Mating-Type Locus of Cryptococcus neoformans: a Step in the Evolution of Sex Chromosomes

Klaus B. Lengeler, Deborah S. Fox, James A. Fraser, Andria Allen, Keri Forrester, Fred S. Dietrich, and Joseph Heitman*

Department of Molecular Genetics and Microbiology, Howard Hughes Medical Institute, Duke University, Durham, North Carolina 27710

Received 27 June 2002/Accepted 11 July 2002

The sexual development and virulence of the fungal pathogen Cryptococcus neoformans is controlled by a bipolar mating system determined by a single locus that exists in two alleles, α and α. The α and a mating-type locus from two divergent varieties were cloned and sequenced. The C. neoformans mating-type locus is unique, spans >100 kb, and contains more than 20 genes. MAT-encoded products include homologs of regulators of sexual development in other fungi, pheromone and pheromone receptors, divergent components of a MAP kinase cascade, and other proteins with no obvious function in mating. The α and a alleles of the mating-type locus have extensively rearranged during evolution and strain divergence but are stable during genetic crosses and in the population. The C. neoformans mating-type locus is strikingly different from the other known fungal mating-type loci, sharing features with the self-incompatibility systems and sex chromosomes of algae, plants, and animals. Our study establishes a new paradigm for mating-type loci in fungi with implications for the evolution of cell identity and self/nonself recognition.

Self/nonself recognition events underlie the function of the major histocompatibility locus in defense against infection and organ transplant rejection, the self-incompatibility systems that prevent inbreeding in plants, and the production of offspring by sexual reproduction. During sexual reproduction, specialized genomic regions promote self/nonself interactions. Sex-determining regions include the mating-type loci in fungi and the sex chromosomes in plants and animals. Dimorphic sex chromosome systems independently evolved in animals, mosses, and dioecious plants. A related but distinct sexual incompatibility system is found in many lower eukaryotes, including algae, protozoans, monoecious plants, and fungi. In these organisms, multiallelic mating-type (MAT) loci monitor cell interactions for sexual compatibility, and if inbreeding is detected, mating is aborted (17, 18, 50, 56).

A common theme of sex determinants is the need to be transmitted as a single unit, and recombination within sex-determining regions is suppressed to avoid generating self-fertile or sterile offspring. Several mechanisms operate to suppress recombination. In the fungal MAT loci, extensive sequence divergence prevents recombination between different alleles. In the case of sex chromosomes, both sequence divergence and chromosomal rearrangements suppress recombination. These rearrangements affect nearly the entire sex chromosome in humans or mice, whereas in lower vertebrates and certain dipterous insects, only a limited region of the sex chromosomes is rearranged. These findings suggest that the dimorphic sex chromosomes evolved via accumulation of chromosomal aberrations.

Fungal mating-type loci serve as paradigms for understanding gene regulation during sexual development and the determination of cell fate and identity (7, 16, 32, 34, 37, 40, 54). Sexual development of ascomycetous fungi is commonly controlled by a bipolar mating system involving a single mating-type locus. In these cases, the MAT locus spans only a few thousand base pairs and exists in two unrelated alleles that control cell identity by encoding transcription factors that act on distant target genes (16, 40, 54).

In contrast to mating in ascomycetes, mating in basidiomycetes is commonly regulated by two independent, unlinked loci, resulting in tetrapolar mating systems (7, 37, 40). Both mating-type loci can be multiallelic, giving rise to thousands of different mating types in some mushroom fungi (38). The structure of mating-type loci in basidiomycetes has been determined for several model systems, including the mushrooms Coprinus cinereus (41, 52, 53) and Schizophyllum commune (66–68, 70) and the maize pathogen Ustilago maydis (4, 27, 39, 62). Similar to ascomycete mating-type loci, one locus encodes a pair of homeodomain transcription factors that controls a subset of developmental processes involved in sexual reproduction (A locus in C. cinereus and S. commune and b locus in U. maydis). The second locus (B in C. cinereus and S. commune and a in U. maydis) encodes a G protein-coupled pheromone receptor linked to one or more pheromone genes.

The opportunistic human fungal pathogen Cryptococcus neoformans is an encapsulated yeast that is distributed worldwide in association with pigeon guano and trees (6). This pathogen has increased in medical importance over the past several decades because of its ability to cause fatal meningoencephalitis in immunocompromised hosts. In contrast to many basidiomycetes, C. neoformans has a bipolar mating system with two opposite mating types, MATα and MATα.

A portion of the C. neoformans MATα locus was initially identified by a difference cloning approach and was found to contain the MFα1 pheromone gene (49). Subsequent work
revealed that the *C. neoformans* MAT locus is unusual in size and gene composition, spanning an ~55-kb region (35) and containing several additional α-specific genes, including STE12α and STE20α (10, 46, 69, 71, 73). Recent studies have revealed the following: (i) the MAT locus is larger than previously suspected (C. M. Hull, R. C. Davidson, and J. Heitman, submitted for publication), (ii) the α and α alleles encode divergent alleles of related genes (9, 46), and (iii) the architectures of the two MAT alleles may differ. Here we present our study on the *C. neoformans* mating-type locus that establishes a novel paradigm for the structure of mating-type loci with implications for fungal evolution and the evolution of specialized sex chromosomes.

MATERIALS AND METHODS

Strains. The strains used for construction of bacterial artificial chromosome (BAC) libraries and analysis of the mating-type loci were *C. neoformans* serotype A var. *grubii* strains H99 (MATα) and 125.91 (MATa), and *C. neoformans* var. *neoformans* congenic strains JEC21 (MATα of JEC21 and MATα of JEC21) and JEC20 (MATa of JEC20 and MATa of JEC20) (31, 41, 42, 46). The serotype D strains used to analyze recombination in the MAT region (Fig. 6) were derived from crosses between MATα strain DSF51 (znf1α::NAT1 ste12α::URA5 ade2) or RDC20-5 (ste12α::URA5 ade2) and *MATa* strain JEC53 (ura5 lys1). The stability of the MAT loci through several crosses (see Fig. 4) was analyzed using the serotype D strains NIH12, NIH433, B3501, B3502, JEC20, and JEC21 (31, 42). Structural analysis of MAT loci in the population of *C. neoformans* var. *neoformans* was conducted using the unrelated serotype D strains CDC92-18, CDC92-27, MMRL760 (all MATα) and #11 (MATα).

BAC and subgenomic libraries. To obtain high-quality chromosomal DNA from *C. neoformans*, protoplasts were isolated as described previously (46). In the present study, the lysis of protoplasts was prolonged to 48 h and proteinase K was added to the lysis buffer at a final concentration of 4 mg/ml. Fresh buffer was added after a 24-h incubation. Plugs containing lysed protoplasts were washed twice with ice-cold Tris-EDTA (TE) buffer containing 0.2 mM phenylmethylsulfonyl fluoride and subsequently washed four to five times with ice-cold TE buffer for 1 h each. Plugs could be stored indefinitely at 4°C in 50 mM EDTA.

In collaboration with Research Genetics (Huntsville, Ala.), the chromosomal DNA was partially digested with *Hin*dIII, and 100-kb fragments were isolated after separation of digested DNA via pulsed-field gel electrophoresis. Genomic fragments were cloned into the BAC vector pBeloBAC11, and clones with inserts were identified using standard blue/white screening techniques. BAC clones of interest were identified by Southern hybridization with mating-type-specific gene probes by using colony lift membranes (Research Genetics).

To close the remaining gaps in strains H99 and 125.91 not covered by the analyzed BAC clones (Fig. 1), desired fragments were identified by Southern blotting and isolated from subgenomic libraries or generated by PCR.

**FIG. 1.** Structures of the serotype D (MATα of JEC20 and MATα of JEC21) and serotype A (MATα of 125.91 and MATa of H99) α and a mating-type alleles and adjacent genomic regions. The mating-type-specific regions are shown as thick bold lines, and flanking regions are shown as thinner black lines. Sequences were analyzed using BLASTX, and identified genes are shown as arrows in the direction of transcription. Genes encoding pheromone response pathway elements are shown as black arrows, locus-specific genes are shown as white arrows, and all other genes are shown as grey arrows. Bars above the mating-type alleles represent the BAC clones, genomic fragments, and PCR products analyzed.
BAC sequencing strategy. To generate high-quality BAC DNA, plasmid DNA was subjected to cesium chloride equilibrium centrifugation. The plasmid DNA of individual BAC clones was isolated from 500 ml of cultures by using alkaline lysis. The DNA was resuspended in 15 ml of TE buffer containing ethidium bromide (400 µl of a 10 mg/ml stock), and cesium chloride was added to a final density of 1.4 mg/ml. The DNA-CsCl solution was transferred into a 15 ml ultracentrifuge tube, and the sample was centrifuged in an NVTi5.1 rotor at 65,000 rpm for ~24 h at room temperature. The lower, plasmid-containing DNA band was removed using a 5 ml syringe with a 20 gauge needle and introduced into a 4 ml ultracentrifuge tube, which was then filled with CsCl solution to a final density of 1.4 mg/ml. The sample was subjected for an additional 24 h at 70,000 rpm. The DNA was removed from the second, ethidium bromide band, extracted several times with salt-saturated isopropanol, and the DNA was dialyzed against 5 liters of TE buffer for several hours. After adding 1/10 volume of 3 M sodium acetate, BAC DNA was precipitated with 0.6 volume of isopropanol (~20°C), washed with 70% EtOH, and resuspended in TE buffer.

Three to five micrograms of BAC DNA was sheared with a Hydro shears device (Gene Machines) to generate ~1.5- to 3-kb DNA fragments. Sheared fragments were subsequently subjected to standard blunting and fill-in reactions, and double-stranded adapters, provided by the Duke Center for Genome Technology (CGT), were ligated in 100-fold excess to the blunted DNA fragments. Fragments were separated from free adapters by agarose gel separation, and the DNA was cloned into a special, pUC18-based linearized vector provided by the genbank. The resulting sequence data were analyzed by comparing BAC sequences to sequences in the GenBank database by using the BLASTX algorithm (1).

Cloning and sequencing the mating-type locus of C. neoformans. We set out to clone and sequence the mating-type locus of C. neoformans to test the following hypotheses. First, does the MAT locus encode homoeodomain transcription factors that govern cell identity as in other fungi? Second, did the α and a alleles of the MAT locus diverge from a common ancestral region of DNA, which we proposed earlier based on the identification of the divergent STE28α and STE28α genes (46, 69)?

RESULTS

Cloning and sequencing the mating-type locus of C. neoformans. We set out to clone and sequence the mating-type locus of C. neoformans to test the following hypotheses. First, does the MAT locus encode homoeodomain transcription factors that govern cell identity as in other fungi? Second, did the α and a alleles of the MAT locus diverge from a common ancestral region of DNA, which we proposed earlier based on the identification of the divergent STE28α and STE28α genes (46, 69)?

TABLE 1. Primer pairs used in recombination and structural analysis of the C. neoformans MAT locus

| Primer pair | Sequence | Tm (°C) |
|-------------|----------|---------|
| JOHE7607 (6) | ATAGACATCCTCACAATGCTC | 66 |
| JOHE7608 | AAGTTCAGCTGCTGAACGATC | 66 |
| JOHE7609 (5) | TTGAGCGCTATATTGGTCTAGC | 66 |
| JOHE7610 | GAAGACGCCATCATACACAGAC | 66 |
| JOHE7611 (8) | GGAGCAGACTGTCACAATCATC | 66 |
| JOHE7611 (7) | GAACGACACTGTCACAATCATC | 66 |
| JOHE7612 (8) | GAAATCGCACCGTGACGATCG | 66 |
| JOHE7613 (5) | GATTGTCGAGGATAGTGGTAGG | 66 |
| JOHE7615 (3) | ATCCGCTCTCCTCATACGTTT | 66 |
| JOHE7617 (4) | TTCCGACCTGCTGATAGCTTCC | 66 |
| JOHE7618 | TGCTTACGCTGAAAGGAGGAC | 66 |
| JOHE7619 (1) | GATTCACCTCCACTCAGTACTAGG | 66 |
| JOHE7620 | GTGAGGAAATGTGGAGGAGGAGGAG | 66 |
| JOHE7621 (2) | CCATTCAACTGCTGATAGCTTCC | 66 |
| JOHE7622 | GTGAGGAAATGTGGAGGAGGAGGAG | 66 |
| Pairs used in structural analysis | | |
| JOHE5396 | GATTATCACCGGACACCG | 60 |
| JOHE5397 | ACAGTCATATCGCAATCGAC | 60 |
| JOHE5398 | CTCCTGATCATCTCATCTGACTCC | 64 |
| JOHE5399 | CGACGCAACAACTGCTGTC | 64 |
| JOHE5400 | TACCACCTGAGGACACAC | 60 |
| JOHE5401 | CGATAGGACTACGAGGACAC | 60 |
| JOHE5402 | ATCCGAGTCCTCAGTCTACAC | 62 |
| JOHE5403 | CGGATCATCACGCTGACTAC | 62 |
| JOHE5404 | TTCCGTCTGAGGACACACCA | 60 |
| JOHE5405 | TCTGCTGATCATCTCATCTG | 60 |
| JOHE5406 | TCTGCGATTTCATCTGACTAC | 60 |
| JOHE5407 | TTGAGCGTCATATTGGTCTAGC | 66 |
| JOHE5408 | TACCGGTGTGAACTGGGGAG | 62 |
| JOHE5409 | TACCGGTGTGAACTGGGGAG | 62 |
| JOHE5410 | ATCCGCTCTCCTCATACGTTT | 66 |
| JOHE5411 | TTCCGACCTGCTGATAGCTTCC | 66 |
| JOHE5412 | TGCTTACGCTGAAAGGAGGAC | 66 |
| JOHE5413 | GATTCACCTCCACTCAGTACTAGG | 66 |
| JOHE5414 | GTGAGGAAATGTGGAGGAGGAGGAG | 66 |

a Tm annealing temperature. 

b Numbers in parentheses correspond to those shown for the primer combinations in Table 1.

c Listed according to their position spanning the MAT locus from left to right (Fig. 7).

Third, has the MAT locus been conserved or rearranged during the evolution of this pathogen?

To determine the complete structure of the α and a mating-type alleles of C. neoformans, genomic BAC libraries were generated from a congeneric pair of serotype D α and a strains...
(JEC21 and JEC20) as well as from the serotype A α and a strains H99 and 125.91. Probes to known mating-type-specific genes were used to identify BAC clones spanning each allele of the mating-type locus. Shotgun libraries were produced from two or three BAC clones encompassing each allele and sequenced (Fig. 1). In the case of the α locus from serotype D, the ~38-kb mating-type region defined by Moore and Edman was analyzed using PCR products and existing cosmid clones (49).

To establish gene order in this region and to rule out possible rearrangements of the BAC clones chosen for sequencing, hybridization-based BAC maps were generated (Fig. 2). An initial screen was conducted using probes to the STE12α and STE20α genes and sequences flanking the previously iden-
Hybridizing BAC clones were subjected to further analysis with additional probes, including the *SXI1*/H9251 and *RUM1*/*H9251* genes (Fig. 2). The resulting BAC map confirmed the gene order for the initial 38- and 55-kb mating-type regions previously analyzed (35, 49) and extended this map to include numerous additional genes. No aberrant recombination events appear to have occurred during the construction of the BAC library. This was further confirmed by comparison of the sequences of the *MAT*/H9251 loci generated in this study with BAC fingerprint maps for the *H9251* strains JEC21 and H99 generated at the Genome Center of the University of British Columbia (61).

Data generated during both mapping processes revealed that the *MAT* locus of strain H99 was not completely covered in a library of ~6,000 available BAC clones. This finding was confirmed during our sequencing efforts. The 10-kb gap in the H99 *MAT* locus was closed by identifying and sequencing the following: (i) genomic clones spanning *MAT*-specific genes, (ii) *MAT*-specific plasmid clones from the H99 shotgun sequencing project, and (iii) a 1.6-kb PCR product spanning the final remaining gap that proved recalcitrant to recovery in *Escherichia coli* (Fig. 2B). This 10-kb region spans three pheromone genes and the *ZNFIα* and *PRT1α* genes and includes several large inverted repeats that may render this region difficult to clone. To provide deeper sequence coverage, sequences for strains H99 (serotype A) and JEC21 (serotype D) that were available from GenBank and public genome sequencing projects were entered in the assembly. The overall region of double-stranded DNA that was bidirectionally sequenced was 245 and 210 kb for the serotype D *MAT* and *MAT* a alleles, respectively, and 225 and 150 kb for the serotype A *MAT* and a alleles, respectively, for a total of 830 kb of genomic sequence.

Mapping the borders of the mating-type locus. A portion of the serotype D *MAT* a mating-type allele was identified by Moore and Edman in 1993, and subsequent work in several labs has contributed to further define the structure of the *MAT* locus (46, 49, 69, 71). Karos and coworkers reported a map of the serotype D *MAT*/H9251 locus that spans an 55-kb region between the *RPO41* and *NOG2* genes (35). Here we present evidence that redefines the left border of the mating-type locus, demonstrating that the *MAT* locus spans an additional 50-kb region upstream of the *RPO41* and *NOG2* genes (35). Here we present evidence that redefines the left border of the mating-type locus, demonstrating that the *MAT* locus spans an additional 50-kb region upstream of the *RPO41* gene and defining the authentic left junction between genomic DNA and the *MAT* locus.

The junctions between the *MAT* locus and neighboring genomic DNA were identified by comparing the serotype D *MAT* a and a allele sequences. The serotype D *MAT* a strain analyzed (JEC21) was generated by backcrossing an a strain 10 times to an *MAT* a strain (JEC20), resulting in a congenic strain pair that should differ only at the *MAT* locus (31, 42). Hence, sequences bordering the *MAT* locus should be identical or nearly so, whereas sequences within *MAT* should be distinct. A DNA sequence comparison of the a and a alleles by dot plot analysis...
revealed that flanking sequences on one side and within the UAP1-FAO1 and IKS1-NOG2 genes are nearly identical, whereas on the other side, the sequences diverge, defining the left and right junctions between the MAT locus and surrounding genomic DNA (Fig. 3, left and right panels). In addition, the order of genes surrounding the UAP1-FAO1 and IKS1-NOG2 genes in the flanking regions is identical between the α and a alleles up to the proposed junctions and then diverges in the opposite mating-type alleles (Fig. 1). In Southern analysis, probes specific to the sequences outside the predicted junctions yielded identical restriction patterns in α and a strains (Fig. 4), whereas MAT-specific probes yielded mating-type specific patterns (Fig. 4). Our findings indicate that the mitochondrial RNA polymerase gene RPO41 originally reported to define the left border of the MAT locus is in fact part of the mating-type locus and not a flanking gene. The finding that the gene order and sequence both diverge on either side of the RPO41 gene further supports this conclusion (Fig. 1 and 3, middle panel). An RPO41-specific probe also yielded different restriction patterns for the α and a strains JEC21 and JEC20 (Fig. 4).

The sequences flanking the MAT loci in the α and a strains JEC21 and JEC20 share ~99% identity for several kilobases before reaching 100% identity, reflecting the position of the most recent recombination between the mating-type junctions and surrounding genomic DNA. A small ~100-bp region just upstream of the left MAT locus junction shares limited similarity between the α and a strains and may reflect an ancient recombination event between the alleles.

Gene order is nearly identical in the regions flanking the mating-type locus in the serotype A and D strains, whereas the central region spanning the MAT locus itself is extensively rearranged. In the left flanking regions, all four mating-type alleles share synteny. In contrast, in the right flanking region, the NOG2, PAN6, HSP12, and APG9 genes all share synteny but the gene that immediately flanks the MAT locus in serotype D (IKS1) is an integral component of the mating-type locus in both the α and a alleles in serotype A (Fig. 1). The IKS1 gene does not appear to be a component of the MAT locus in serotype D, as it is embedded in sequences that share ~99% identity between strains JEC21 and JEC20. As discussed further below, the IKS1 gene may have entered the MAT locus in serotype A (gene capture model) or exited the locus in serotype D (gene egress model). Comparison of the IKS1 gene sequences reveals that the serotype A IKS1α and IKS1α alleles are dramatically divergent (52% identity), whereas the IKS1 genes flanking the serotype D MAT alleles share 99% identity with each other and significant identity (85%) with the serotype A IKS1α allele. These findings support a model in which the IKS1 gene was lost from the locus and fixed in the flanking region by inversion and recombination events, with concomitant loss of the IKS1α gene in the serotype D lineage.

In conclusion, based on synteny and sequence comparisons, our study demonstrates that the mating-type locus of C. neoformans is significantly larger than previously proposed, spanning an ~105- to 130-kb region that lies between the FAO1 and IKS1-NOG2 genes in serotype D and the FAO1 and NOG2 genes in serotype A. The serotype D α and a alleles span 105,656 and ~117,308 bp, respectively, whereas the serotype A α and a alleles span ~102,764 and ~127,082 bp, respectively. Thus, the α alleles of the MAT locus are larger than the α alleles.

**Genes contained in the mating-type locus.** Approximately 20 genes contained in the MAT locus were identified when the BLASTX algorithm was used to compare the MAT locus sequence with sequences in GenBank (Fig. 1). Table 2 summarizes the genes identified within the mating-type alleles and flanking sequences. Transcripts corresponding to several of these genes are present in expressed sequence tags derived from cDNA from strain H99 and the serotype D strain B3501, a precursor to strain JEC21 (University of Oklahoma Health Science Center [http://www.genome.ou.edu/cneo.html]). The four alleles of the mating-type locus have been annotated with respect to the exon-intron structure of the genes contained and expressed sequence tags corresponding to genes within the MAT locus. This information is available electronically (http://cneo.genetics.duke.edu/mating-type/). Repetitive sequences and transposon remnants have also been annotated (Fig. 5).

Previous studies on the C. neoformans mating-type locus had suggested that key regulators of sexual differentiation found in other basidiomycetous fungi might be missing. However, in the newly defined MAT locus, we identified homologs of both key mating-type components from model basidiomycetes. First, a pheromone receptor (STELα/a) and several pheromone precursor genes (MFAα/a) were identified in the locus, similar to those present in the a mating-type loci of U. maydis and U. hordei and the B loci of C. cinereus and S. commune and in accord with several recent reports (14, 47, 63). In contrast to those in the model basidiomycetes, the genes for the pheromones and pheromone receptor are not tightly linked to one another but are instead dispersed throughout the C. neoformans MAT locus (Fig. 1). Second, a gene encoding a novel homeodomain protein (SXIα) was identified in both the serotype A and D α mating-type alleles but not within the a mating-type alleles. This homeodomain homolog is analogous to the components found in the b mating-type loci of U. maydis and U. hordei and the A locus of C. cinereus and S. commune. The pheromones, pheromone receptor, and Sxiα transcription factor have all been linked to roles in the sexual development of C. neoformans (14, 63; Hull et al., submitted).

The sexual development of fungi is regulated by a pheromone-activated mitogen-activated protein (MAP) kinase signaling cascade. In C. neoformans, several elements of the MAP kinase pathway are encoded by genes in the MAT locus and exist in divergent forms in the α and a alleles. These include homologs of the p21-activated protein kinase Ste20, the MEK kinase Ste11, and the transcription factors Ste12 and Znf1, which function in the sexual development and virulence of this organism (9, 10, 14, 15, 69, 73). The link between the components of the pheromone response pathway and the MAT locus is novel, and the biological importance of this unusual gene clustering for the organism is unknown but may involve the unique properties associated with the MATa locus that promote haploid fruiting and virulence.

In addition to mating-specific genes, several other genes are contained within the MAT locus that have no obvious role in sexual development (Table 2). However, based on their similarity to genes identified in other organisms, the functions of the products of a few of these genes can be predicted. For
FIG. 4. Mating-type locus is stable through several genetic crosses. The strains and backcrossing scheme used during the construction of the congenic pair of serotype D strains JEC21 and JEC20 are shown on the left. The strains used in the analysis are indicated in boldface type. Mating type is indicated as α or a. Strains in boldface type were subjected to restriction enzyme digestion (BamHI, HindIII, and PstI) and Southern blotting using the mating-type-specific and nonspecific probes indicated. No differences were apparent between JEC21 and JEC20 and the ancestral strains NIH12, NIH433, B3501, and B3502. The relative positions of the probes used are indicated below in the corresponding mating-type locus.
example, Rum1 is a retinoblastoma binding protein 2-like corepressor that corepresses genes regulated by the MAT locus. (55). By analogy, the Rum1 encoded homeodomain transcription factors bE and bW in U. maydis (3, 36, 72). Another interesting protein is Cap1, which shares homology with a Neurospora crassa protein kinase. (3, 36, 72). Cap1 might therefore play a role in the synthesis of the capsular polysaccharide that is essential for virulence.

The MAT locus contains transposon remnants and many repetitive sequences. Multiple transposon-related sequences were identified in each allele of the MAT locus (Fig. 5). Most of these sequences represent decayed versions of long terminal repeats associated with a ubiquitous family of retrotransposable elements that inhabits the genome of C. neoformans (28). These include several copies of LTR11, LTR14, Cnirt3, and Cnirt4. In general, the position of these elements was not conserved with respect to the locus borders or neighboring genes, suggesting that these elements were recently acquired by each allele. There are three examples of particular interest. First, the region between the SXIIα and CAP1α genes in strain JEC21 differs from that in the serotype A strain H99 in which a Cnirt3 element has inserted and replaced intervening sequences. A second complete copy of the Cnirt3 element is also present between the STE3α and MFα genes. The two Cnirt3 elements are in a direct orientation, but because each is flanked by ~50-bp inverted repeats, homologous recombination events could occur between the distal or internal ends of the two elements and transpose the intervening genes. A second interesting case is the serotype D-specific gene NCP1α, which shares homology with a Neurospora crassa protein of unknown function but is missing sequences homologous to the N-terminal region and instead contains a fragment of the Cnirt4 transposable element. Finally, several different transposase-related genes were identified in the α alleles, implying that one or more copies of Tc1/mariner-type transposons were present in the locus and might have contributed to structural rearrangements during the evolution of the MAT locus. For example, local transposition of an inserted element could create inverted sequence repeats and promote inversions by homologous recombination.

The mating-type locus was also found to contain a surprising number of repetitive sequences, including simple sequence repeats. As shown in Fig. 5, all four alleles contained multiple copies of several different simple tri- and tetranucleotide re-
peats. A particularly notable example was 71 imperfect copies of a tetranucleotide repeat contained within the serotype D STE11a gene that were not present in any of the other STE11 genes. In addition, the pheromone precursor genes are often encoded by divergent pairs of genes that were embedded in regions that constitute large inverted repeats. This may give rise to unique mechanisms by which the genes are duplicated, rearranged, and lost as the alleles of the mating-type locus diverged from their common ancestors. For example, inversions between the identical MFα1/H92511 and MFα1/H92512 genes in the serotype D/H9251 allele would transpose the order and direction of the intervening genes (PRT1/H9251, ZNF1/H9251, RPL39/H9251, and MFα3/H92513).

Recombination is suppressed in the mating-type region. An important feature of mating type is stable inheritance as a single unit, and recombination is suppressed in these regions to avoid generation of sterile or self-fertile offspring. We used PCR analysis with α and a allele-specific primers to test whether the sequences we defined as the MAT locus faithfully cosegregate with mating type and whether recombination occurs in this locus (Fig. 6). Twenty-four progeny derived from two defined crosses between multiply marked strains were tested by PCR to test whether recombination occurred between the ends of the MAT locus. In addition, mating type was scored by genetic backcrosses. No recombination was observed in the mating-type region, and mating-type-specific sequences faithfully cosegregated with the corresponding mating types as determined by mating assays with tester strains. Forche and coworkers recently reported an amplified fragment length polymorphism-based physical map for C. neoformans and established the recombination frequency for the mating-type chromosome at ~24 kb/centimorgan, demonstrating that recombination readily occurs elsewhere on this chromosome (24). In addition, the CNB1 gene resides on the mating-type chromosome but is completely unlinked to the MAT locus in genetic crosses (data not shown) (25), providing additional evidence for recombination events distal to MAT.

Sequences flanking the mating-type locus are nearly identical, but a few sequence polymorphisms between the α strain JEC21 and the a strain JEC20 are present immediately upstream and downstream of the MAT locus. We used primers

FIG. 5. Multiple transposon remnants and repetitive sequences are embedded in the MAT locus. Transposable element-related sequences are depicted for the four alleles of the MAT locus. Complete element copies are indicated in a larger font size and boldface type. In addition, local sequence repeats were identified and annotated for each allele.
designed for these sequences and the same set of meiotic progeny to test whether recombination occurred just outside of the borders of the \textit{MAT} locus. No recombination events were observed, providing additional evidence for the integrity of the locus and its correct assignment (Fig. 6).

The $\alpha$ and $\alpha$ alleles of the \textit{MAT} locus are stably inherited. The $\alpha$ and a congenic pair of serotype D strains JEC21 and JEC20 was generated by a series of 10 backcrosses (Fig. 4) (31, 42). One concern was whether the mating-type alleles might have rearranged during the process of strain construction, possibly as a result of increased recombination during meiosis. Southern analysis was used to compare the genomic structure of the $\alpha$ and $\alpha$ alleles of strains JEC21 and JEC20 with those of their ancestors by using probes to sequences within and flanking the \textit{MAT} locus (Fig. 4). No differences in restriction patterns were observed for any of the genes analyzed. Thus, the structure of the mating-type locus has been stably inherited through multiple generations, providing additional evidence that recombination is suppressed in this genomic region.

\textbf{Structure of the serotype D \textit{MAT}/H9251 mating-type locus is conserved in nature.} The serotype D $\alpha$ and $\alpha$ strains JEC21 and JEC20 and their derivatives are widely used because of their congenic background and because of the ability to conduct classical genetic experiments with them (31). This was one of the major reasons why these strains were chosen to determine the structure of the mating-type locus in serotype D. However, an important issue is whether the structure of the mating-type locus of these lab strains is representative of unrelated serotype D strains. We addressed this by a PCR-based approach using primers that amplify overlapping fragments spanning the original $\sim$38-kb serotype D \textit{MAT}/H9251 mating-type locus proposed by Moore and Edman. Fragments of identical sizes were obtained with 9 of the 10 primer pairs using as templates DNA from the unrelated serotype D strains JEC21, CDC92-18, CDC92-27, and MMRL760 (Fig. 7). Only one primer combination produced a larger, $\sim$14-kb PCR product from strain MMRL760 (Fig. 7), compared with an $\sim$10-kb PCR product from JEC21 and the two CDC strains. Further PCR analysis revealed that an insertion of $\sim$4 kb had occurred between the \textit{STE11} and \textit{MFa1} genes of the \textit{MAT} locus of this atypical yet still fertile strain (Fig. 7).
Sequence analysis of this region of the MAT locus of strain MMRL760 revealed that a novel mariner-related transposable element had inserted into the locus. Compared with that of strain JEC21, an additional 3,906 bp are present in the MAT locus of strain MMRL760, and this novel sequence is flanked by 136-bp inverted repeats that are identical at 135 of 136 positions. In addition, the element is inserted at a TA sequence and created a TA-TA duplication at the insertion site. The right half of this element encodes an open reading frame that might represent a transposase gene. Importantly, by comparison with the results of the ongoing genome project, this region of the element was found to share significant sequence identity with five distinct regions of the genome of serotype D strain JEC21. Curiously, the MAT locus of strain JEC21 contains a region of several hundred base pairs that shares identity with the left end of this element. Thus, this element may have either transposed into a remnant of itself, or strain JEC21 contains a fragment of the element as a result of a previous excision event.

Structural rearrangements during evolution and divergence of the MAT alleles. Our findings reveal that the C. neoformans mating-type locus is significantly larger than previously suspected. In serotype D, the α allele spans ~105 kb and the a allele spans ~117 kb, whereas in serotype A, the α and a alleles span ~103 and ~127 kb, respectively. Thus, in both serotypes, the a allele is larger than the α allele. The number of genes identified within the locus ranges from 19 (MATα in serotypes A and D) to 23 (MATα in serotype A). While some of the genes encoded by the MAT locus have already been shown or predicted to function in the pheromone response pathway that regulates mating (Fig. 1, black arrows), other genes have no obvious function with respect to sexual development.

The gene order is strikingly different between different alleles of the mating-type locus. Genes outside the mating-type locus exhibit synteny in both serotype A and serotype D (Fig. 1 and 8A), whereas gene order inside the mating-type locus has been dramatically remodeled (Fig. 8A and data not shown). In addition, a few genes are present in either the α or the a mating-type allele but not in both, including SXI1α, RPL39α, and NCPlα in serotype D and SXI1α and NAD4α in serotype A (Fig. 1 and 8A) (Table 2). When the α or a mating-type alleles were compared between serotypes A and D, the rearrangement of the locus was even more striking (Fig. 8B). Furthermore, several genes identified are unique to the mating-type allele of only one serotype (Table 2). Interestingly, the JKS1 gene flanks the mating-type locus in serotype D but is located within the mating-type locus in serotype A, possibly as the result of a DNA inversion. With this exception, the order of the genes outside the MAT locus is conserved between the two mating types and varieties. Interestingly, the orders of the genes just inside the left ends of the MATα mating-type loci of serotype A strain H99 and serotype D strain JEC21 are similar, with the exception of one inversion (SPO14α) and two small insertions (Cnir3α and NAD4α) (Fig. 8B).

In summary, our findings reveal that the α and a mating-type alleles diverged from a common ancestral region of DNA by a process involving rearrangements, inversions, and nucleotide substitutions. Moreover, the α and a alleles have both undergone extensive rearrangements as the serotype A and serotype D strains evolved into varieties or even distinct species.

DISCUSSION

We have analyzed the structure of four mating-type alleles of the fungal pathogen C. neoformans, including the α and a alleles of the serotype A and D varieties grubii and neoformans. The MAT locus of C. neoformans is considerably larger than previously reported (35) and spans ~105 to 130 kb. While the gene order outside the locus is largely conserved, even between the two serotypes, genes inside the mating-type locus have been subject to extensive rearrangements. This is true not only for the two opposite mating-type alleles in a given serotype but also for a single MAT allele compared between serotypes. In addition, a few genes were identified that are present in only one or the other allele. Recombination in the mating-type locus and the surrounding genomic region is suppressed, and the mating-type alleles are stable through multiple genetic crosses without rearrangement. The basic structure of the mating-type locus in C. neoformans is largely conserved within the population of this organism, but the detection of a transposable element in the α locus of an atypical serotype D strain reveals that genetic alterations can occur in the population.

When compared to those of other model ascomycetes or basidiomycetes, the mating-type locus of C. neoformans has a unique structure in terms of both size and gene composition. The MAT locus in most ascomycetes is limited in size and encodes transcription factors that determine mating type and cell identity. In basidiomycetes with tetrapolar mating systems, one mating-type locus resembles ascomycete mating-type loci in size and gene composition. The second locus encodes pheromone and pheromone receptor systems and can extend up to 20 kb via gene duplications. Only a few other fungal mating-type loci have been found to contain genes lacking an obvious function in mating (33, 54, 64). The mating-type locus of C. neoformans is the largest single-copy MAT locus known, and the locus contains a striking number of genes, including ones that function in mating and others with no predicted role in sexual differentiation.

In contrast to what occurs in other basidiomycete mating-type loci, the C. neoformans genes encoding pheromones (MFα1-3 and MFα1-3) and pheromone receptors (STEα3α and STEα3α) are not adjacent to each other but rather dispersed throughout the locus (Fig. 1). The MFα1-3 pheromone and STEα3α pheromone receptor genes were identified in the previously published C. neoformans MAT locus (35), but no transcriptional regulators of the homeodomain or HMG domain family were previously known. In our studies, BLASTX analysis of the complete α and a mating-type sequences identified a gene close to the left end of the α mating-type alleles that exhibited weak similarity to other homeodomain transcription factors involved in mating and cell identity in other fungi (Fig. 1). As will be presented elsewhere, deletion analysis of the SXI1α (sex inducer 1 α) gene reveals a role for Sxi1α in sexual development (Hull et al., submitted). No SXI1α-related gene is present in either a allele studied, and no cross-hybridizing genes are present in a-specific DNA by Southern analysis (Hull et al., submitted). The identification of the mating-type-specific homeodomain transcription factor Sxi1α brings the mating-type system of C. neoformans closer to those of other basidiomycetes with respect to the main regulators involved in sexual development than previously suspected. Our findings reveal
that both transcriptional regulators and a pheromone and pheromone receptor system are present in the *C. neoformans* MAT locus, but the arrangement of the locus is distinct compared to those of other model basidiomycetes in which the two regulators are unlinked.

In addition to these regulatory genes, ~15 other genes were identified in the different mating-type alleles (Table 2). Some encode components of the pheromone response pathway that regulates mating, fruiting, or virulence of *C. neoformans* (14, 15, 35, 45–47, 49, 63, 69, 71, 73). Interestingly, a similar unusual cluster of genes that may be involved in pheromone signaling was recently reported in another opportunistic human fungal pathogen, *Pneumocystis carinii* (65). Genome sequencing revealed a locus that shares similarities with the mating-type locus of *C. neoformans* and contains genes encoding components of a putative pheromone response pathway (65). Whether this region represents a true mating-type locus of *P. carinii* is not known, and no sexual cycle has been described for...
this pathogenic fungus. The finding that the basidiomycete C. neoformans and the ascomycete P. carinii share similarly arranged mating-type loci raises the question of whether the MAT locus plays a role in the virulence of P. carinii, as has already been established for the MATα locus of C. neoformans.

Our studies reveal that three different types of mating-type loci exist in fungi. The first comprises the classical MAT loci of ascomycetes, in which mating type is determined by specialized transcription factors encoded by a single, compact locus. The second comprises the tetrapolr mating systems of the basidiomycetes, in which mating type is determined by two distinct, unlinked loci encoding transcriptional regulators and pheromone and pheromone receptor systems. The third is the novel mating-type locus in C. neoformans and a related region in P. carinii, in which mating-specific transcription factors, a pheromone and pheromone receptor system, and elements of the pheromone-activated MAP kinase cascade are part of a single, contiguous multigene locus. Because C. neoformans is a basidiomycete and P. carinii is an ascomycete, this class of MAT locus either evolved prior to the divergence of the two major fungal phyla or resulted from convergent evolution.

Unlike most model basidiomycetes but similar to C. neoformans, U. hordei has a bipolar mating system and recombination in the mating-type region is suppressed. Two opposite mating-type and pathogenicity alleles, MAT-1 and MAT-2, have been identified and have been found to span 500- and 460-kb regions, respectively (44). MAT-1 and MAT-2 include one locus encoding mating-type-specific transcription factors and a second locus containing tightly linked pheromone and receptor genes. Both loci reside on the same chromosome and are separated by 450 to 500 kb of intervening DNA in which recombination is suppressed. These findings explain at a molecular level how a tetrapolar mating system can be converted into a bipolar system by linking of the commonly found mating-type loci on a single chromosome and the involvement of mechanisms that suppress recombination across the intervening sequences (2, 44).

An interesting question is how recombination is suppressed across the MAT loci of C. neoformans and U. hordei. The sequence of the interval between the two loci of U. hordei is being determined and contains many repetitive sequences and transposable elements that may contribute to the suppression of recombination (J. Kronstad, personal communication). Our analysis of the C. neoformans mating-type alleles reveals two factors that may also play a role. First, mating-type-specific alleles of several genes vary from 5 to ~50% in sequence (46), and some genes are unique to one or the other allele. Second, gene positions in the mating-type alleles are extensively rearranged (including inversions). For example, the RPO41α and RPO41α genes are almost identical (97%) but are oriented in opposite directions in serotype D. Thus, crossover events between these two alleles would result in one acentric and one dicentric chromosome, both of which would be unstable. These sequence and structural differences likely prevent proper alignment of this chromosomal region during meiosis and thereby suppress recombination.

Interestingly, for the ascomycete Neurospora tetrasperma, genetic and cytological studies have shown that during meiosis the chromosomes containing the mating-type loci are unpaired over a large interval that includes the MAT locus, and recombination is suppressed in this region. In addition, specific sites flanking this region trigger recombination events that may function to ensure proper chromosome segregation during meiosis (26, 48). These observations suggest that the chromosomes containing the fungal MAT loci share features with mammalian sex chromosomes.

The mating-type-determining region of C. neoformans shares features with both the self-incompatibility locus that governs pollen recognition in species of the crucifer plant Brassica (50) and the mating-type locus of the green alga Chlamydomonas reinhardtii. For example, the multiallelic S locus in Brassica spp. is composed of divergent and rearranged sequences linking the SRK and SCR genes involved in pollen-stigma interactions (5). In Chlamydomonas, the mating-type locus is located in a region of ~830 kb in which recombination is suppressed (23). In addition, a 190-kb core region thought to contain the mating-type determining factors is highly rearranged via several translocations, inversions, duplications, and deletions (21–23). These chromosomal aberrations are thought to be responsible for suppressing recombination in the core region and the flanking 640 kb of genomic DNA. The mating-type region in C. reinhardtii is located close to one end of linkage group VI (23). This is similar to C. neoformans because analysis from the ongoing genome project reveals that the MAT locus resides ~170 kb from one telomere of this 1.8-Mb chromosome. Whether chromosomal location has any impact on the function of the mating-type loci in these organisms is not known, but it is interesting that the HML and HMR silent mating-type cassettes in S. cerevisiae are also located near the ends of yeast chromosome III.

Sex determination in higher eukaryotes is often accompanied by the presence of dimorphic sex chromosomes. An interesting model that explains the evolution of sex chromosomes is based on the initial requirement for genetic differences in multiple loci for the definition of sexual identity. Since the generation of self-fertile or sterile progeny is unfavorable, mechanisms had to evolve to ensure tight linkage between the genes involved (11, 12), possibly including the evolution of nonhomologous genes and chromosomal rearrangements. Once established, these mechanisms suppress the exchange of genetic material in these regions, and genetic divergence between genomic regions results in the evolution of a “diallelic” sex chromosome system. It has been proposed that animal sex chromosomes evolved from autosomes that were initially homologous except for a small sex-determining region (30). Following suppression of recombination in this region, subsequent divergence between the two “autosomes” resulted in the evolution of the sex chromosomes responsible for the hetero- (XY) and homogametic (XX) sexes in mammals (13, 43, 57, 58). The pseudoautosomal region on the mammalian Y chromosome may reflect its ancestral autosomal origin.

The ~1.8-Mb mating-type chromosome in C. neoformans shares features with mammalian sex chromosomes. Although the sex-determining region comprises only ~7% of this fungal chromosome, recombination is suppressed in the mating-type region but does occur in more distal regions of the chromosome. While recombination is suppressed between most of the sex chromosomes of mammals, recombination does occur in the pseudoautosomal region and is thought to be essential for proper chromosome segregation. Similar to mammalian sex
chromosomes, the MAT locus of *C. neoformans* is characterized by nonhomologous genes and extensive rearrangements. In addition, the lack of genetic exchange favors the accumulation of repetitive sequences and transposable elements within the sex-determining region, which favors intrachromosomal rearrangements and drives divergence. The MAT loci in the green alga *C. reinhardtii* and the fungi *T. thermophila*, *P. carinii*, and *U. hordei* all share similar features with sex chromosomes. Since sex is thought to have originally evolved in lower eukaryotes, such as yeasts and algae, it is intriguing that the sex-determining systems of several unicellular eukaryotes share features resembling an early step in the evolutionary pathway to the dimorphic sex chromosomes of multicellular eukaryotes.

**ACKNOWLEDGMENTS**

We thank Christina Hull, Robin Wharton, and John Perfect for advice and comments and Jim Kronstad for providing high-density BAC filter arrays and BAC data.

This study was supported by R01 grant AI51013 and P01 grant AI44975 (NIADDK) to the Duke mycology research unit. Joseph Heitman is a Burroughs Welcome Scholar in molecular pathogenic mycology and an associate investigator of the Howard Hughes Medical Institute.

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