Transcriptional Regulation of MDR1, Encoding a Drug Efflux Determinant, in Fluconazole-Resistant Candida albicans Strains through an Mcm1p Binding Site

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Constitutive, high-level transcription of the gene encoding the drug efflux facilitator Mdr1p is commonly observed in laboratory and clinical strains of Candida albicans that are resistant to the antifungal drug fluconazole (FLC). In five independently isolated FLC* laboratory strains, introduction of a wild-type MDR1 promoter fragment fused to the yeast enhanced green fluorescent protein (yEGFP) reporter gene resulted in high-level expression of GFP, demonstrating that overexpression of MDR1 is dependent on a trans-acting factor. This study identified a 35-bp MDR1 promoter element, termed the MDRE, that mediates high-level MDR1 transcription. When inserted into a heterologous promoter, the MDRE was sufficient to mediate high-level expression of the yEGFP reporter gene specifically in MDR1 trans-activation strains. The MDRE promoted transcription in an orientation-independent and dosage-dependent manner. Deletion of the MDRE in the full-length promoter did not abolish MDR1 trans-activation, indicating that elements upstream of the MDRE also contribute to transcription of MDR1 in these overexpression strains. Analysis of the MDRE sequence indicated that it contains an Mcm1p binding site very similar in organization to the site seen upstream of the Saccharomyces cerevisiae MFA1 and STE2 genes. Electrophoretic mobility shift analysis demonstrated that both wild-type, FLC-sensitive and MDR1 trans-activated, FLC-resistant strains contain a factor that binds the MDRE. Depletion of Mcm1p, by use of a strain in which MCM1 expression is under the control of a regulated promoter (44), resulted in a loss of MDRE binding activity. Thus, the general transcription factor Mcm1p participates in the regulation of MDR1 expression.

Candida albicans is a commensal organism of the human gastrointestinal tract and genitourinary tract but can become an important opportunistic pathogen, especially in immunocompromised individuals (32). Depending on the underlying immune defect, C. albicans causes a variety of disease states, including superficial mucosal infections and more serious disseminated infections that exhibit approximately 40% attributable mortality (24). C. albicans has been associated with the AIDS epidemic from the outset (21). Oropharyngeal candidiasis, or thrush, in seemingly healthy individuals is often the first indication of human immunodeficiency virus (HIV) infection, and most AIDS patients have one or more episodes of Candida infection (22, 52). Fluconazole is the drug of choice for treating C. albicans in their oral cavities (35). Fluconazole-resistant (FLCR) isolates, deletion of MDR1 confers increased resistance to some compounds (28). Despite the frequency with which MDR1-overexpressing, FLC*R strains are isolated, the molecular mechanisms leading to high-level expression of MDR1 are not well understood (37). In some FLC*R strains, high-level expression of MDR1 is due to the effects of an undefined trans-acting factor(s) (54). Recent work by Hiller et al. indicates that three independent regions of the MDR1 promoter are capable of contributing to MDRI expression in a FLC* strain overexpressing MDR1 and that the portion of the MDR1 promoter they term region 2 (~588 to ~500) mediates benzoyl induction of MDR1 transcription in the FLC-sensitive (FLC*) laboratory strain CAI4 (29). In another study, analyzing the FLC* C. albicans laboratory strain

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CA18, Harry et al. identified two or more cis-acting promoter regions that contribute to MDR1 expression (25). Under the conditions used by Harry et al., the more-proximal cis-acting element (−399 to −299) was responsible for benomyl-induced transcription of MDR1 while the more-distal cis-acting element (−601 to −500) was implicated in MDR1 induction by oxidizing agents. The most-distal region identified by both groups (−601/−588 to −500) contains a sequence that resembles and functions like the YAP1-responsive element of Saccharomyces cerevisiae (25). Yap1p is a member of the bZIP family of transcription factors and regulates the S. cerevisiae FLR1 gene, encoding a multidrug efflux protein that is similar to C. albicans Mdr1p (1). The homologous C. albicans gene is termed CAP1 (for C. albicans AP-1) (2). Surprisingly, deletion of CAP1 in C. albicans did not lead to FLC hypersensitivity (2). When the CAP1 gene was disrupted in a laboratory-derived FLCR strain that overexpresses MDR1, increased levels of MDR1 mRNA were observed, indicating that Cap1p acts as a negative regulator of MDR1 in this strain and is not responsible for the preexisting MDR1 overexpression. Therefore, other uncharacterized protein factors probably bind to elements within the MDR1 promoter and trans-activate the MDR1 gene in FLCR strains.

Expression of the other drug efflux transporters, Cdr1p and Cdr2p, is also regulated through a trans-activating factor that binds a specific sequence within their promoters (10, 11). Cdr1p and Cdr2p are ATP binding cassette (ABC) transporters that are highly similar to each other in sequence (46). Increased expression of these genes has been observed in FLCR clinical strains, and deletion of the CDR1 gene in C. albicans results in hypersensitivity to the azole drugs fluconazole, ketoconazole, and itraconazole and to other drugs such as terbinafine and amorolfine (47). The CDR2 gene also confers resistance to the azoles, terbinafine, and amorolfine (46). Sanglard and coworkers identified a transcription factor, Tac1p, that binds an element within the CDR1/CDR2 promoter, the drug resistance element (DRE), and demonstrated its involvement in the high-level constitutive expression of these genes seen in some FLCR strains (10). Mutations within the TAC1 open reading frame (ORF) that lead to hyperactive trans-activation of CDR1 and CDR2 have been identified in FLCR strains (9). Therefore, mutations that result in increased activity of trans-activators occur commonly in FLCR strains.

In this study, we demonstrate that a promoter element termed the MDRE (for “MDR1 drug resistance element”) mediates MDR1 trans-activation in five independent FLCR, MDR1-overexpressing strains, indicating a common mechanism for trans-activation. The MDRE acts in a dosage-dependent and orientation-independent manner to increase transcription of MDR1 in drug-resistant, trans-activation strains. The protein Mcm1p binds the MDRE, and mutations within the MDRE that abolish Mcm1p binding abolish MDR1 trans-activation. Extracts from MDR1 trans-activation strains contain higher levels of MDRE binding activity. The sequence of MCM1 is wild type in these FLCR strains, indicating that the activity rather than the structure of Mcm1p is altered in the trans-activating mutants. Therefore, these results demonstrate that Mcm1p participates in the regulation of expression from the MDR1 promoter at the MDRE.

### MATERIALS AND METHODS

#### Strains, plasmids, and media.

C. albicans strains are listed in Table 1. Parent strains were CA14 (Δura3/Δura3) (17) and CAPR306 (Δura3/Δura3 Δcrd1/Δcrd1) (42). The crdl deletion strain CAPR306 was used as a parent in these studies because the crdl mutation provides a convenient genetic marker for experiments such as protoplast fusion. To select FLCR strains, independent pools of the parental strains were exposed to increasing levels of fluconazole during liquid growth in batch culture in Sabouraud’s dextrose broth (Difco). Briefly, the two drug-sensitive parental strains were grown in six replicate populations each in 2 μg/ml fluconazole. Upon reaching saturation, the cultures were diluted 1:1,000 in fresh medium again containing 2 μg/ml fluconazole. Upon reaching saturation, these populations were diluted into fresh medium containing 4 μg/ml fluconazole. This selection was repeated in 4 μg/ml fluconazole, then 8 μg/ml fluconazole (twice), then 16 μg/ml fluconazole (twice), then 32 μg/ml fluconazole (twice), and finally 64 μg/ml fluconazole (once). A representative isolate from each final population was chosen for detailed analysis. Antifungal susceptibility testing was performed by the standard CLSI (formerly NCCLS) microdilution method (5, 15). Strains were routinely grown in yeast extract-peptone-dextrose, CM-uridine, and synthetic dextrose (SD) (43).

#### DNA manipulations and analysis.

Plasmid isolation, PCR, restriction digestion, cloning, gel electrophoresis, and Southern hybridization analysis were performed by standard methods (45). C. albicans genomic DNA was isolated by a glass bead disruption procedure (43). Automated DNA sequencing was performed by Michael Berne and coworkers at the Tufts University Core Facility.

#### RNA analysis.

Cells for RNA isolation were grown in SD liquid medium at 30°C to an optical density at 600 nm (OD<sub>600</sub>) of 1.0. Uridine was added as needed at 100 μg/ml. For RNA extraction, cells were lysed with glass beads and phenol (4). Twenty micrograms of total RNA per sample was separated on a formaldehyde-agarose gel, transferred to a Nytran-plus membrane, and probed by standard Northern hybridization methods (45). The MDR1 probe was generated with a PCR product that corresponded to the MDR1 ORF. The CDR1 and CDR2 probes were generated by PCR and spanned the nucleotide regions −109 to +280 and −112 to +274, respectively, with regard to their ORFs (46). Probes

### Table 1. _Candida albicans_ strains used in this study

| Strain | Genotype<sup>a</sup> | Reference or source |
|--------|----------------------|---------------------|
| CA14   | Δura3::imm434/Δura3::imm434; congenic to clinical isolate SC5314 | 16 |
| CAPR306| CA14, crdl::hisG/crdl::hisG | 42 |
| CAPR507| CA14, FLCR, CDR1/CDR2 trans-activation | This study |
| CAPR510| CA14, FLCR, MDR1 trans-activation | This study |
| CAPR513| CAPR306, FLCR, MDR1 trans-activation | This study |
| CAPR514| CAPR306, FLCR, MDR1 trans-activation | This study |
| CAPR515| CAPR306, FLCR, MDR1 trans-activation | This study |
| CAPR517| CAPR306, FLCR, MDR1 trans-activation | This study |
| CAPR518| CAPR306, FLCR, CDR1/CDR2 trans-activation | This study |
| MRCan42| ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434 | 44 |
|        | ENO1::ENO1-tetR-SchHAP4AD-HA4::ADE2 CaCM1::myc-URA3::97t::CaCM1/CaneCM1::FRT |

<sup>a</sup> HA, hemagglutinin.
were labeled with [α-32P]dATP by the random priming method using the Stratagene Prime-it kit.

Construction of MDR1 and CDR2 promoter transcriptional fusions to the yEGFP reporter gene. The plasmids constructed for this study are described in Table 2. The integrating plasmid pLIB1 (T. Volkert and C. A. Kumamoto, unpublished data) encodes the yeast enhanced green fluorescent protein gene (yEGFP) flanked by 10 bp of the ACT1 5' untranslated region (UTR) and 389 bp of the ACT1 3' UTR. Sall and EcoRV sites upstream of the ACT1 5' UTR sequence were used for cloning (8). pLIB1 also carries a fragment of the CaUD2 gene to provide homology for integration and the CaUD3 gene as a selectable marker.

Template DNA for PCR amplification of MDR1 or CDR2 promoter fragments was from strain CAI4, a naïve strain that had never been exposed to fluconazole (17). Various MDR1 promoter fragments were PCR amplified with the primers listed in Table 2, digested with the restriction enzyme SphI, and ligated into EcoRV-digested pLIB1. CDR2 promoter fusions were generated similarly, except that the restriction enzyme EcoRV was used to digest PCR-amplified CDR2 fragments prior to ligation into the pLIB1 EcoRV site. All MDR1 and CDR2 promoter fusion constructs were sequenced. For the heterologous expression of MDR1, pPRC2-4 contains a 556 bp MDR1 promoter fragment and ligated into the SalI site of pLIB1. The plasmid pPRC2-5 contains the region of the MDR1 promoter from 187 to 7 (DRE mutation) and ligated into the SalI site of pLIB1. The plasmid pPRM10 D6/MDRPR contains the region of the MDR1 promoter from 501 to 7 (DRE mutation) and ligated into the SalI site of pLIB1.

Template DNA for PCR amplification of CDR2 promoter fragments was from strain CAI4, a naïve strain that had never been exposed to fluconazole (17). Various CDR2 promoter fragments were PCR amplified with the primers listed in Table 2, digested with the restriction enzyme SphI, and ligated into EcoRV-digested pLIB1. CDR2 promoter fusions were generated similarly, except that the restriction enzyme EcoRV was used to digest PCR-amplified CDR2 fragments prior to ligation into the pLIB1 EcoRV site. All MDR1 and CDR2 promoter fusion constructs were sequenced. For the heterologous expression of CDR2, pPRC2-4 contains a 556 bp CDR2 promoter fragment and ligated into the SalI site of pLIB1. The plasmid pPRC2-5 contains the region of the CDR2 promoter from 187 to 7 (DRE mutation) and ligated into the SalI site of pLIB1. The plasmid pPRM10 D6/MDRPR contains the region of the CDR2 promoter from 501 to 7 (DRE mutation) and ligated into the SalI site of pLIB1.

Transformation of C. albicans. Candida cells were transformed by the lithium acetate method of Gietz et al. and selected for uridine prototrophy on CM-uridine (19). yEGFP reporter plasmids were digested with the restriction enzyme BamHI to direct their integration into the ADE2 locus.

Microscopy. Cells were visualized by using an Olympus BX60 microscope with a 1.4NA 100× objective lens. Images were collected with a Hamamatsu (Bridge- water, NJ) model C4742-95 cooled charge-coupled-device camera and analyzed with OpenLab software. Relative fluorescence of the MDR1 trans-activation

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**Table 2. yEGFP reporter plasmids used in this study**

| Plasmid | Primer pair used to generate insert | Region of promoter amplified |
|---------|---------------------------------|----------------------------|
| pPRM1   | MDRPF/MDRPR                     | -1108 to -7                |
| pPRM2   | M2PFW/MDRPR                     | -857 to -7                 |
| pPRM3   | M3PFW/MDRPR                     | -622 to -7                 |
| pPRM4   | M4PFW/MDRPR                     | -423 to -7                 |
| pPRM5   | M5PFW/MDRPR                     | -226 to -7                 |
| pPRM6   | D1/MDRPR                        | -407 to -7                 |
| pPRM7   | D2/MDRPR                        | -385 to -7                 |
| pPRM8   | D3/MDRPR                        | -356 to -7                 |
| pPRM9   | D4/MDRPR                        | -335 to -7                 |
| pPRM10  | D5/MDRPR                        | -309 to -7                 |
| pPRM11  | D6/MDRPR                        | -282 to -7                 |
| pPRM9A1 | D4DEL1/MDRPR                    | -335 to -7 (Δ309-303)      |
| pPRM9A2 | D4DEL2/MDRPR                    | -335 to -7 (Δ302-296)      |
| pPRM9A3 | D4DEL3/MDRPR                    | -335 to -7 (Δ295-289)      |
| pPRM9A4 | D4DEL4/MDRPR                    | -335 to -7 (Δ288-282)      |
| pPRM10A5| D5DEL5/MDRPR                    | -309 to -7 (Δ281-275)      |
| pPRM10A6| D5DEL6/MDRPR                    | -309 to -7 (Δ274-268)      |
| pPRM10A7| D5DEL7/MDRPR                    | -309 to -7 (Δ267-261)      |
| pPRM11A8| D508/MDRPR                      | -295 to -7 (Δ260-254)      |
| pPRM12A9| D509/MDRPR                      | -295 to -7 (Δ253-247)      |
| pPRC2-1 | C2PFW/C2PRV                     | 841 to -7                  |
| pPRC2-4 | C2PFW/C2PRV                     | -230 to -7                 |
| pPRC2-5 | C2PFW/C2PRV                     | -230 to -7 (DRE mutation)  |
| pPRC2-4mut| C2PF2mutFW/C2PRV                | -187 to -7 (no DRE)        |
| pPR2hetFW| C2PF2FW/C2PRV (plus MDRE into Sall site) | -187 to -7 (no DRE) |
| pPR2hetRV| C2PF2mutFW/C2PRV (plus two copies of MDRE into Sall site, forward orientation) | -187 to -7 (no DRE) |
| pPR2hetRV| C2PF2mutFW/C2PRV (plus two copies of MDRE into Sall site, reverse orientation) | -230 to -7 (DRE mutation) |
| Oligonucleotide | Sequence |
|----------------|----------|
| For cloning    |          |
| MDRP          | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| MDRR          | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| MP3FW         | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| MP4FW         | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5038         | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5DEL5        | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| GSF           | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| C2P4FW        | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| C2P3FW        | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| CDR2PF        | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D4GSR         | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D4DEL5        | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D6       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D4DEL1       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D4DEL2       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D4DEL3       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5DEL4       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5DEL5       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5DEL6       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5DEL7       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5EL8        | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5EL9        | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5EL10       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| MDRSF        | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| MDRSR        | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| GSR           | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| GSF           | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| C2P4FW        | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| GSR           | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5DEL5       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5DEL6       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5DEL7       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5DEL8       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| D5DEL9       | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| GSR           | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| GSF           | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| C2P4FW        | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| GSR           | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |
| C2P4FW        | 5'-CGGAATTTTCATATTCTTGTAGGACGAGTCCTC-3' |

TABLE 3. Oligonucleotides used in this study
FIG. 1. Expression of the GFP reporter gene in an MDR1 trans-activation strain. (A) Diagram of the yEGFP reporter plasmid pPRM1 integrated at the ADE2 genomic locus (not drawn to scale). The MDR1 promoter (hatched box) is transcriptionally fused to the yEGFP ORF (white box) plus 389 bp of the ACT1 3′ UTR (black box). The URA3 gene (gray box) provides a selectable marker for integration into CAI4 derivatives. The plasmid backbone is denoted as a thin line. The stippled boxes indicate the incomplete fragments of the ADE2 gene generated by the integration with BamH-digested pPRM1. (B) Cells of strains containing the chromosomally integrated P_{MDR1}(1108)-GFP fusion plasmid pPRM1 were grown on agar medium without fluconazole and resuspended in phosphate-buffered saline. Nomarski (left) and corresponding fluorescence (right) micrographs of cells are shown. Exposure for the fluorescence micrographs was 800 ms.

The gel was prerun at 100 V for 90 min at 4°C with buffer recirculation. After sample loading, electrophoresis was continued at 30 to 35 mA for approximately 2 h until the bromphenol blue marker dye exited the gel. After electrophoresis, gels were transferred to Whatman paper, dried, and exposed for autoradiography.

RESULTS

Phenotypic characterization of FLCR strains overexpressing drug efflux transporters. In order to define the molecular events that lead to trans-activation of the drug efflux transporter genes in FLCR strains, C. albicans strains highly resistant to fluconazole (MIC$_{50}$ > 64 µg/ml) were generated by exposure of strains CAI4 (ura3/ura3) and CAPR306 (ura3/ura3 cdl1/crd1) to increasing concentrations of fluconazole during liquid growth, as described in Materials and Methods. The two drug-sensitive parental strains were grown in six replicate populations each and were exposed to stepwise increases of fluconazole. After drug selection, all 12 independent populations exhibited a fluconazole MIC$_{50}$ of >64 µg/ml, as determined by the CLSI microdilution assay (15). Parental populations cultured in the same manner except without drug exhibited a fluconazole MIC$_{50}$ of approximately 0.25 µg/ml, identical to that of the original parental isolates. A representative isolate from each of the independent populations was chosen for further analysis.

To determine whether the drug-resistant isolates carried mutations in trans-acting factors that increased expression of MDR1 or CDR2, the promoters of these two genes were PCR amplified from the chromosome of the drug-sensitive strain CAI4 and cloned upstream of the promoterless yEGFP gene in the vector pLIB1 (Fig. 1A). These promoter fusions did not exhibit detectable GFP expression when introduced into the parental drug-sensitive strains (Fig. 1B and Table 4). When the MDR1 fusion plasmid pPRM1 was introduced into the drug-resistant isolates, 5 of the 12 independent isolates exhibited high levels of GFP expression in both the presence and absence of drug (Table 4), indicating that these strains contained trans-acting mutations that increased MDR1 transcription (e.g., strain CAPR514, an MDR1 overexpressor [OE], shown in Fig. 1B). Two of 12 independent drug-resistant isolates exhibited increased levels of P$_{CDR2}$(641-847)-GFP expression, indicating that these strains contained trans-acting mutations that increased CDR2 expression (Table 4). FLCR and yEGFP expression were stably maintained in the absence of fluconazole selection for 60 generations (Table 4, column 4). To confirm that the strains with increased P$_{MDR1}$-GFP and P$_{CDR2}$-GFP expression truly overexpressed MDR1 or CDR2, respectively, the expression of these genes in the FLCR strains was analyzed by Northern blotting. As shown in Fig. 2 and Table 4, each strain exhibiting trans-activation of MDR1 or CDR2 had increased levels of MDR1 mRNA or CDR2 mRNA, respectively. Strains exhibiting CDR2 trans-activation also coordinately overexpressed CDR1, as has been previously described for strains that contain hyperactive TAC1 alleles (9). This report focuses on the mechanism(s) whereby MDR1 is trans-activated in FLCR strains; mutations leading to trans-activation of CDR2 will be described in detail elsewhere.

Identification of an MDRE within the MDR1 promoter. To delimit the site(s) within the MDR1 promoter that is important for its expression in the MDR1 trans-activation strains, additional promoter fusions were constructed, as shown in Fig. 3. yEGFP was transcriptionally fused to the following fragments
from the *MDR1* promoter: −852 to −7, −622 to −7, −423 to −7, and −226 to −7. All of the *MDR1* promoter fusion constructs, except for the smallest fusion (−226 to −7), exhibited strong fluorescence when introduced into the five *MDR1* trans-activation strains (Fig. 3A). This result indicates that an MDRE(s) lies within 423 bp upstream of the initiation codon. Additionally, the five independently isolated *MDR1* overexpression mutants delimited the same region of the *MDR1* promoter as important for high-level trans-activation, suggesting that the same mechanism for *trans*-activation was utilized in all of these strains.

To determine the 5′ end of the putative MDRE, further promoter fusions were generated that had 5′ ends between 429 and 226 bp upstream of the *MDR1* initiation codon. Constructs containing promoter fragments with 5′ ends at −407, −385, −356, −335, and −309 all exhibited strong fluorescence, similar to the −1108 fusion, when integrated into *MDR1* trans-activation strains; however, *MDR1* trans-activation strains containing fusions with a 5′ end lying at −282 or −254 exhibited a complete loss of fluorescence (Fig. 3A). Harry et al. determined that the *MDR1* transcriptional start point was at −65 and identified a putative TATA binding site at −200, based on sequence analysis (26); therefore, the region mediating trans-activation is well upstream of these core promoter elements.

To define the 5′ and 3′ boundaries of the MDRE, 7-bp deletions were constructed that spanned the region −309 to −245, as shown in Fig. 3B. *MDR1* promoter fusions with deletions of −309 to −303 (i.e., Δ1) and −302 to −296 (Δ2) exhibited expression in *MDR1* trans-activation strains similar to the full-length promoter fusion seen in pPRM1. However, deletions in the region from −295 to −261 (Δ3, Δ4, Δ5, Δ6, and Δ7) led to a drastic drop in the ability of the promoter fragments to mediate reporter gene expression. The promoter construct with a deletion from −260 to −254 (Δ8) exhibited a moderate defect in the ability to be *trans*-activated, while a promoter construct deleted for bp −253 to −247 (Δ9) was similar in GFP expression to the full-length promoter construct pPRM1. The ability of the Δ9 promoter construct to express GFP as well as the full-length promoter construct pPRM1 defined the 3′ end of the MDRE and indicated that these deletions were upstream of the basal transcriptional sites, such as the TATA factor binding site required for promoter function. As shown in Fig. 3C, deletion of a portion of the MDRE in the context of the full-length (−1108) *MDR1* promoter did not abolish *trans*-activation in *MDR1* OE strains, indicating that upstream elements were capable of mediating *MDR1* trans-activation in the absence of the functional MDRE. These upstream elements are unrelated to the MDRE and may correspond to regions identified by Harry et al. and Hiller et al. (25, 29). The sequence conferring high-level *MDR1* trans-activation, termed the MDRE, encompassed bp −295 to −261 relative to the *MDR1* initiation codon (Fig. 4). The MDRE is relatively large, between 23 and 35 bp in length, suggesting that it may define more than one protein binding site (i.e., a regulatory protein and its coregulator). Inspection of the sequence indicated an inverted repeat (11 of 12 bp) that matches the *Saccharomyces cerevisiae* Mcm1p binding site (59).

The MDRE mediates high-level *trans*-activation when cloned into a heterologous promoter. To determine whether the MDRE can function as an independent element outside of the context of the *MDR1* promoter, the MDRE was cloned into a nonfunctional promoter. Derivatives of the *CDR2* pro-
moter containing a DRE point mutation or a deletion of the DRE were used because, when fused to yEGFP, these constructs did not express GFP in MDR1 trans-activation strains.

The \( /H11002 \) to \( /H11002 \) region of the MDR1 promoter was cloned into the SalI site of pPRC2-4mut (mutation in DRE) and pPRC2-5 (deletion of DRE). The plasmids pPRhetFW and pPR2xhetFW contained one or two copies, respectively, of the MDR1 promoter adjacent to the nonfunctional CDR2 promoter of pPRC2-5 (Fig. 5). The plasmid pPR2xhetRV contained two copies of the MDR1 promoter adjacent to the nonfunctional CDR2 promoter of pPRC2-4mut, but the MDREs are in the orientation opposite that seen in the MDR1 promoter (Fig. 5).

All of these constructs failed to express GFP in a FLC strain background, as expected (Fig. 5). When the previously inactive CDR2 promoter fusion plasmids contained one or more copies of the MDR1 promoter (i.e., pPRhetFW, pPR2xhetFW, or pPR2xhetRV), these plasmids specifically mediated trans-activation in the MDR1 trans-activation strain (CAPR514) but not in the FLC (CAPR306) or CDR1/CDR2 trans-activation strain (CAPR518) (Fig. 5). The MDR1 promoter acted in a dosage-dependent

FIG. 3. Deletion of portions of the MDR1 promoter results in defective expression of the GFP reporter. (A) The hatched box labeled MDR1 promoter represents the full-length MDR1 promoter encompassing bp \(-1108\) to \(-7\) relative to the start of the ORF (not drawn to scale). The MDR1 promoter (see the text) is shown as a black box. Numbers above and below the hatched box indicate the end points of smaller fusions extending from the numbered line to residue \(-7\). The thick black arrow denotes the transcription initiation site at \(-65\), and the asterisk indicates the putative consensus TATA element at \(-200\) (26). Fusions indicated above the hatched box were fully active in MDR1 trans-activation. Fusions indicated below the box were completely inactive. (B) Internal 7-bp deletions (\( \Delta 1 \) to \( \Delta 9 \), indicated by white boxes) were constructed in fusions extending from \(-335\), \(-309\), or \(-295\) to \(-7\). Internal deletions correspond to the following regions of the MDR1 promoter: \( \Delta 1 \) (\(-309\) to \(-303\)), \( \Delta 2 \) (\(-302\) to \(-296\)), \( \Delta 3 \) (\(-295\) to \(-289\)), \( \Delta 4 \) (\(-288\) to \(-282\)), \( \Delta 5 \) (\(-281\) to \(-275\)), \( \Delta 6 \) (\(-274\) to \(-268\)), \( \Delta 7 \) (\(-267\) to \(-261\)), \( \Delta 8 \) (\(-260\) to \(-254\)), and \( \Delta 9 \) (\(-253\) to \(-246\)). GFP expression from these constructs is indicated at right. +++, full activity; ++, 40 to 70% of full activity; +, 5 to 20% of full activity; -, no activity. (C) Internal deletion of region \( \Delta 5 \) (\(-281\) to \(-275\)) in the full-length 1,108-bp MDR1 promoter fragment. +++++ indicates full activity.

FIG. 4. The MDR1 contains an Mcm1p binding motif. The sequence of the region conferring a strong MDR1 trans-activation phenotype, termed the MDR1 promoter, is shown. Regions indicated by brackets and labeled \( \Delta 3 \) to \( \Delta 7 \) correspond to the deletions shown in Fig. 3 and described in Materials and Methods. The lower sequence represents the MMS12-Mcm1p and Ste12p binding sites found in the S. cerevisiae STE2 promoter. Sequences in bold correspond to the MCM1 binding site, \( 5'\)CCYWWNNNGG-G\( 3'\). Boxed regions correspond to the TATA sequence of the MMS12 promoter binding site. Underlined sequence corresponds to the Ste12p (Cph1p) binding site, \( 5'\)ATGAACA-3'. Vertical lines between the two sequences indicate identity; dashes indicate matches to the \( \alpha 2\)-Mcm1 consensus binding site as described by Zhong and Vershon (5'-TGTANW\( \alpha 2\),CCN,G GW\( \alpha 3\),NTACA-3'); X indicates deviation from the S. cerevisiae consensus for these binding sites (57).
construct pPR2xhetRV mediated an orientation-independent effect in that the promoter fusion to the dosage-dependent effect, the MDRE also exhibited which contained a single copy of the MDRE (Fig. 5). In addition, the MDRE to mediate transcriptional enhancement in an orientation-independent manner is similar to that of an upstream MDR1 trans-activation sequence (23).

To identify factors that mediate MDR1 trans-activation through binding of the MDRE, EMSA was undertaken with protein extracts from FLC and MDR1 trans-activation strains. As can be seen in Fig. 6A, lanes 2 to 4 and 6 to 8, whole-cell protein extracts from both CAPR306 (FLC) and CAPR514 (MDR1 OE) contained a factor(s) that bound to and retarded the mobility of the MDRE probe. This binding was specifically competed with a DNA fragment containing the 7-bp deletions (76-bp (59)). There are also symmetrically placed binding sites for the homeobox protein Ste12p (31, 39). There are also symmetrically placed binding sites for the homeobox protein Ste12p (31, 39). Therefore, regions 4 and 5 of the MDRE, which are necessary for MDR1 trans-activation, are also necessary for factor binding. The sequence from regions 4 and 5 matches the S. cerevisiae consensus binding site for Mcm1p (Fig. 4) (59).

Whole-cell extracts from a strain depleted for Mcm1p failed to shift the MDRE. The MCM1 gene is an essential gene in C. albicans; however, its function can be studied by placing MCM1 under the control of a regulated promoter (44). In the strain MRcan42, expression of the MCM1 gene is under the control of the doxycycline-repressible promoter Prr. Whole-cell extracts were made from the strain MRcan42 with cells exposed to repressive conditions for 0, 2, 4, or 6 h. Substantial reduction of Mcm1p occurred by 2 h, with Mcm1p no longer detectable by 4 h (44). Whole-cell extracts from cells of strain CAPR514 (MDR1 OE) exposed to doxycycline retained the ability to shift the MDRE probe after 6 h of doxycycline treatment (Fig. 7, lanes 4, 5, and 6), indicating that the presence of doxycycline did not inhibit interaction between the MDRE and the factor(s) that leads to gel mobility shifts. Whole-cell extracts of cells from strain MRcan42 specifically shifted the MDRE under nonrepressive conditions (Fig. 7, lane 7). However, exposure of MRcan42 cells to doxycycline quickly led to the loss of the factor that shifted the MDRE probe. By 2 h of doxycycline exposure, extracts of the MRcan42 cells exhibited a dramatic reduction in the ability to shift the MDRE probe (Fig. 7, lane 8), and by 4 h of doxycycline exposure, extracts of the MRcan42 cells no longer shifted the MDRE probe (Fig. 7, lane 9). These results strongly suggest that Mcm1p is the factor that binds the MDRE.

The region 288 to 275 of the MDRE is required for factor binding. DNA fragments containing the 7-bp deletions that genetically define the MDRE (Fig. 3B) were tested for the ability to compete the binding of the labeled MDRE probe. The MDRE probe was specifically shifted when no competitor was present (Fig. 6B). However, when a 100-fold excess of a 76-bp (Δ76) fragment that spans the MDRE was added to the binding reaction, binding was effectively competed and no shift of the MDRE probe was seen (Fig. 6B). As shown in Fig. 6B, regions 2, 3, 6, 7, 8, and 9 were not required for binding, as 69-bp fragments that were missing these regions still effectively competed with the labeled MDRE probe for factor binding. In contrast, regions 4 and 5 (288 to 275) were necessary for specific competition with the labeled MDRE probe (Fig. 6B).
pheromone. As shown in Fig. 4, the MDRE is highly similar in sequence to regulatory sequences in the 5′ UTRs of the a genes STE2 and MFA1 (59). In Fig. 4, the core TGTA binding sites that symmetrically flank the Mcm1p binding site are boxed in the S. cerevisiae STE2 promoter sequence (48, 58). The symmetrical boxed regions of the MDRE sequence (GGTA/TACC) are similar to the 2 binding site and maintain the spacing of 3 or 4 bp that is important for repression in S. cerevisiae (48). However, the 5′ T residue that is thought to be critical for α2p binding is replaced by a G residue (49, 58). Activation of a genes such as STE2 and MFA1 in haploid a cells of S. cerevisiae requires Ste12p, which binds to the consensus sequence ATGAAACA (59), underlined in Fig. 4 (12, 14). A similar sequence, 5′-ATGACACA-3′, with one mismatch from the consensus, is shown underlined in the MDRE. The location of this putative Ste12p binding site adjacent to the Mcm1p binding site of the MDRE raises the possibility that the C. albicans homolog of Ste12p, Cph1p, may play a role in MDR1 trans-activation.

**DISCUSSION**

Wirsching et al. demonstrated that in two independent FLCR clinical strains of C. albicans, MDR1 overexpression was due to mutations in an unidentified trans-regulatory factor(s) (54). We have demonstrated that in five independently isolated FLCR laboratory strains, MDR1 overexpression was also achieved through trans-activation of MDR1, suggesting that this may be the most common mechanism for MDR1 overexpression.

Recent work from the Morschhäuser laboratory has implicated three regions of the MDR1 promoter as important for trans-activation in the MDR1-overexpressing strain F5U4, a Ura− strain derived from a FLC R clinical isolate: region 1 (397 to 300), region 2 (588 to 500), and region 3 (287 to 209) (29). We demonstrated that in our five independently isolated MDR1 trans-activating laboratory strains, a small region of the MDR1 promoter, the MDRE, was sufficient to mediate high-level trans-activation of MDR1. The MDRE (295 to 261) shares a 26-bp overlap with the 78-bp region (287 to 209) described by Hiller et al. and may identify the same cis-acting element. These results raise the possibility that the MDRE contributes to MDR1 expression in both laboratory-isolated and clinically isolated FLCR strains.

Although our reporter gene methodologies were quite similar, some differences were seen between the results described in this report and the publication by Hiller et al. Using the MDR1 trans-activation strain F5U4, Hiller and coworkers constructed 5′ MDR1 promoter deletions to −495 without any

**FIG. 6.** MDRE binding activity detected in cell extracts. (A) EMSA with whole-cell protein extracts from a FLC S parental strain and a representative MDR1 trans-activation strain, CAPR514. The probe is a 5′ end-labeled dsDNA fragment corresponding to bp −299 to −254 with respect to the MDR1 initiation codon and was generated by annealing oligonucleotides MDRSF and MDRSR (Table 3). Lanes 5 and 9 also contain a 100-fold excess of unlabeled probe DNA. The radiolabeled probe was incubated with protein extracts and analyzed on a polyacrylamide gel, and the resulting autoradiogram or phosphorimagery scan is shown. Samples were free probe (lane 1); CAPR306 (wild-type [WT]) extract with 1 μg (lane 2), 2 μg (lane 3), 3 μg (lane 4), and 3 μg (lane 5) total protein; and CAPR314 (MDR1 OE) extract with 1 μg (lane 6), 2 μg (lane 7), 3 μg (lane 8), and 3 μg (lane 9) of total protein. (B) EMSA with competitor fragments that have deletions throughout the MDRE region. Reactions were carried out as described for panel A. Full-length unlabeled competitor (Δ0) is a 76-bp DNA fragment that contains the 35-bp MDRE plus 21 bp upstream and 20 bp downstream and was generated by annealing oligonucleotides GSF and GSR (Table 3). Competitor fragments (Δ2 to Δ9) have 7-bp deletions of the numbered regions corresponding to those shown in Fig. 3. Competitor fragments were added at a 100-fold molar excess.
effect on promoter activity while deletion to −397 resulted in an approximately two-thirds reduction of promoter activity but maintained constitutive overexpression. Further 5′ deletion to −300 abolished all promoter activity (29). In contrast, we observed that constructs beginning at −295 exhibited full promoter activity in the MDR1 trans-activation strains CAPR510, CAPR513, CAPR514, CAPR515, and CAPR517 (Fig. 3). Hiller et al. observed that small internal deletions in the region from −355 to −209 had little or no effect on promoter activity when sequences upstream of −495 were present (29). Similarly, we found that deletion of the MDRE in the context of the full-length (−1108) promoter did not affect MDR1 expression, indicating that there was a region(s) upstream of the MDRE that was functionally redundant with the MDRE and could contribute to MDR1 trans-activation. If the promoter fusion constructs of Hiller and coworkers contained any two of their three regions, high-level reporter gene expression was detected in MDR1 OE strains (29). In contrast, in the MDR1 trans-activation strains described in this study, promoter fusions containing only the MDRE (−299 to −254), which may correspond to region 3 of Hiller et al., were capable of mediating expression equal to that of full-length (−1108) promoter constructs.

One possible interpretation of these differing results is that multiple mechanisms control the expression of MDR1 and that the importance of the various cis-acting regions for MDR1 overexpression differs depending on the strain. Anderson et al. showed that, in S. cerevisiae, different fluconazole selection regimens led to the selection of different types of resistant mutants (3). Therefore, MDR1 trans-activating strains selected under different conditions may differ in the relative contributions of various transcription factors to the expression of MDR1. In addition, the constructs used by Hiller et al. were integrated at the ACT1 locus while our constructs were integrated at the ADE2 locus. The local context of the integration site may affect expression from the promoter; therefore, the differences in integration sites may contribute to the differences in our results.

The MDRE is distinct from sites identified thus far as important for benomyl-induced expression of MDR1 in the congenic FLC strains CAI4 and CAI8. The findings of Hiller et al. indicated that region 2 (−588 to −500) of the MDR1 promoter mediates benomyl induction of MDR1 in strain CAI4 (29). In contrast, work by Harry et al., using the related strain CAI8, identified another region of the MDR1 promoter (−399 to −299) as being responsible for benomyl-induced expression (25). It is unclear why these two research groups identified different regions of the MDR1 promoter as important for benomyl induction in these related laboratory strains, but Hiller et al. suggest that this may be due to the differences in benomyl exposure used by the two groups (29). These two regions identified thus far as mediating benomyl induction of MDR1 are clearly distinct from the MDRE identified in this study with FLC strains; therefore, these findings underscore the conclusion that the MDR1 promoter is complex and subject to regulation through multiple pathways.

Several lines of evidence indicate that the MDRE is bound by the general transcription factor Mcm1p and that this binding is important for MDR1 trans-activation in FLC strains. The most obvious feature of the MDRE is the exact match of the MDRE is under the control of the doxycycline-repressible promoter Pir, or cells of strain CAPR314 (MDR1 OE) were exposed to doxycycline for between 0 and 6 h. Extracts of these cells were incubated with radiolabeled probe and analyzed as described for Fig. 6. Lane 3 contains a 100-fold molar excess of cold competitor fragment (+cc). Lane 1, free MDRE probe; lanes 2 to 6, 3 μg of protein extract from strain CAPR514 (MDR1 OE) exposed to doxycycline for 0, 2, 4, and 6 h, respectively; lanes 7 to 10, 3 μg of protein extract from MRcan42 (Pir-MCM1) cells that had been exposed to doxycycline for 0, 2, 4, and 6 h, respectively.
Therefore, the mechanism of fluconazole resistance described in this communication which is mediated through MCM1-dependent overexpression of an efflux determinant may not be shared with *S. cerevisiae*.

In addition to containing an MCM1 binding site, the MDRE has sequence similarity to regulatory regions upstream of the *S. cerevisiae* most highly characterized genes. Repression of a-specific genes in α haploid cells and α/α diploid cells requires cooperative binding of α2p and MCM1p to adjacent binding sites and subsequent recruitment of the Snf6-Tup1 repression complex. In contrast, no α2p is present in a cells and therefore no Snf6-Tup1 complex is recruited; in this situation, MCM1p cooperates with the transcription factor Ste12p to activate transcription. In *S. cerevisiae*, the only binding sites for coregulators that flank the MCM1p binding site and are in opposite orientation are α2 boxes with the core sequence 5’-TGTA-3’. The 5’ T residue is thought to be important for binding, although one of the α2 boxes of *S. cerevisiae* ASG7 contains a 5’ C residue rather than a T residue. However, in the MDRE, these boxes contain the sequence 5’-GGTA-3’.

A sequence similar to a Ste12p binding site partially overlaps the boundary of the MDRE. In some strains of *C. albicans*, e.g., clinical strain B792, this sequence is identical to the consensus Ste12p binding site. Observations suggest that *C. albicans* Cph1p, the Ste12p homolog, participates in regulating the expression of *MDR1*. Preliminary analyses indicate that the CPH1 ORFs from the MDRE trans-activation strains are wild type in sequence (Riggle and Kumamoto, unpublished). Thus, the role, if any, of Cph1p in *MDR1* trans-activation requires further study.

The data presented in this paper suggest the working model presented in Fig. 8. MCM1p is capable of binding to the MDRE of the *MDR1* promoter in both wild-type and *MDR1* trans-activation strains. In *trans*-activation strains, the *MCM1* ORF is wild type in sequence and is transcriptionally expressed at wild-type levels, as determined by Northern hybridization analysis (Riggle and Kumamoto, unpublished). However, MCM1p activity is modified in these strains so that MCM1p binding is increased. This increased binding and/or a unique interaction with a coregulator leads to increased transcription of the *MDR1* gene. Future studies will focus on determining the mechanism of enhanced *MDR1* trans-activation in FLC*R* strains.

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ADDENDUM IN PROOF

A similar cis-acting region has recently been described (B. Rognon, Z. Kozovska, A. T. Coste, G. Pardini, and D. Sanglard, Microbiology, in press).

REFERENCES

1. Alarco, A. M., I. Balan, D. Talibi, N. Mainville, and M. Raymond. 1997. API-mediated multidrug resistance in *Saccharomyces cerevisiae* requires FLR1 encoding a transporter of the major facilitator superfamily. J. Biol. Chem. 272:19304–19313.
2. Alarco, A. M., and M. Raymond. 1999. The hZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. J. Bacteriol. 181:700–708.
3. Anderson, J. B., C. Sirajinsingh, A. B. Parsons, C. Boone, C. Wickens, L. E. Cowen, and L. M. Kohn. 2003. Mode of selection and experimental evolution of antifungal drug resistance in *Saccharomyces cerevisiae*. Genetics 163:1287–1298.
4. Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl (ed.). 1989. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
5. Barchiesi, F., A. L. Colombo, D. A. McGough, and M. G. Rinaldi. 1994. Comparative study of broth macrodilution and microdilution techniques for *in vitro* antifungal susceptibility testing of yeasts by using the National Committee for Clinical Laboratory Standards’ proposed standard. J. Clin. Microbiol. 32:2494–2500.
6. Ben-Yaacov, R., S. Knoller, G. A. Caldwell, J. M. Becker, and Y. Koltin. 1994. *Candida albicans* gene encoding resistance to benomyl and methotrexate is a multidrug resistance gene. Antimicrob. Agents Chemother. 38:648–652.
7. Como, J. A., and W. E. Dismukes. 1994. Oral azole drugs as systemic antifungal therapy. N. Engl. J. Med. 330:263–272.
8. Cormack, B. P., G. Bertram, M. Egerton, N. A. Gov, S. Falkow, and A. J. Brown. 1997. Yeast-enhanced green fluorescent protein (yEGFP) a reporter gene of expression in *Candida albicans*. Microbiology 143:303–311.
9. Coste, A., V. Turner, F. Ischer, J. Morschhäuser, A. Forche, A. Selmecki, J. Berman, J. Bille, and D. Sanglard. 2006. A mutation in Tac1p, a transcription factor regulating *CDR1* and *CDR2*, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in *Candida albicans*. Genetics 172:2139–2156.
10. Coste, A. T., M. Karababa, F. Ischer, J. Bille, and D. Sanglard. 2004. TAC1: transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of Candida albicans ABC transporters CDR1 and CDR2. Eukaryot. Cell 3:1639–1652.

11. de Brullé, M., M. B. Tebbe, C. Schueller, and D. Sanglard. 2002. A common drug-resistant element mediates the upregulation of the Candida albicans ABC transporters CDR1 and CDR2, two genes involved in antifungal drug resistance. Mol. Microbiol. 43:1197–1214.

12. Dolan, J. W., C. Kirkman, and S. Fields. 1989. The yeast STE12 protein binds to the DNA sequence mediating pheromone induction. Proc. Natl. Acad. Sci. USA 86:5703–5707.

13. Dubois, E., and F. Messenguy. 1991. In vitro studies of the binding of the yeast transcriptional activator Mcm1 to its promoter. Mol. Cell. Biol. 11:2162–2168.

14. Errede, B., and G. Ammerer. 1989. STE12, a protein involved in cell-type-specific transcription and signal transduction in yeast, is part of protein-DNA complexes. Genes Dev. 3:1349–1361.

15. Gudlaugsson, O., S. Gillespie, K. Lee, J. Vande Berg, J. Hu, S. Messer, L. Passmore, G., T. Maine, R. Elble, C. Christ, and B. K. Tye. 1988. Saccharomyces cerevisiae protein involved in plasmid maintenance is necessary for mating of MATa alpha cells. J. Mol. Biol. 204:593–606.

16. Fling, M. E., J. Kopf, A. Tamarkin, J. A. Gorman, H. A. Smith, and Y. Koltin. 1991. Analysis of a Candida albicans gene that encodes a novel mechanism for resistance to triazoles and azoles. Mol. Genet. 227:318–329.

17. Fonzi, W. A., and M. Y. Irwin. 1993. Isogenic strain construction and gene mapping in Candida albicans. Genetics 134:717–728.

18. Gallant, J. E., R. D. Moore, and R. E. Chaisson. 1994. Prophylaxis for opportunistic infections in patients with HIV infection. Ann. Intern. Med. 120:932–944.

19. Gietz, R. D., R. H. Schiestl, A. R. Willems, and R. A. Woods. 1995. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:841–851.

20. Goldway, M., D. Telf, R. Schmidt, A. B. Oppenheim, and Y. Koltin. 1995. Multidrug resistance in Candida albicans: disruption of the BEn gene. Antimicrob. Agents Chemother. 39:422–426.

21. Gottlieb, M. S., R. Schroff, H. M. Schanker, J. D. Weisman, P. T. Fan, R. A. Wolf, and A. Saxon. 1981. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N. Engl. J. Med. 305:1425–1431.

22. Graybill, J. R. 1988. Systemic fungal infections: diagnosis and treatment. I. The opportunistic agents. Infect. Dis. Clin. Am. 2:805–825.

23. Guarente, L., and E. Hoar. 1984. Upstream activation sites of the CYCI gene of Saccharomyces cerevisiae are active when inverted but not when placed downstream of the ‘TATA box’. Proc. Natl. Acad. Sci. USA 81:7860–7864.

24. Guillaumsohn, O., S. Gillespie, K. Lee, J. Vande Berg, J. Hu, S. Messer, L. Passmore, M. Martinez, D. Calabrese, D. Sanglard, and T. F. Patterson. 2001. Prevalence of molecular mechanisms of resistance to azole antifungal agents in Candida albicans strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrob. Agents Chemother. 44:2965–2971.

25. Riggle, P. J., and C. A. Kumamoto. 2000. Role of a Candida albicans P1-type ATPase in resistance to copper and silver ion toxicity. J. Bacteriol. 182:4899–4908.

26. Herwaldt, M. Pfaller, and D. Diekema. 1984. Upstream activation sites of the MDA1 gene, a new multidrug ABC transporter gene. Microbiology 119:1203–1214.

27. Singlard, D., F. Ischer, M. Monod, and J. Bille. 1997. Cloning of Candida albicans genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug transporter ABC gene. Microbiology 143:405–416.

28. Singlard, D., F. Ischer, M. Monod, and J. Bille. 1996. Susceptibilities of Candida albicans multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. Antimicrob. Agents Chemother. 40:2300–2305.

29. Smith, D. L., and A. D. Johnson. 1992. A molecular mechanism for combinatorial control in yeast: MCM1 protein sets the spacing and orientation of the homeodomains of an alpha 2 dimer. Cell 68:133–142.

30. Tan, S., and T. J. Richmond. 1998. Crystal structure of the yeast MATalpha2/MCM1/DNA ternary complex. Nature 391:660–666.

31. Tsong, A. E., M. G. Miller, R. M. Raisner, and A. D. Johnson. 2003. Evolution of a combinatorial transcriptional circuit: a case study in yeasts. Cell 115:389–399.

32. Wandschke, H., F. Dromer, I. Improvisi, M. Lozano-Chiu, J. H. Rex, and D. Sanglard. 1998. Antifungal drug resistance in pathogenic fungi. Med. Mycol. 36:119–128.

33. Wandschke, H., D. W. Warnock, B. Dupont, D. Kerridge, S. Sen Gupta, L. Imperisio, P. Marichal, F. C. Odds, F. Provost, and O. Ronin, 1994. Mechanisms and clinical impact of antifungal drug resistance. J. Med. Vet. Mycol. 32:189–202.

34. Zhong, H., R. McCord, and A. K. Vershon. 1999. Identification of target sites of the alpha2-Mcm1 repressor complex in the yeast genome. Genome Res. 9:1040–1047.

35. Zhong, H., and A. K. Vershon. 1997. The yeast homeodomain protein MATalpha2 shows extended DNA binding specificity in complex with MCM1. J. Biol. Chem. 272:8402–8409.

36. Zhu, J., and M. Q. Zhang. 1999. SCPD: a promoter database of the yeast Saccharomyces cerevisiae. Bioinformatics 15:607–611.