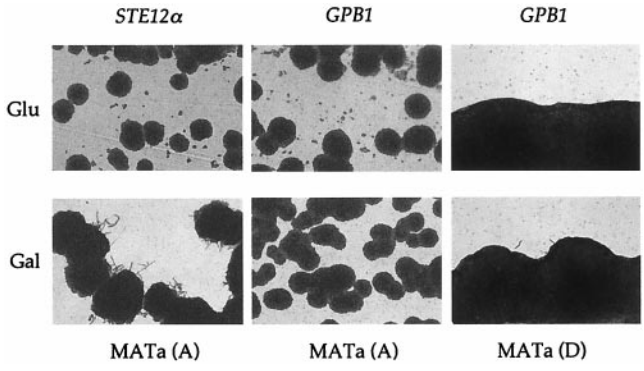

Fig. 5. Overexpression of Ste12α induces filament formation in strain 125.91. A 5-FOA-resistant *ura5* derivative of strain 125.91 [MATa (*A*)] was transformed with plasmids containing a galactose-inducible allele of the transcription factor *STE12α* or the G-protein β-subunit *GPB1*. Strains were grown on solid filamentation medium containing 0.5% glucose (Glu) or 2% galactose (Gal), and photographed after 5 days of incubation at 30°C at ×10 magnification. In contrast to strain 125.91, overexpression of *GPB1* induced filament formation in the serotype D MATa strain JEC34 [MATa (*D*)].

H99, 100% mortality was delayed until day 50 in the serotype A MATa strain 125.91 (Fig. 6). These findings suggest that the virulence of serotype A MATa strains may be reduced compared with MATα strains, as is known to be the case with congenic serotype D strains of *C. neoformans* (8). Confirmation of this postulate will require construction of congenic pairs of MATα and MATa strain in serotype A, which is not possible at present because of the mating defect of the MATa strain 125.91.

Discussion

C. neoformans has a defined sexual cycle, and the MATα mating-type locus has been linked to the ability to undergo haploid fruiting (33) and to virulence of this organism (8). MATa strains are less virulent than congenic MATα strains in a murine tail vein injection model (8). Therefore, structural analysis of both mating-type loci should provide insight into the virulence of this fungal pathogen.

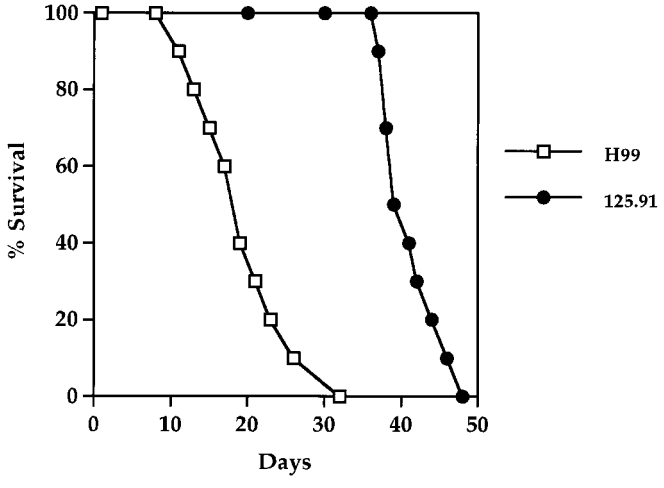

Fig. 6. Virulence of serotype A MATa strain 125.91. Ten BALB/c mice each were inoculated via lateral tail vein injection with 10^6 cells of the wild-type serotype A strain H99 (MATα) or the Tanzanian serotype A isolate 125.91 (MATa). Survival was plotted against days postinoculation and results were significantly different for animals infected with strain H99 compared with strain 125.91 ($P < 0.01$ Kruskal-Wallis test).

Most studies have focused on the virulence-associated MATα mating-type locus, a portion of which was identified by Moore and Edman (9). The mating-type locus of *C. neoformans* shows unusual features in comparison to other basidiomycetes. First, although the locus contains a pheromone/receptor system, the size of the locus (≈55 kb) is substantially larger compared with other fungi (34, 35). In addition, several genes encoding components of a putative pheromone response pathway are present within the locus (10, 11, 32). Surprisingly, these genes encoded by the MATα mating-type locus do not cross-hybridize with DNA from MATa strains. Thus, either the MATa locus lacks these genes or contains quite divergent alleles. It is interesting to note that the MTLa and MTLα mating-type-like loci in *Candida albicans* are also quite divergent (36).

In this report, we identify the first MATa-specific gene, *STE20a*, and show that it is allelic to its MATα counterpart *STE20α.* Ste20a shows only 70% overall similarity to Ste20α, which explains the lack of cross-reactivity in low-stringency Southern analysis. We are currently using the *STE20a* gene as a probe to identify mating-type-specific bacterial artificial chromosomes from a JEC20 library to characterize the entire structure of the MATa mating-type locus.

The ability to perform genetic crosses makes *C. neoformans* an excellent molecular model system for fungal pathogenesis. A congenic pair of serotype D strains, JEC20 and JEC21, has been constructed that differs only at the mating-type loci (8). However, serotype A strains are the most common clinical isolates throughout the world, especially in AIDS patients where 99% of isolates are serotype A (37). Therefore, it would be of great value for pathogenesis studies to establish a congenic pair of serotype A MATa/MATα strains. However, it has been difficult to identify any serotype A MATa strain, and it was thought that this mating type might have become extinct in serotype A. In contrast, we show that a clinical isolate from Dar Es Sallam, Tanzania (125.91) contains a novel *STE20a* allele. By PCR analysis, DNA sequence comparison, and capsular antibody reactivity, strain 125.91 is clearly serotype A. This is the first description of a serotype A MATa strain in *C. neoformans*. However, the serotype A MATa strain 125.91 was sterile under all laboratory conditions tested. This may in part explain the difficulty in identifying serotype A MATa strains, because mating type has been largely determined by classic genetic backcrosses.

This raises the question of whether other serotype A MATa strains in nature might all be sterile. Our recent finding that some unusual clinical serotype AD isolates contain the serotype A MATa locus suggests that not all such strains are sterile (25). Serotype AD strains have arisen through intervariety crosses between *C. neoformans* var. *neoformans* and *grubii,* indicating that fertile serotype A MATa strains might still exist in nature, but the ecological niche of these strains remains unknown. Protoplast fusion may provide an approach to cross the sterile MATa strain 125.91 and recover fertile serotype A MATa strains. If the sterility of these strains is not caused by mutations within the mating-type locus, this should permit construction of a congenic pair of serotype A MATa and MATα strains.

During the evolution of pathogenicity and ability to proliferate in distinct environmental niches, *C. neoformans* may be evolving to be asexual. Interfertile MATa and MATα strains still exist in nature in the serotype D variety *neoformans* strains. In contrast, in the divergent serotype A variety *grubii* strains that are the predominant form of clinical and environmental isolates, the majority of isolates are MATα mating type but poorly fertile with MATa serotype D strains, and in both the laboratory and nature, give rise to unusual diploid serotype AD strains (25). The serotype A MATa strain we have identified does not mate with either serotype A or serotype D MATα strains. Thus, in the serotype A lineage, MATa and MATα strains may have become

genetically isolated. We propose that as the serotype A lineage has evolved to be more prevalent in the environment and clinical cases, the ability to mate has become impaired or lost. These observations may be similar to the findings that another human fungal pathogen, *Candida albicans*, is an obligate diploid that is primarily clonal (38) and may only rarely employ its newly discovered sexual cycle (39, 40). These findings suggest an association between virulence and an asexual life cycle, possibly to prevent recombination events that would reassort multiple unlinked genes that are required for virulence or survival in certain environmental niches.

Note Added in Proof. Nucleotide sequence alignments showing the similarity and divergence between the serotype A and D *STE20a* and *STE20α* genes (Figs. 7–10), and the chromoblot demonstrating that the serotype A MATa strain 125.91 lacks transposable elements that are common in the genome of serotype D strains (Fig. 11) have been posted at http://www.duke.edu/~lengeler/PNAS.html.

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