

## Subtelomeric Silencing of the *MTL3* Locus of *Candida glabrata* Requires yKu70, yKu80, and Rif1 Proteins<sup>∇†</sup>

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*Candida glabrata* is a haploid opportunistic fungal pathogen that is phylogenetically related to *Saccharomyces cerevisiae*. Even though *C. glabrata* has no known sexual cycle, it contains, like *S. cerevisiae*, three mating type-like loci (*MTL*) called *MTL1*, *MTL2*, and *MTL3*, as well as most of the genes required for mating, meiosis, and sporulation. *MTL1* is localized at an internal position on chromosome B and is thought to be the locus corresponding to the *MAT* locus in *S. cerevisiae*. *MTL2* and *MTL3* are localized close to two telomeres on different chromosomes (29.4 kb from Chr E-L and 10.5 kb from Chr B-L, respectively). By using *URA3* reporter gene insertions at the three *MTL* loci, we found that in contrast to the case for *S. cerevisiae*, only *MTL3* is subject to transcriptional silencing while *MTL2* is transcriptionally active, and this is in agreement with previously reported data. We found that the silencing of *MTL3* is nucleated primarily at the left telomere of chromosome B and spreads over 12 kb to *MTL3*, rather than nucleating at flanking, closely positioned *cis*-acting silencers, like those flanking *HMR* and *HML* of *S. cerevisiae*. Interestingly, the silencing of *MTL3* absolutely requires the yKu70, yKu80, and Rif1 proteins, in sharp contrast to the silencing of the *HM* loci of *S. cerevisiae*. In addition, we found that several cell type-specific genes are expressed in *C. glabrata* regardless of the presence, or even absence, of mating type information at any of the *MTL* loci.

*Candida glabrata* is a haploid yeast found as a normal part of the mammalian microflora, but in recent years it also has emerged as a common opportunistic pathogen of humans, and now it accounts for about 12% of all *Candida* infections worldwide, second only to *Candida albicans* (29, 36, 37, 39, 50). *C. glabrata* and *Saccharomyces cerevisiae* are closely related phylogenetically, and both genomes conserve a high degree of synteny, whereas *C. albicans* is more distantly related (6, 14).

Sexual reproduction has been documented in many fungal species; however, several human fungal pathogens either do not reproduce sexually or very rarely do so (33). As the genomes of more fungal species are sequenced it has become clear, however, that the vast majority of the species have highly conserved genes involved in sexual reproduction, and yet some species have not been observed to mate (5, 6, 16). In some cases, a cryptic sexual cycle and even same-sex mating have been discovered recently, as is the case for *C. albicans* and *Aspergillus fumigatus* (1, 34). *C. glabrata* has no known sexual cycle even though it contains the vast majority of the genes required for mating (6, 14), and some mating type identity is maintained (32).

In many fungal species, mating and cell type identity are

determined by the information encoded by a locus called *MAT* (mating type) in the budding yeast *S. cerevisiae* or *MTL* (mating type like) in other fungi. The information contained in the *MAT* locus of *S. cerevisiae* can be either type **a** or type  $\alpha$  and consists of three transcriptional regulators that control the sexual cycle. Mating type **a** cells (*MATa* cells) contain the gene *a1*, and *MAT $\alpha$*  cells contain the genes  $\alpha1$  and  $\alpha2$ . Haploid *MATa* cells can mate only with cells carrying the *MAT $\alpha$*  information (and *vice versa*) to form diploid **a/ $\alpha$**  cells that are unable to mate (21, 26). In addition to the *MAT* locus, the *S. cerevisiae* chromosome III contains two silent loci, *HMR* and *HML*, located near each telomere (~22.7 and ~13.0 kb, respectively). The *HMR* and *HML* loci usually contain identical copies of *MATa* and *MAT $\alpha$* , respectively, that are normally maintained efficiently repressed through a chromatin-based mechanism called silencing (21, 44). The silencing of these loci (*HM*) is essential for maintaining cell type identity and the occurrence of a sexual cycle. The *HM* loci are each flanked by two small regulatory sites (~150 bp) called silencers, and each one contains binding sites for the proteins Rap1, the origin of recognition complex (ORC), and Abf1 in different combinations. These silencer-binding proteins recruit the four Sir proteins (Sir1 to -4) to nucleate silencing. Once the Sir proteins are at the silencer, Sir2, an NAD<sup>+</sup>-dependent histone deacetylase (25, 46), removes acetyl groups from the N-terminal tails of histones H3 and H4 of the adjacent nucleosomes.

The Rif1 and Rif2 proteins of *Saccharomyces cerevisiae* compete with Sir3 and Sir4 for Rap1 binding, and *S. cerevisiae rif1 $\Delta$*  mutants display increased silencing at subtelomeric regions and a modest decrease in silencing at the *HM* loci (20, 55). These phenotypes presumably are due to the titration of the

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available Sir proteins that are recruited efficiently by Rap1 to the longer telomeres that *rif1* $\Delta$  mutants display.

The Sir2-4 complex is absolutely required for the establishment and maintenance of silent chromatin, whereas Sir1 is not essential and contributes only modestly to establish the silent chromatin at the *HM* loci (17, 38, 44). It is thought that the yeast Ku complex (yKu70/80) is not required for silencing at the *HM* loci, although it is essential for silencing at the subtelomeric regions (4, 28, 43, 49). Recently, however, it was found that Sir1 has a small, redundant role with yKu70 and yKu80 for silencing at *HM* loci and that the yKu complex binds these loci in a process dependent on Sir4 (35, 53).

*C. glabrata*, like *S. cerevisiae*, contains three mating type-like loci called *MTL1*, *MTL2*, and *MTL3*, which correspond to *MAT*, *HMR*, and *HML*, respectively (47). Initially it was thought that only *MTL1* was transcriptionally active while *MTL2* and *MTL3* were maintained in a silent state, like *HMR* and *HML*. Recently, it was reported that unlike the case for *S. cerevisiae*, the *MTL2* locus is transcriptionally active and some cell type identity is maintained through the cell type-specific regulation of the expression of the  $\alpha 1$  and  $\alpha 2$  genes and the cell type-specific processing of the *a1* transcript, which contains two introns (32). In this regard it should be noted that *C. glabrata* does not have an *SIR1* orthologue (15), which in *S. cerevisiae* contributes to establishing the silent chromatin at the *HM* loci (38).

To better understand how *C. glabrata* achieves the mating type-specific expression of the  $\alpha$  genes and to determine unambiguously from which of the *MTL* loci the transcription of the *a1* gene comes in our *C. glabrata* strain, we constructed a collection of single-deletion mutants of each *MTL* locus, as well as all of the combinations of double mutants and the triple-deletion mutant, which contains no mating type information. By using these mutants and *URA3* reporter gene insertions throughout the three *MTL* loci, we found four major differences between *C. glabrata* and *S. cerevisiae* with respect to the regulation of the expression of mating type-specific genes. First, we found, in agreement with Muller et al. (32), that *MTL2*, unlike *HMR* in *S. cerevisiae*, is transcriptionally active. Second, *C. glabrata* does not seem to maintain cell type identity like *S. cerevisiae*. Third, *MTL3* is subject to subtelomeric silencing rather than silencing mediated by discrete, *cis*-acting silencer elements. Finally, we found that unlike silencing at *HML* of *S. cerevisiae*, *MTL3* silencing absolutely requires Rif1, yKu70, and yKu80 proteins. Taken together, our results highlight several critical differences between the regulation of the expression of the genes located at the *MTL* loci, as well as some genes involved in the maintenance of cell type identity and sexual reproduction, compared to that of *S. cerevisiae*.

#### MATERIALS AND METHODS

**Strains, plasmids, and primers.** All strains, plasmids, and primers used in the present study are listed in Tables S1, S2, and S3, respectively, in the supplemental material.

**Media.** Yeast cells were grown in standard yeast media as described previously (45), and 2% agar was added for plates. Synthetic complete (SC) medium contained 1.7 g/liter yeast nitrogen base [without (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and amino acids], 5 g/liter (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.6% Casamino Acids, and 2% glucose. Yeast extract-peptone-dextrose (YPD) medium contained 10 g/liter yeast extract, 20 g/liter peptone, 2% glucose, and 25 mg/liter uracil. When required, YPD plates were supplemented with 420  $\mu$ g/ml hygromycin (Invitrogen, Carlsbad, CA). To score

5-fluoroorotic acid resistance (5-FOA; Toronto Research Chemicals, North York, Canada), 0.9 g/liter 5-FOA and 25 mg/liter uracil were added to the SC plates (5-FOA plates).

Bacteria were grown in Luria-Bertani (LB) medium as described previously (2) with 1.5% agar added for plates. LB medium contained 5 g/liter yeast extract, 10 g/liter tryptone, and 5 g/liter NaCl, and when needed, it was supplemented with either 50  $\mu$ g/ml carbenicillin or 50  $\mu$ g/ml carbenicillin plus 30  $\mu$ g/ml kanamycin. All plasmid constructs were introduced into strain DH10B by electroporation.

**Cloning and sequencing of the three *MTL* loci of *C. glabrata*.** To clone the three mating type loci of our wild-type strain, BG14 (*ura3 $\Delta$ ::Tn903* G418<sup>r</sup>) (11), we amplified by PCR three different fragments encompassing the three regions where the *MTL* loci are located, using primers specific for each *MTL* locus containing added BamHI sites. These fragments were cloned into the cloning vector pAP502 (12) or pCR2.1 TOPO (Invitrogen, Carlsbad, CA) and sequenced.

Sequence analysis revealed that our wild-type strain (BG14) contains information at *MTL1* and *MTL2* and  $\alpha$  information at *MTL3*. The 2.997-kb PCR fragment containing the *MTL1* locus was amplified with primers 160 and 161 (see Table S3 in the supplemental material) and was cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) to generate pIC122. pIC122 then was digested with NcoI, blunted with Klenow fragment of DNA polymerase I, and then digested with MscI to delete most of the kanamycin resistance gene to generate pSD10. A 2.734-kb PCR fragment containing the *MTL2* locus was amplified with primers 2434 and 2435 (see Table S3) and was cloned into pAP502 (12) to generate pAP699. A 3.319-kb PCR fragment containing the *MTL3* locus was amplified with primers 2438 and 2439 (see Table S3) and was cloned into pAP502 to generate pAP695.

**Construction of *URA3* insertions throughout the three *MTL* loci.** To assess whether the *MTL* loci are transcriptionally silenced, we inserted a *URA3* reporter gene at different positions throughout the *MTL1*, *MTL2*, and *MTL3* loci using a modified Tn7 transposon carrying the *URA3* gene (9). For this, we mutagenized *in vitro* each of the plasmids containing each *MTL* locus (pSD10, pAP699, and pAP695) with Tn7 UKR as described previously (9). We obtained hundreds of independent insertions for each plasmid transformed in *Escherichia coli*. Insertions first were mapped by colony PCR to identify insertions within each *MTL* locus using primers that anneal with the ends of the Tn7 (oriented outwards) transposon and primers annealing at different positions within the cloned *MTL* fragment. In this way, we picked for further study a total of 5 independent *URA3* insertions throughout *MTL1* (pSD plasmids), 7 along *MTL2*, and 10 in the *MTL3* locus (pRZ plasmids). All of these insertions (a total of 22) were sequenced, and the results are described in Table S4 in the supplemental material.

All of the 22 insertions were introduced into the *C. glabrata* genome by homologous recombination at their respective loci by the one-step gene replacement method as described previously (10, 12). The homologous recombination of each insertion was verified by PCR using locus-specific genomic primers that hybridized outside the region cloned in the plasmids carrying the insertion and primers annealing to the ends of Tn7. Because the transposition of Tn7 occurs *in vitro* and subsequently selects transposition reactions in *E. coli*, only one Tn7 (*URA3*) insertion is obtained in *C. glabrata* after homologous recombination has occurred following transformation with linearized plasmids. Once it has recombined in *C. glabrata*, Tn7 is stable and does not transpose, since it does not contain the transposase genes. To show that *C. glabrata mtl::URA3* strains contain only one copy of the *URA3* gene at the indicated locus, we used three different insertions at each *MTL* locus and replaced the relevant *mtl::URA3* insertion with the hygromycin cassette we used to generate *mtl* $\Delta$  mutants. We used the knockout plasmids described in supplemental Table S2 (pSD7, pRZ15, and pRZ17) to transform the different *URA3* insertions and selected resistance to hygromycin. Correct transformants (where the hygromycin cassette was integrated by homologous recombination at each *MTL*) were screened for the *Ura*<sup>-</sup> phenotype. All of the correct Hyg<sup>r</sup> transformants were uracil auxotrophs and resistant to 5-FOA, indicating that *mtl1,2,3::URA3* insertions contain only one insertion at the indicated *MTL* locus (data not shown). We also performed Southern blotting with three *URA3* insertions at each *MTL* locus using an internal *URA3* fragment as the probe. Genomic DNA from each strain was digested with SspI, and 80  $\mu$ g of DNA was run in a 0.8% agarose gel and transferred to a Hybond membrane. The membrane was hybridized overnight with peroxidase-labeled probe for 12 h and washed under stringent conditions. The Southern blot for the nine insertions showed that there is only one band of the expected size corresponding to only one *URA3* insertion in each strain (data not shown).

**Reporter *URA3* gene expression assays (5-FOA sensitivity assays).** To assess the degree of silencing of the *URA3* gene inserted at different positions through-

out the *MTL* loci, we carried out 5-FOA growth assays as described previously (42). Briefly, strains containing the different *URA3* insertions along the *MTL* loci were grown in YPD to stationary phase for around 36 h. The cultures were adjusted to an optical density at 600 nm ( $OD_{600}$ ) of 1 with sterile water, and 10-fold serial dilutions were made in 96-well plates. Five  $\mu$ l of each dilution was spotted onto three different plates, YPD, SC without uracil (SC-ura), and SC plus 5-FOA, after which the plates were incubated at 30°C for 48 h and photographed.

**Generation of strains with deletion of silencing genes.** To generate derivatives with the deletion of silencing genes in strains with three representative *URA3* insertions along the *MTL3* silent locus, we followed the one-step gene replacement procedure using disruption plasmids for each gene to be deleted (*SIR2*, *SIR3*, *SIR4*, *HDF1*, *HDF2*, and *RIF1*) (42) (see Table S1 in the supplemental material). Disruption plasmids contain a hygromycin resistance cassette flanked by 5'- and 3'-flanking regions of each gene (all of the plasmids used for allele replacements are listed in Table S2 in the supplemental material). Each disruption plasmid was digested with enzymes that cut at both ends, within the 5' and 3' cloned flanking regions, generating ends homologous to each specific gene to be deleted in the *C. glabrata* genome (of each insertion strain to be deleted). The released fragment was used to transform *C. glabrata* selected on plates supplemented with 420  $\mu$ g/ml hygromycin. Homologous recombination at each locus was verified by PCR with a primer that anneals in the sequences external to the cloned 5' and 3' fragments and a primer annealing within the hygromycin cassette. We also verified the absence of each gene deleted by the inability to PCR amplify a small internal fragment from each deleted gene (<300 bp). Each of these derivatives was used for 5-FOA plate growth assays as described above.

**Construction of strains with deletions of two *MTL* loci.** To construct strains that carry only one type of mating information, we set out to construct strains containing deletions of two *MTL* loci (leaving only one *MTL* locus). The first deletion was generated by allele replacement as described above. To construct double or triple mutants, the hygromycin cassette used to generate the first deletion was excised by expressing *ScFLP1* product from plasmid pMZ18 (see Table S2 in the supplemental material). *ScFLP1* encodes a site-specific recombinase that recognizes two direct repeats, the Flp1 recognition target (FRT) sites that flank the hygromycin cassette of the disruption plasmids. The Flp1 recombinase then induces recombination between FRT sites and the hygromycin marker is excised from the chromosome, resulting in an unmarked deletion of the corresponding *MTL* locus and leaving one copy of the FRT site. Unmarked deletion strains are identified as Hyg<sup>r</sup> colonies on YPD hygromycin plates, and the deletion is confirmed by PCR using primers annealing in the chromosome outside the 5' and 3' regions used for the disruption.

The resulting Hyg<sup>r</sup> strains then are used as recipients for double or triple deletions using the same hygromycin resistance cassette and selecting for Hyg<sup>r</sup> colonies.

**RT-PCR.** RNA was extracted from stationary-phase cells (36 h in YPD) using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and treated with DNase I (Sigma-Aldrich, St. Louis, MO). The synthesis of cDNA and PCR was carried out using the AccessQuick reverse transcription-PCR (RT-PCR) system (Promega, Madison, WI). The RT primers used for each gene are listed in Table S3 in the supplemental material. The cDNA synthesis reaction was carried out at 45°C for 45 min for all of the genes. The PCR was carried out at 57°C for *a1*, *STE3* (CAGL0M08184g), *STE12* (CAGL0H02154g), *STE20* (CAGL0K02673g), and *ACT1* (CAGL0K12694g); 53°C for *MF(ALPHA)* (CAGL0H03135g) and *HOG1* (CAGL0M11748g); 55°C for *MFA1* (CAGL0C01919g); 50°C for  $\alpha 1$  (CAGL0B1243g),  $\alpha 2$  (CAGL0B1265g), and *STE2* (CAGL0K12430g); and 58°C for *BARI* (CAGL0J02288g) and *HO* (CAGL0G05423g). A reaction mixture without reverse transcriptase was included as a negative control in all of the RNA samples and with every pair of primers. No bands were obtained, indicating that the RNA preparations had no DNA contamination.

## RESULTS

**The *MTL3* locus is the only silent mating type-like locus in *C. glabrata*.** *C. glabrata* possesses three *MTL* loci arranged in a configuration similar to that of the mating type loci of *Saccharomyces cerevisiae* (47). *MTL1* is the only one localized at an internal position within chromosome B (approximately 113 kb from the left telomere of this chromosome), while *MTL2* and *MTL3* loci are located close to two telomeres, at approximately 29.4 and 10.5 kb from the left telomeres of chromosomes E

(Chr E-L) and B (Chr B-L), respectively (Fig. 1A). We have shown previously that genes in subtelomeric regions of several chromosomes in *C. glabrata* are subject to chromatin-based transcriptional silencing in a process that depends on Sir2, Sir3, Sir4, Rap1, the Ku70/80 complex, and Rif1 (10, 12). To assess whether *MTL2* and *MTL3* are subject to silencing, we constructed strains containing *URA3* reporter gene insertions at different positions throughout the three *MTL* loci; the precise insertion site for each one is described in Table S4 in the supplemental material. Strains containing *URA3* insertions were plated on medium containing the drug 5-fluoroorotic acid (5-FOA) and on plates lacking uracil (SC-ura plates) to assay for cells expressing *URA3*. The amount of growth on medium containing 5-FOA reflects the extent of the transcriptional silencing of *URA3*.

As shown in Fig. 1B, we isolated five *URA3* reporter insertions throughout the *MTL1* locus. As expected, all of the insertions at *MTL1* are transcriptionally active as measured by the lack of growth on 5-FOA plates and robust growth on SC-ura plates. The parental strain (BG14 *ura3Δ::Tn903*), used as a control, is not able to grow in the absence of uracil, but it grows well on plates containing 5-FOA. Unlike what has been reported for *S. cerevisiae* (19, 40, 48), seven different *URA3* reporter insertions along the *MTL2* locus (homologous to *HMR*) show that the chromatin in this locus is transcriptionally active, as evidenced by their ability to grow on SC-ura plates and the failure to do so on 5-FOA plates (Fig. 1C). *MTL2* has been reported to be transcriptionally active in *C. glabrata* as assayed by the quantitative PCR of the *a1* gene in several strains (32).

At the *MTL3* locus, however, the first eight *URA3* reporter gene insertions throughout the locus are subject to transcriptional silencing, as shown by the robust growth on 5-FOA plates (Fig. 1D). A significant proportion of the cells from cultures containing these reporter insertions at *MTL3* also are able to grow on media lacking uracil, indicating the expression of *URA3* in these cells. The leakiness of the silencing at *MTL3* contrasts sharply with the silencing observed at the homologous *HML* locus of *S. cerevisiae*, where no expression of reporter genes introduced at this site is detected (3, 40). The last two insertions in *MTL3*, located at the C-terminal end or downstream from the  $\alpha 2$  gene, show a decreased level of silencing (Fig. 1D).

**Native genes contained at *MTL3* are not expressed, while genes at *MTL1* and *MTL2* are expressed.** The complete processing of the *a1* gene is achieved only when transcribed from *MTL1*. To determine whether the native *a1*,  $\alpha 1$ , and  $\alpha 2$  genes from the three *MTL* loci are expressed, we constructed a series of null mutants in each *MTL* locus, all of the combinations of double mutants and the triple knockout (Fig. 2A), and performed RT-PCR experiments on all of them. As shown in Fig. 2B,  $\alpha 1$  and  $\alpha 2$  genes are not expressed under the conditions tested in any of the strains derived from the BG14 background (*MTL1a*, *MTL2a*, and *MTL3 $\alpha$* ), where  $\alpha$  information comes from *MTL3* only (but see below). However, a transcript is clearly detectable from the reference, sequenced strain ATCC2001 (CBS138), where the  $\alpha$  genes are located at both *MTL1* and *MTL3* (<http://www.genolevures.org/cagl.html>). In our strain background, the *a1* gene, which contains two introns, is expressed from both loci, *MTL1a* and *MTL2a*, but it is



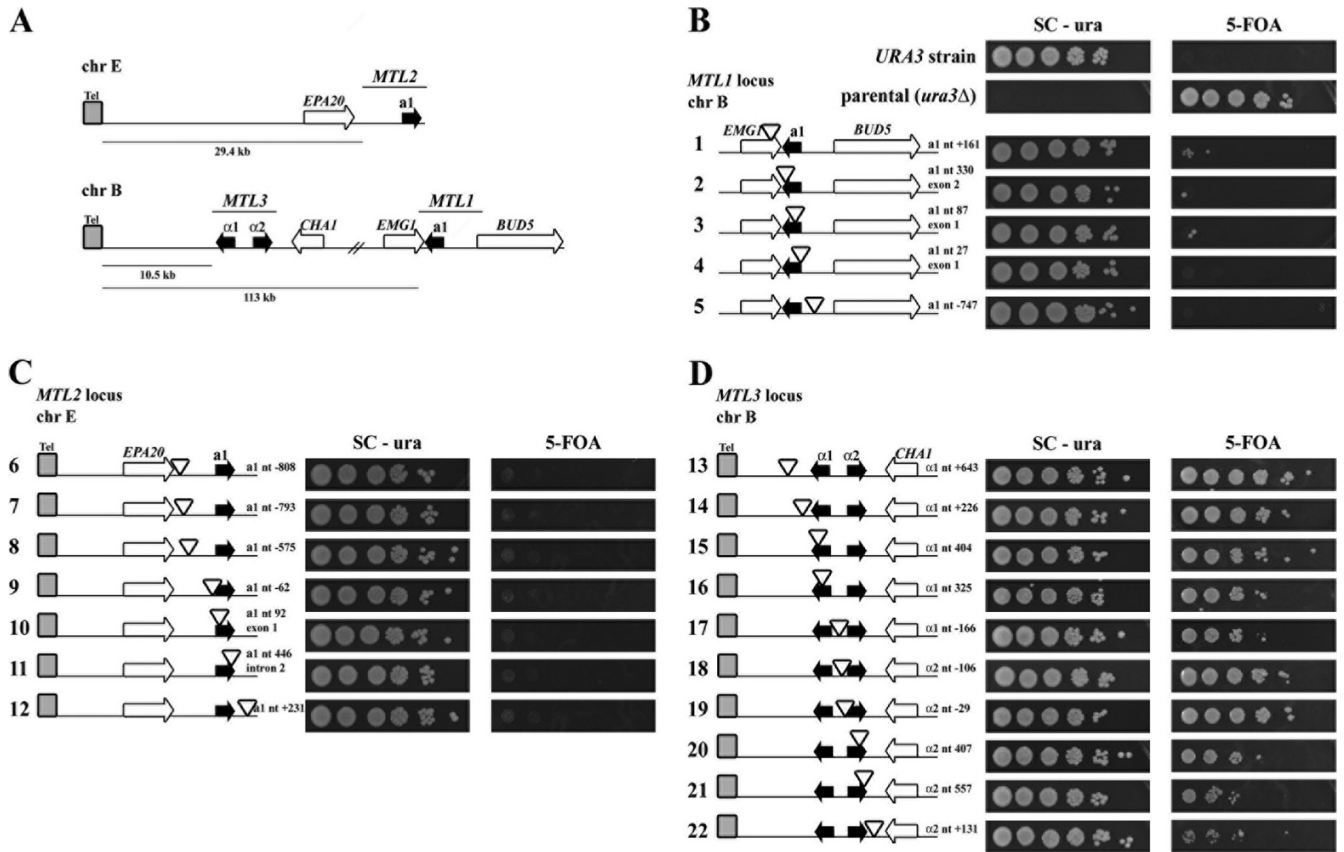


FIG. 1. *MTL3* is the only *MTL* locus subject to silencing in *Candida glabrata*. (A) Schematic representation of the *MTL1*, *MTL2*, and *MTL3* loci of *C. glabrata*. *MTL1* and *MTL3* are located on chromosome B. *MTL3* is placed 10.5 kb away from the left telomere of chromosome B (ChrB-L), and *MTL1* is at an internal position on this chromosome (113 kb away from ChrB-L). *MTL2* is located 29.4 kb from the left telomere of chromosome E (ChrE-L). (B, C, and D) Plate growth assay to determine the expression of the *URA3* reporter gene (shown as empty triangles) inserted across the three *MTL* loci in *C. glabrata*. To the left of each panel is shown a schematic representation of each *MTL* locus and the relative positions of the reporter gene insertions with respect to the *a1*,  $\alpha 1$ , or  $\alpha 2$  gene (represented by black arrows that indicate the direction of transcription). The exact position within the *a1*,  $\alpha 1$ , or  $\alpha 2$  gene of each insertion is indicated. Strains of *C. glabrata* containing 5 *URA3* insertions throughout the *MTL1* locus (B), 7 in the *MTL2* locus (C), or 10 in the *MTL3* locus (D) were grown to stationary phase in YPD, and 10-fold serial dilutions in sterile water were made. Equal numbers of cells of each dilution were spotted onto SC-ura and SC plates containing 5-FOA. Plates were incubated at 30°C for 48 h and photographed.

processed (both partially and completely) only when the transcript is expressed from *MTL1*. Figure 2B, lanes 2 and 6, shows that for strains *mtl1* $\Delta$  and (*mtl1,mtl3*) $\Delta$ , where *a1* is transcribed only from *MTL2a*, only the unprocessed form of the *a1* transcript is detected, while in strains *mtl2* $\Delta$  and (*mtl2,mtl3*) $\Delta$ , where *a1* is transcribed only from *MTL1*, the partially and totally processed forms can be seen (Fig. 2B, lanes 3 and 7). In the CBS138 strain, only the unprocessed form of the *a1* transcript is detected, since in this strain type *a* information is present only at the *MTL2* locus (Fig. 2B, lane 9). These results are in agreement with previously reported data for *C. glabrata* (32) and indicate that in contrast to *S. cerevisiae*, *C. glabrata* does not maintain repression at *MTL2*, which is the orthologue of *HMR*.

***C. glabrata* cells containing different mating type information at the *MTL* loci express both *a*-specific and  $\alpha$ -specific genes.** Because of the imperfect silencing at *MTL3*, it is possible that some cells containing *a* information at *MTL1* and  $\alpha$  at *MTL3* express both types of mating information (RT-PCR using increased number of cycles shows detectable  $\alpha 1$  and  $\alpha 2$

transcripts from *MTL3*; data not shown). It also is possible that a very small fraction of the *a1* transcript expressed from *MTL2* is processed correctly, such that in cells containing  $\alpha$  information at *MTL1* there would be the expression of both types of mating information in a given cell. To maintain cell type identity in *S. cerevisiae*, *MATa* or *MAT $\alpha$*  cells use a regulatory circuit controlled by the mating type genes *a1*,  $\alpha 1$ , and  $\alpha 2$  in which *a* cells express specific genes present only in *MATa* cells (*a*-specific genes, or *asg*) and *MAT $\alpha$*  cells express a set of genes present only in these cells ( $\alpha$ -specific genes, or  $\alpha$ *sg*). In diploid cells (after mating; *MATa*/ $\alpha$  cells), a heterodimer repressor is made consisting of the *a1*/ $\alpha 2$  proteins that represses the transcription of several genes expressed only in haploid cells (haploid-specific genes, or *hsg*). We asked whether some of the known *asg*,  $\alpha$ *sg*, or *hsg* in *S. cerevisiae* are regulated in the same way in *C. glabrata*. For this, we used the strains containing all of the deletion combinations of the *MTL* loci, so that there are strains that contain only one type of information. We performed RT-PCR for three orthologues of the *S. cerevisiae* *asg* (*STE2*, *MFA1*, and *BARI*), two of the  $\alpha$ *sg* [*STE3*, *MF(ALPHA)*], and four

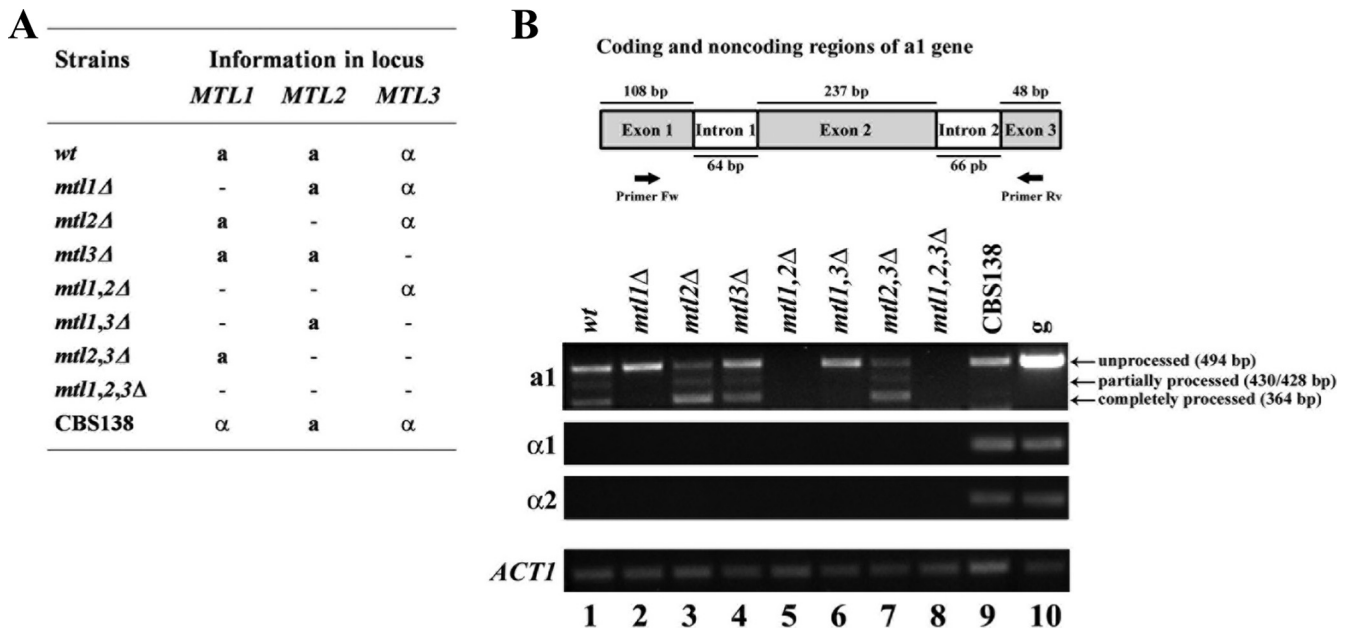


FIG. 2. *a1* transcript, which contains two introns, is processed only when transcription comes from *MTL1* but not when it is initiated at *MTL2*. (A) Description of the mating type information present at each *MTL* locus in the collection of strains containing single, double, and triple deletion mutations in the *MTL* loci. (B) Locus-specific processing of the *a1* transcript. Top panel, schematic representation of the *a1* gene showing the two introns and the positions of the primers used for the PCR (black arrows). Bottom panel, the indicated strains containing deletions in the *MTL* loci were grown to stationary phase and RNA was extracted and used for RT-PCR (see Materials and Methods). *a1* transcript was detected using primers indicated for RT-PCR in the top panel. The unprocessed transcript is 494 bp long, the partially processed transcripts are 430 and 428 bp long, and the completely processed transcript is 364 bp long. Note that the complete processing of the *a1* gene can be detected only when transcription comes from the *MTL1* locus [lanes 3 and 7; *mtl2Δ* and (*mtl2, mtl3Δ*)] but not when transcription is initiated at *MTL2* [lanes 2 and 6; *mtl1Δ* and (*mtl1, mtl3Δ*)]. Strains containing deletions of both *MTL1* and *MTL2* [lanes 5 and 8; (*mtl1, mtl2Δ*) and (*mtl1, mtl2, mtl3Δ*)] were used as negative controls, since there is no *a1* gene. Lane 9, RNA from strain CBS138 (reference strain ATCC2001 containing α information in *MTL1*) was used as a positive control for the transcription of the *α1* or *α2* gene. Lane 10, genomic DNA from the wild-type strain (g) was used as a positive control for PCR. *ACT1* RT-PCR was used as an internal control. Negative control experiments where no reverse transcriptase was added did not amplify any bands (data not shown).

of the *hsg* (*STE12*, *STE20*, *HOG1*, and *HO*). Figure 3 shows that all of the genes we tested are expressed at relatively moderate levels in all of the *MTL* mutant strains, including the strain that contains no mating type information [*mtl(1,2,3)Δ*] (Fig. 3, lane 8) and also the parental strain and CBS138 that contain a and α information, respectively, at *MTL1*. This suggests that *C. glabrata* does not keep a cell type identity, as has been described for *S. cerevisiae*.

**Silencing at the *MTL3* locus requires Sir2, Sir3, Sir4, yKu70, yKu80, and Rif1.** The yKu70 and yKu80 proteins are absolutely required for silencing at the subtelomeric regions in *S. cerevisiae* (telomere position effect, or TPE) (44), while at the silent *HM* loci these proteins play only a minor role, performing an overlapping function with Sir1 at establishing the silent chromatin (35, 53).

Silencing at the subtelomeric regions of *C. glabrata* requires the Sir proteins (Sir2-4), Rap1, yKu70, and yKu80 and Rif1 (10, 12). To determine whether the silencing proteins also are required for silencing at *MTL3*, we made deletion/insertion mutations in each of the *SIR2*, *SIR3*, *SIR4*, *HDF1*, *HDF2*, and *RIF1* genes in three different *C. glabrata* strains containing the *URA3* reporter insertions at representative locations within *MTL3* (Fig. 4). As expected, the silencing of the reporter *URA3* at the three positions tested in *MTL3* absolutely requires the Sir2 to -4 proteins, as measured by the lack of growth on

5-FOA plates (Fig. 4A to C). Surprisingly, silencing at this locus also requires yKu70, yKu80, and Rif1 proteins (Fig. 4A to C). We determined by RT-PCR that the expression of the native genes *α1* and *α2* in each of the mutants in silencing proteins is strongly derepressed (Fig. 4D).

In *S. cerevisiae* the silencers flanking the silent mating loci contain binding sites for Rap1, ORC, and Abf1. These silencers are not conserved in *MTL2* and *MTL3* of *C. glabrata* (although there are two conserved putative Abf1 binding sites flanking *MTL3* as well as one ORC putative binding site). The absence of conserved silencers at *MTL3* in *C. glabrata* could explain the leaky silencing observed that depends on the nucleation and spreading of the silent chromatin from the telomere.

**Silencing at *MTL3* is nucleated predominantly at the telomere and spreads through the *MTL3* locus.** The dependence of silencing at the *MTL3* locus on yKu70, yKu80, and Rif1 is reminiscent of the subtelomeric silencing we have observed at other telomeres in *C. glabrata* (10, 12, 42). To determine whether silencing at *MTL3* is nucleated at the left telomere of chromosome B (which is only 10.5 kb away) and then propagates into the *MTL3* locus, we introduced this locus carrying each of the three representative *URA3* insertions into a region away from any telomere. We chose a large, internal, intergenic region 224 kb away from the left telomere of chromosome L

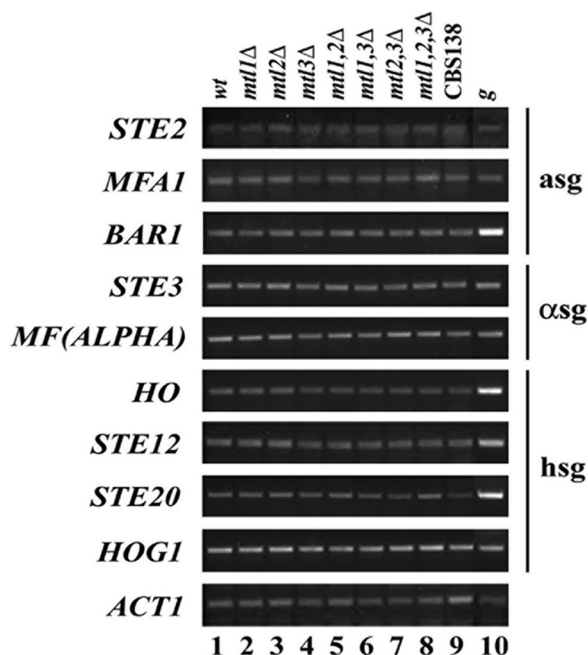


FIG. 3. Analysis of the expression of several cell type-specific genes by RT-PCR. Genes analyzed are *STE2*, *MFA1*, and *BARI* as examples of  $\alpha$ -specific genes (asg); *STE3* and *MF(ALPHA)* are  $\alpha$ -specific genes ( $\alpha$ sg), and *HO*, *STE12*, *STE20*, and *HOG1* are haploid-specific genes (hsg). RNA was extracted from stationary-phase cultures of the collection of single, double, and triple mutants in the *MTL* loci as indicated. *C. glabrata* strain CBS138 (ATCC2001) was used as a positive control for the expression of  $\alpha$ -specific genes. *ACT1* RT-PCR was used as an internal control and genomic DNA from the wild-type strain (g) as a positive control for the PCR. Negative-control experiments where no reverse transcriptase was added did not amplify any bands (data not shown).

that is not subject to silencing to integrate the entire *MTL3* locus with the reporter insertions. These constructs consist of a 3.313-kb fragment (1.262 kb downstream from  $\alpha 1$  and 0.531 kb downstream from  $\alpha 2$ ) containing the *MTL3* locus as well as the flanking sequences containing the conserved putative Abf1 and ORC binding sites and each of the three *URA3* reporter insertions. As shown in Fig. 5A to C, moving *MTL3* away from the left telomere of chromosome B, where it normally resides, into an internal location of chromosome L results in an almost-complete loss of silencing as measured by the lack of growth on 5-FOA plates (compare growth on 5-FOA of strains with *URA3* insertions at chromosome L with that of their counterpart strains with *URA3* insertions integrated at the subtelomeric region of chromosome B). The fact that there is no silencing of the *MTL3::URA3* reporter system when moved to a nonsubtelomeric region suggests that the flanking regions of *MTL3* do not contain a *cis*-acting silencer and that the silencing observed at this locus is nucleated mainly at the telomere and propagates into *MTL3* from there. In agreement with the expression of the reporter *URA3* at this location, we could detect the expression of the  $\alpha 1$  and  $\alpha 2$  genes at this internal position (Fig. 5D).

## DISCUSSION

The occurrence of a sexual cycle in human pathogenic fungi is uncommon, and it has been suggested that mating sorts out

specific combinations of genes required for virulence or survival in the host (33). However, in several cases of human fungal pathogens, a cryptic sexual cycle has been found. One example is the obligate diploid *Candida albicans*, where the vast majority of strains are heterozygous at the mating type-like locus (*MTL*) containing both types of information ( $\alpha/\alpha$ ). In this human pathogen, mating was shown to occur if two requirements are fulfilled: first, the information present at the *MTL* locus needs to be homozygous (cell expressing either  $\alpha/\alpha$  or  $\alpha/\alpha$  information), and second, cells must switch to the mating-competent type or opaque cell type. Under these conditions, a parasexual cycle can be detected where opaque cells expressing only one type of information can mate with another opaque cell expressing only the opposite type of information (23, 24, 30, 31). Furthermore, it was shown recently that *C. albicans* also can undergo same-sex mating between homozygous strains in a way similar to what has been reported for some other medically relevant fungi (1).

*Candida glabrata* has never been shown to mate, and all isolates analyzed to date are haploids. Using a population genetics-based analysis it was determined that the population structure is predominantly clonal, with some evidence for recombination (13).

The genes that control mating and cell type identity in fungi are in the *MAT* locus in *S. cerevisiae* and *MTL* in other fungi. *C. glabrata*, like *S. cerevisiae*, contains three mating type loci, *MTL1*, *MTL2*, and *MTL3*, and only *MTL1* was thought to be transcriptionally active, corresponding to the functional homologue of the *MAT* locus, so that the information present at *MTL1* is thought to determine the mating type of the cell (32, 47). In a recent report, Muller et al. (32) found that  $\alpha 1$  and  $\alpha 2$  are expressed in a mating type-specific way while  $\alpha 1$  is transcribed in both mating types, and mating type identity was proposed to be maintained in *C. glabrata* through the mating type-specific processing of the  $\alpha 1$  gene (32). In this work, we looked at the transcriptional activity of the three *MTL* loci and determined that *MTL1* and *MTL2* are expressed and only *MTL3* is silenced; in fact, *MTL3 $\alpha$*  (an orthologue of *HMR $\alpha$* ) is imperfectly silenced in *C. glabrata*, most likely through silencing nucleated at the telomere that propagates more than 11 kb to *MTL3*. Surprisingly, the silencing of *MTL3 $\alpha$*  absolutely depends on the silencing proteins yKu70, yKu80, and Rif1p. In *S. cerevisiae*, these proteins do not have a predominant role in the silencing of the mating type silent cassettes, and only in the absence of Sir1 could a small effect of the Ku proteins in silencing the *HM* loci be detected (35, 53).

***C. glabrata* *MTL1a* and *MTL2a* loci are transcriptionally active.** By introducing multiple insertions of the reporter *URA3* gene at various positions along *MTL1* and *MTL2* loci, we found, in agreement with Muller et al. (32), that both *MTL1a* and *MTL2a* (the functional homologue of *HMRa*) are transcriptionally active (Fig. 1B and C). This is in sharp contrast to *S. cerevisiae*, where both *HMRa* and *HML $\alpha$*  are very efficiently silenced (3, 22, 27).

**Locus-specific processing of the  $\alpha 1$  transcript.** By using a collection of single, double, and triple *MTL* deletion strains to unambiguously determine whether the transcription of the native  $\alpha 1$  gene comes from *MTL1* or *MTL2* (since both contain this gene), we determined that this gene is transcribed from both loci, but there is a locus-specific processing of the  $\alpha 1$



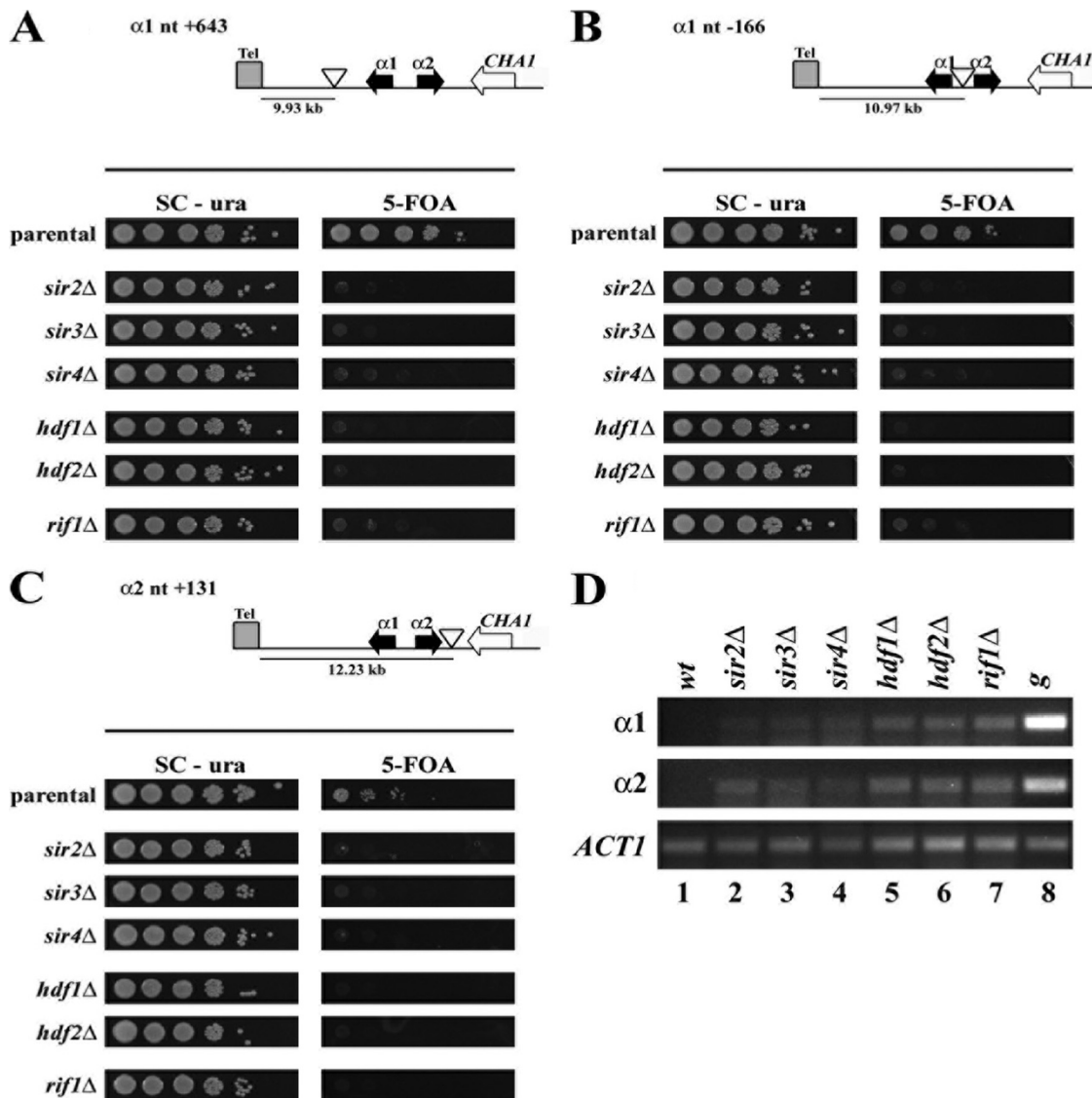


FIG. 4. Silencing at *MTL3* depends on the silencing proteins Sir2, Sir3, Sir4, yKu70, yKu80, and Rif1. The top parts of A, B, and C show three representative *URA3* insertions across the *MTL3* locus (indicated by empty triangles) that were selected to be introduced independently in each one of the deletion/insertion mutations in *SIR2*, *SIR3*, *SIR4*, *HDF1* (yKu70), *HDF2* (yKu80), and *RIF1*. Stationary-phase cultures of strains of *C. glabrata* containing the shown *URA3* insertions and the indicated deletion alleles, *sir2* $\Delta$ , *sir3* $\Delta$ , *sir4* $\Delta$ , *hdf1* $\Delta$ , *hdf2* $\Delta$ , and *rif1* $\Delta$ , were diluted in sterile water. Equal numbers of cells of each dilution were spotted onto SC-ura and SC plates containing 5-FOA. Plates were incubated at 30°C for 48 h and photographed. (A) Strains of *C. glabrata* containing the indicated silencing mutations and the *URA3* insertion closest to the ChrB-L telomere (9.93 kb from the telomere repeats). (B) Strains containing the indicated silencing mutations and the *URA3* insertion between  $\alpha 1$  and  $\alpha 2$  of *MTL3* (10.97 kb from the telomere). (C) Strains containing the indicated silencing mutations and the *URA3* insertion farthest away from the *MTL3* telomere (12.23 kb). (D) Analysis of the expression of  $\alpha 1$  and  $\alpha 2$  genes from *MTL3* by RT-PCR. Strains containing deletion alleles in *SIR2*, *SIR3*, *SIR4*, *HDF1* (yKu70), *HDF2* (yKu80), and *RIF1* were grown to stationary phase, and RNA was extracted to perform RT-PCR. *ACT1* RT-PCR was used as an internal control. Control experiments where no reverse transcriptase was added did not amplify any bands (not shown). Genomic DNA from the wild-type strain (g) was used as a positive control for the PCR (lane 8).

transcript. When the transcription of *a1* comes from *MTL2*, as in strains *mtl1* $\Delta$  and (*mtl1*, *mtl3*) $\Delta$  (Fig. 2B, lanes 2 and 6), we cannot detect the processing of the transcript, whereas when transcription comes from *MTL1*, as in strains *mtl2* $\Delta$  and (*mtl2*, *mtl3*) $\Delta$  (Fig. 2B, lanes 3 and 7), we can detect both the fully processed transcripts and the intermediates of the partially processed transcript. These results are consistent with previously reported data and are thought to demonstrate the mechanism by which *C. glabrata* maintains some cell type identity

(32). The sequences of both *a1* genes at *MTL1* and *MTL2* loci in our background are identical for up to 500 bp on the 5'-flanking sequence, except for two mismatches at positions -375 and -360 from the start codon. At the 3' region, the identity extends for 370 bp downstream from the stop codon; further upstream or downstream the sequences are completely different. It is possible that the two differences in the 5' region or the sequences upstream from -500 bp or downstream from +370 lead to differential splicing. Another possibility is that

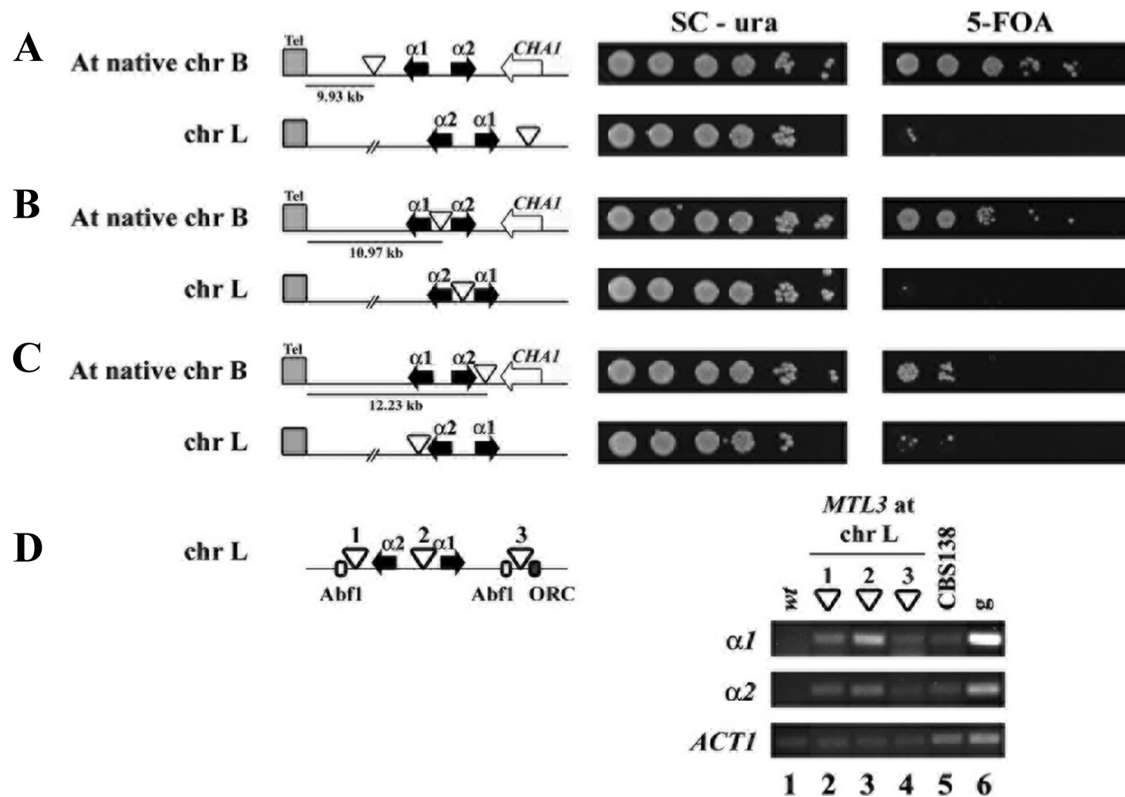


FIG. 5. Silencing at *MTL3* requires the telomere context. The entire *MTL3* region containing the three representative *URA3* insertions at *MTL3* and the flanking sequences that contain Abf1 and ORC putative binding sites (indicated by white and black small rectangles, respectively) were integrated at an internal, large intergenic region in chromosome L, and silencing at these positions was assayed by the plate growth assay as described for Fig. 1. (A) Silencing of the *URA3* insertion closest to the telomere, at its native location 9.93 kb from the left telomere of chromosome B (top row) and when integrated at an internal position on chromosome L (bottom row). (B) Silencing of the *URA3* insertion between  $\alpha 1$  and  $\alpha 2$  genes, at its native location 10.97 kb from the left telomere of chromosome B (top row) and when integrated at an internal position on chromosome L (bottom row). (C) Silencing of the *URA3* insertion farthest from the telomere at its native site 12.23 kb from the left telomere of chromosome B (top row) and when integrated at an internal position on chromosome L (bottom row). (D) Expression measured by RT-PCR of  $\alpha 1$  and  $\alpha 2$  genes from *MTL3* when inserted at an internal location on chromosome L.

there are as-yet-unidentified mechanisms, possibly chromatin based or others, that detect where the  $\alpha 1$  transcript originates and only process it when it comes from *MTL1*, but the identification of this regulation needs further studies.

***C. glabrata* cells express a-specific,  $\alpha$ -specific, and haploid-specific genes regardless of the mating type information present at *MTL* loci.** Surprisingly, we found that unlike *S. cerevisiae*, *C. glabrata* expresses at least some of the genes that are cell type specific in *S. cerevisiae*. For example, three *S. cerevisiae* a-specific genes (*STE2*, *MFA1*, and *BARI*) as well as two of the  $\alpha$ -specific genes [*STE3* and *MF(ALPHA)*] are expressed at moderate levels in *C. glabrata* independently of the presence or absence of mating type information at any of the *MTL* loci (Fig. 3). The transcription of four of the *S. cerevisiae* haploid-specific genes (*STE12*, *STE20*, *HOG1*, and *HO*) (18, 26) also was detected in all of the strains tested, including the triple mutant strain (*mtl1*, *mtl2*, *mtl3*) $\Delta$  (Fig. 3, lane 8). This suggests that *C. glabrata* does not regulate the expression of cell type-specific genes in the same way that *S. cerevisiae* regulates these genes, and this probably leads to a lack of cell type identity of *C. glabrata*. This is also different from *C. albicans*, where the expression of a-specific genes is detected only when a cells are induced with alpha pheromone (51), while in *C.*

*glabrata* expression is detected in the absence of pheromone and in all *mtl* mutant strains. In this regard, both *MFA1* and *BARI* promoter regions contain relatively conserved putative Mcm1 and  $\alpha 2$  recognition sequences while *STE2* is less conserved, and the spacing differs from that of *S. cerevisiae* (52). For the  $\alpha$ sg, the promoter region of *STE3*, but not *MF(ALPHA)*, contains putative Mcm1 and  $\alpha 1$  recognition sequences.

Genes negatively regulated by the  $\alpha 1/\alpha 2$  heterodimer are a subset of the haploid-specific genes in *S. cerevisiae*, and of the four hsg we assayed, only the *HO* promoter region contains an  $\alpha 1/\alpha 2$  heterodimer recognition sequence. The result, however, is the same for all of the genes we tested, since all were expressed at moderate levels in all of the strains, regardless of the information at the *MTL* loci and of the presence or absence of conserved binding sites for the different transcription factors. It is not known if the  $\alpha 1$ ,  $\alpha 1$ , and  $\alpha 2$  proteins are functional in *C. glabrata*, or whether they control a different set of genes. It is possible that *C. glabrata* has undergone a rewiring of the transcriptional regulators of the mating pathway, and that they do not regulate sexual reproduction or cell type identity genes. It also should be noted that both  $\alpha 1$  and  $\alpha 2$  are



not closely conserved, sharing only 27 and 38% identity, respectively, across the entire lengths of the proteins.

***MTL3* locus is subject to subtelomeric silencing that depends on yKu70, yKu80, and Rif1.** Unlike *MTL2*, *MTL3* is subject to transcriptional silencing as shown by growth on 5-FOA plates of all the strains with the insertions we isolated across the entire locus (Fig. 1D). The degree of silencing decreases as the distance between the *URA3* insertions and the telomere increases (the farthest insertion is located 12.233 kb from the telomere [insertion no. 22; Fig. 1D, last row]). Relieving subtelomeric silencing by making deletions of each of the genes coding for silencing proteins results in the expression not only of the reporter *URA3* gene but also of the native  $\alpha 1$  and  $\alpha 2$  genes at *MTL3* (Fig. 4D).

It should be noted that in *S. cerevisiae*, the functional orthologous locus *HML $\alpha$*  is very efficiently silenced in a process depending on Sir1 to -4, Rap1, ORC, and Abf1, but not on the yKu proteins (4, 28, 43, 49). However, a small effect of yKu70 and yKu80 on both of the *HM* silent loci that is redundant with Sir1 recently was discovered in *S. cerevisiae* (35, 53). Rif1 also was shown to contribute to silencing but only at a weakened *hmr $\Delta$ A* silencer (20, 55).

In contrast, in *C. glabrata* the silencing of *MTL3* absolutely requires Sir2 to -4, Rif1, yKu70, and yKu80 (Fig. 4). In this regard, silencing at *MTL3* is reminiscent of the silencing we have found at telomeres Chr C-L and Chr I-R, in which silencing appears to be nucleated at the telomere and propagates over long distances to some *EPA* genes (like *EPA7*, *EPA4*, and *EPA5*). A consequence of this type of silencing is that *MTL3* is not as efficiently silenced as the *HM* loci of *S. cerevisiae*, resulting in a sizable proportion of the population in each culture expressing, at least transiently, the reporter gene (Fig. 1). Sir1 is required for the establishment of silencing at the *HM* loci in *S. cerevisiae*, and *C. glabrata* does not contain the *SIR1* gene (15). This could explain in part the lack of silencing at *MTL2* and the imperfect silencing at *MTL3*. In contrast, silencing at the *HM* loci in *S. cerevisiae* is nucleated at discrete silencers flanking each locus that depend on Rap1, ORC, and Abf1 but not on the yKu proteins. At *MTL3* in *C. glabrata*, however, the sequences of the silencers are not conserved, although there are two putative Abf1 binding sites flanking  $\alpha 1$  and  $\alpha 2$  genes and an ORC consensus site downstream from  $\alpha 1$ . These conserved, putative binding sites, however, are not sufficient to mediate the silencing of the  $\alpha 1$  and  $\alpha 2$  genes independently of the telomere, since when moving three representative *URA3* reporter insertions in the *MTL3* locus (along with its flanking regions and putative binding sites for Abf1 and ORC) to an internal, intergenic region in chromosome L (200 kb away from the telomere), both the *URA3* reporter and  $\alpha 1$  and  $\alpha 2$  genes are expressed (Fig. 5A, B, C and D).

The leaky silencing of *MTL3* could have implications in *C. glabrata*, since strains containing *a1* at *MTL1* could form the *a1*/ $\alpha 2$  heterodimer in at least some cells in any given population. This could lead to the repression of several genes if, in *C. glabrata*, this heterodimer functions in the same way as it does in *S. cerevisiae*, where it is a repressor of many haploid-specific genes. We are investigating this possibility.

Taken together, our results highlight some important differences between *C. glabrata* and *S. cerevisiae* in recruiting silencing machinery to their respective mating loci. There are dif-

ferences in the expression of cell type-specific genes as well that could explain in part the apparent absence of cell type identity and sexual reproduction in *C. glabrata*.

It has been proposed that many human fungal pathogens rarely undergo sexual reproduction, which possibly gives them an advantage to proliferate in the host, but under stressful conditions, a sexual or parasexual cycle can be achieved, allowing for adaptation to hostile environments (33). Genomic data from many fungal species shows that the vast majority of them have conserved almost all the genes needed to reproduce sexually (5, 6), even in those species where no sexual cycle has been found. Although it is not known why they have kept all of these genes, it is possible that some of them have been rewired in the asexual pathogenic species and now play an additional role important to their survival as commensals or pathogens in their hosts. For example, it has been reported that in *C. albicans*, *C. glabrata*, and *C. neoformans* some components of the pheromone response pathway play a role in the virulence of these pathogens, and this implies that the components of the pheromone response pathway, rather than the actual mating, are important for pathogenesis (5, 7, 8, 41, 54). Further studies on the regulation of cell type-specific genes and pheromone response pathway genes will help define whether *C. glabrata* can undergo sexual reproduction and whether some of these genes have been rewired to control other processes important for its survival in the mammalian host.

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