Multiple *cis*-Acting Sequences Mediate Upregulation of the *MDR1* Efflux Pump in a Fluconazole-Resistant Clinical *Candida albicans* Isolate

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Overexpression of the *MDR1* gene, which encodes a multidrug efflux pump of the major facilitator superfamily, is a frequent cause of resistance to the antimycotic agent fluconazole and other metabolic inhibitors in clinical *Candida albicans* strains. Constitutive *MDR1* overexpression in such strains is caused by mutations in as yet unknown *trans*-regulatory factors. In order to identify the *cis*-acting sequences in the *MDR1* regulatory region that mediate constitutive *MDR1* upregulation, we performed a promoter deletion analysis in the genetic background of an *MDR1*-overexpressing clinical *C. albicans* isolate. We found that several different regions in the *MDR1* promoter can mediate *MDR1* overexpression in this isolate. In contrast, deletion of one of these regions abolished benomyl-induced *MDR1* expression in a *C. albicans* laboratory strain. These results suggest that multiple transcription factors control expression of the *MDR1* efflux pump in *C. albicans* and that the mutation(s) that causes constitutive *MDR1* overexpression and drug resistance in clinical *C. albicans* isolates affects the activities of several of these transcription factors.

The yeast *Candida albicans* is a member of the microflora on mucosal surfaces of the gastrointestinal and urogenital tracts in many healthy people, but it can also cause superficial as well as life-threatening systemic infections, especially in immunocompromised patients (21). Oropharyngeal candidiasis, which frequently affects patients infected with the human immunodeficiency virus and AIDS patients, is commonly treated with the antimycotic agent fluconazole, which inhibits the biosynthesis of ergosterol, the major sterol in the fungal cell membrane. *C. albicans* can develop resistance to fluconazole, especially during long-term treatment of oropharyngeal candidiasis (30). Fluconazole resistance is caused by different molecular mechanisms, including alterations in the sterol biosynthetic pathway; overexpression of the *ERG11* gene, which encodes the target enzyme of fluconazole, sterol 14 α -demethylase (Erg11p); mutations in the *ERG11* gene that result in a reduced affinity of Erg11p to fluconazole; and overexpression of genes encoding membrane transport proteins which actively transport fluconazole out of the cell. In clinical *C. albicans* strains, several of these mechanisms are often combined to result in the stepwise development of clinically relevant fluconazole resistance (for a review, see reference 18).

Two types of efflux pumps that mediate resistance to fluconazole and structurally unrelated toxic compounds have been identified in *C. albicans* (6, 23–25). While *CDR1* and *CDR2* belong to the ATP-binding cassette (ABC) transporters, *MDR1* is a member of the major facilitator superfamily which uses the proton gradient across the cytoplasmic membrane as an energy source for transport. In drug-susceptible *C. albicans* strains, *MDR1* is expressed only at low levels in standard laboratory media, but its expression can be induced by some toxic compounds, like benomyl (10, 13, 27). In contrast, many flu-

conazole-resistant clinical *C. albicans* isolates constitutively overexpress *MDR1* (8, 9, 16, 22, 26, 29). Comparison of the *MDR1* promoter sequences in matched pairs of fluconazole-susceptible and *MDR1*-overexpressing, fluconazole-resistant *C. albicans* isolates from the same patient demonstrated that the constitutive *MDR1* upregulation in the resistant isolates was not caused by promoter mutations but was caused by alterations in a *trans*-regulatory factor(s) (31). However, in contrast to the ABC transporters *CDR1* and *CDR2*, whose expression has recently been shown to be controlled by the transcription factor Tac1p, which is mutated in *CDR1/CDR2*-overexpressing *C. albicans* strains (3, 4), the regulatory factors controlling *MDR1* expression and the mutations that are responsible for its constitutive activation in drug-resistant clinical isolates have not yet been identified.

It is likely that transcription factors that normally activate *MDR1* only under inducing conditions are constitutively active in *MDR1*-overexpressing, drug-resistant *C. albicans* isolates. Alternatively, *MDR1* upregulation may also result from relief of repression by a negative regulatory factor. To control *MDR1* expression, such transcription factors must bind to their target sequences within the *MDR1* promoter. Therefore, to gain more insight into the mechanisms that lead to *MDR1* upregulation, we set out to identify the *cis*-regulatory sequences in the *MDR1* promoter which mediate its constitutive overexpression in drug-resistant clinical *C. albicans* isolates. To this end, we generated a series of *MDR1* promoter deletion derivatives and determined their activities in the genetic background of such a clinical isolate.

MATERIALS AND METHODS

Strains and growth conditions. The *C. albicans* strains used in this study are listed in Table 1. All strains were stored as frozen stocks with 15% glycerol at –80°C. The strains were propagated on synthetic defined medium (SD) agar plates containing 6.7 g of yeast nitrogen base without amino acids (YNB; Bio 101, Vista, Calif.), 20 g of glucose, 0.77 g of complete supplement medium without uracil (CSM-URA; Bio 101), and 15 g of agar per liter. For routine growth of the strains, YPD liquid medium (20 g of peptone, 10 g of yeast extract, 20 g of

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TABLE 1. *C. albicans* strains used in this study

Strain	Parent	Relevant genotype or characteristic	Reference
CAI4		<i>ura3Δ::imm434/ura3Δ::imm434</i>	7
F5		<i>MDR1</i> -overexpressing clinical isolate	8
F5U1	F5	<i>URA3/ura3Δ::MPA^R-FLIP^a</i>	This study
F5U2	F5U1	<i>URA3/ura3Δ::FRT</i>	This study
F5U3	F5U2	<i>ura3Δ::FRT/ura3Δ::MPA^R-FLIP</i>	This study
F5U4	F5U3	<i>ura3Δ::FRT/ura3Δ::FRT</i>	This study

^a *MPA^R-FLIP*, the *MPA^R* flipper cassette.

glucose, 15 g of agar per liter) was used. To support the growth of *ura3* strains, 100 μ g ml⁻¹ uridine was added to the medium.

Plasmid constructions. To generate the *P_{MDR1}-GFP* fusion, a 1.1-kb *MDR1* promoter fragment was amplified by PCR from plasmid pGFP50 (31) with primers MDR1p7 and MDR1p9 (see Table 2 for the primer sequences), and the PCR product was digested at the *XhoI* and *BamHI* sites introduced at positions -1101 and -9, respectively, with respect to the position of the *MDR1* start codon. The *GFP* gene from pGFP31 (19) was amplified with primers GFP13 and GFP4, and the PCR product was digested at the *BamHI* site introduced before the start codon (boldface in Table 2) and at an internal *NcoI* site. Sequences from the *ACT1*-coding region (positions +862 to +1547) were amplified from pGFP31 with primers ACT23 and ACT22. The PCR product was digested at the *KpnI* and *XhoI* sites that were introduced and was cloned together with the *P_{MDR1}* fragment and the N-terminal *GFP* fragment into *KpnI/NcoI*-digested pGFP31 to result in pMPG1. *ACT1* upstream sequences (positions -762 to +48) were then amplified with primers ACT24 and ACT25. The PCR product was digested at the *SacII* and *PstI* sites that were introduced and was cloned into *SacII/PstI*-digested pMPG1 to produce pMPG2, in which the *GFP* reporter gene is placed under control of the *MDR1* promoter (see Fig. 1).

Sequential truncations of the *MDR1* promoter starting from position -913, -588, -495, -397, -300, or -286 were generated by amplification of *MDR1* promoter fragments with primer MDR1p9 and one of the following primers: MDR1p16, MDR1p17, MDR1p19, MDR1p20, MDR1p18, or MDR1p21. The PCR products were digested at the *XhoI* and *BamHI* sites that were introduced and were substituted for the full-length *MDR1* promoter in pMPG2 to generate pMPG7, pMPG8, pMPG9, pMPG10, pMPG12, and pMPG13, respectively.

Internal deletions within the *MDR1* promoter region (see Fig. 2) were made as follows. Digestion of pMPG2 at two *XbaI* sites located at positions -916 and -592 within the *MDR1* promoter and religation resulted in pMPG3. To construct pMPG4, a distal *MDR1* promoter fragment (positions -1097 to -600) was amplified with primer pair MDR1p7/MDR1p10, and a proximal *MDR1* promoter fragment (positions -496 to -10) was amplified with primer pair MDR1p9/MDR1p11. The PCR products were digested with *XhoI/EcoRI* and *EcoRI/BamHI*, respectively, and cloned together into *XhoI/BamHI*-digested pMPG2. Additional deletion constructs were made in an analogous fashion: for pMPG5, distal (positions -1097 to -500) and proximal (positions -287 to -10) *MDR1* promoter fragments were amplified with the primer pairs MDR1p7/MDR1p12 and MDR1p9/MDR1p13 and substituted for the *MDR1* promoter fragment in pMPG2. For pMPG6, the primer pairs MDR1p7/MDR1p14 and MDR1p9/MDR1p15 were used in the same way to delete the region from positions -272 to -101. To construct pMPG11, the distal and proximal *MDR1* promoter fragments from pMPG4 and pMPG5, respectively, were fused and substituted for the *MDR1* promoter in pMPG2. For pMPG14, pMPG15, pMPG16, and pMPG17, which contain deletions from positions -312 to -270, positions -355 to -309, positions -275 to -240, and positions -242 to -209, respectively, distal and proximal *MDR1* promoter fragments were amplified with the primer pairs MDR1p7/MDR1p23 and MDR1p9/MDR1p22, MDR1p7/MDR1p25 and MDR1p9/MDR1p24, MDR1p7/MDR1p14 and MDR1p9/MDR1p26, and MDR1p7/MDR1p27 and MDR1p9/MDR1p28, respectively, and substituted for the *MDR1* promoter fragment in pMPG2. To obtain pMPG19 containing a deletion from positions -205 to -101, a distal *MDR1* promoter fragment was amplified with the primers MDR1p7/MDR1p29 and substituted for the corresponding fragment in pMPG6.

Truncated *MDR1* promoter regions containing additional internal deletions (see Fig. 3 and 4) were obtained as described below and substituted for the full-length *MDR1* promoter in pMPG2. For pMPG20, part of the *MDR1* promoter from pMPG5 was amplified with primers MDR1p20 and MDR1p9. To produce pMPG21, the distal *MDR1* promoter fragment from pMPG20 was fused with the proximal *MDR1* promoter fragment from pMPG17. For pMPG22, part of the *MDR1* promoter from pMPG14 was amplified with primers MDR1p21

TABLE 2. Primers used in this study

Primer	Sequence ^a
ACT22	5'-GGTATTTTGACCTCGAGATACCCAATTG-3'
ACT23	5'-GACATAACAATGGTACCGGTATAATTG-3'
ACT24	5'-CTTTAAACATGCGCGGAATATACAG-3'
ACT25	5'-CCAATCAATGCGAGAATCAATATAG-3'
GFP4	5'-TCTGGTAAAAGGACAGGGC-3'
GFP13	5'-ATATAGGATCCAAATGAGTAAAGGAGAAGAACTTTTC-3'
MDR1p7	5'-CGTAAATCTCGAGAAACGGACTCCG-3'
MDR1p9	5'-ATTGTGGATCCCTATGTAAGTAGATGTATTGC-3'
MDR1p10	5'-ATGCTGAATTCCTTTTATTGGCATAGTGGG-3'
MDR1p11	5'-CATCAGAAATTCATTTTAGGAAATTTACCG-3'
MDR1p12	5'-TTCCTAAATGAATTTCTGATGTAACAAA-3'
MDR1p13	5'-ACGGTGAATTCCTAATGGGAAAAATACCG-3'
MDR1p14	5'-CGGTAGAATTCCTCAATAGGATTTTACCGTTG-3'
MDR1p15	5'-GGGATGGGATATCGAATTCAGTTTCCAAC-3'
MDR1p16	5'-CACTACTCGAGAATTAAGGAAATAAGTTGATCG-3'
MDR1p17	5'-ACGGTCTCGAGCCTAATGGGAAAAATACCG-3'
MDR1p18	5'-GCTACCACTCGAGCACACGGTAAAAATCC-3'
MDR1p19	5'-CATCTCGAGTCAATTTAGGAAATTTACCG-3'
MDR1p20	5'-AAAAAGCTCGAGGAAAAAAGCGGATTTAC-3'
MDR1p21	5'-GCCCGTCTCGAGCGCCGTTTTCCTTGGCGTGGC-3'
MDR1p22	5'-GGGAATTCCTACCGAATTAAGGAAATAAG-3'
MDR1p23	5'-TAATTGAATTCACACCCATAATTGTTGTAC-3'
MDR1p24	5'-GGGTGTTGAATTCAGTTAATCACACGGTAAATCC-3'
MDR1p25	5'-AACAAAGAATTCAAAAGTAGCCACGGAAAAATGCCACGGC-3'
MDR1p26	5'-GATTTTGAATTCGGAAATATATTATTCTTC-3'
MDR1p27	5'-AATTTCCGAATTCAAAATCTTAGGTTGTGTATTC-3'
MDR1p28	5'-CGCTTGAATTCATAAACTTCTATCGCGAAATG-3'
MDR1p29	5'-CGCGATAGAAGGAATTCGAAAATAAGCGATGAAG-3'
MDR1p31	5'-CAACTCGAGTGGTAAGTATTGGCGAAAG-3'
MDR1p32	5'-TCTTTCGGAATTCCTTACAGTAGAGTTGT-3'

^a The restriction sites introduced into the primers are underlined; the *GFP* start codon in primer GFP13 is highlighted in boldface.

and MDR1p9. For pMPG23, part of the *MDR1* promoter from pMPG20 was amplified with primers MDR1p31 and MDR1p9. To create pMPG24, the distal *MDR1* promoter fragment from pMPG22 was fused with the proximal *MDR1* promoter fragment from pMPG11. For pMPG26, part of the *MDR1* promoter from pMPG16 was amplified with primers MDR1p21 and MDR1p9. pMPG27 was obtained by amplifying the distal *MDR1* promoter fragment and flanking *ACT1* sequences from pMPG20 with primers ACT23 and MDR1p32 and fusing it with the proximal *MDR1* promoter fragment from pMPG20.

***C. albicans* transformation.** *C. albicans* strains were transformed by electroporation (14) with the following gel-purified, linear DNA fragments: the *SacI*-*SphI* fragment from pSFIU4 (28) to inactivate the *URA3* gene in strain F5 and the *KpnI*-*SacII* fragments from pMPG2 and its derivatives to integrate the *P_{MDR1}-GFP* fusions into one of the *ACT1* alleles of strains F5U4 or CAI4 with the help of the *URA3* marker. Uridine-prototrophic transformants were selected on SD agar plates and mycophenolic acid (MPA)-resistant transformants were selected on SD agar plates containing 10 μ g ml⁻¹ MPA (Sigma, Deisenhofen, Germany), as described previously (20). Single-copy integration of all constructs was confirmed by Southern hybridization.

Isolation of genomic DNA and Southern hybridization. Genomic DNA from *C. albicans* strains was isolated as described previously (17). DNA (10 μ g) was digested with the appropriate restriction enzymes, separated in 1% (wt/vol) agarose gels, and after ethidium bromide staining, transferred by vacuum blotting onto nylon membranes and fixed by UV cross-linking. Southern hybridization with enhanced chemiluminescence (ECL)-labeled probes was performed with the ECL labeling and detection kit from Amersham (Braunschweig, Germany), according to the instructions of the manufacturer.

Fluorescence microscopy and flow cytometry. To detect and quantify *GFP* expression in the reporter strains, cells grown overnight in YPD medium were pelleted, suspended in phosphate-buffered saline to a density of 2×10^5 cells per ml, and kept on ice. To induce the *MDR1* promoter in transformants of strain CAI4, the overnight cultures were mixed with an equal volume of fresh YPD medium containing 50 μ g ml⁻¹ benomyl and incubated for 3 h before harvesting of the cells. Fluorescence microscopy was performed with a Zeiss LSM 510 inverted confocal laser scanning microscope equipped with a Zeiss Axiovert 100 microscope. Imaging scans were acquired with an argon laser with a wavelength

of 488 nm and corresponding filter settings for green fluorescent protein (GFP) and parallel transmission images. The cells were observed with a $\times 63$ immersion oil objective. Fluorescence-activated cell sorter (FACS) analysis was performed with a FACSCalibur cytometry system equipped with an argon laser emitting at 488 nm (Becton Dickinson, Heidelberg, Germany). Fluorescence was measured on the FL1 fluorescence channel equipped with a 530-nm band-pass filter. Ten thousand cells were analyzed per sample and were counted at low flow rate. Fluorescence and forward scatter data were collected by using logarithmic amplifiers. Data analysis was performed on fluorescence intensities that excluded cell autofluorescence and cell debris. The mean fluorescence values were determined with CellQuest Pro (Becton Dickinson) software.

RESULTS

Generation of a *ura3Δ* derivative of *MDR1*-overexpressing, clinical *C. albicans* isolate F5. In previous work we had ectopically integrated the *GFP* reporter gene under the control of the *MDR1* promoter (P_{MDR1}) into the *CDR4* locus in matched pairs of fluconazole-resistant and fluconazole-susceptible clinical *C. albicans* isolates with the help of the dominant MPA^R selection marker to demonstrate that the constitutive *MDR1* overexpression observed in the resistant isolates was caused by alterations in a *trans*-regulatory factor and not by mutation of the *MDR1* promoter (31). As the MPA^R marker seems to integrate preferentially into the *IMH3* gene from which it is derived, many MPA -resistant transformants had to be screened to identify those that contained the P_{MDR1} -*GFP* fusion (31). Therefore, to facilitate integration of a larger number of reporter constructs containing *GFP* under the control of *MDR1* promoter deletion derivatives, we decided to generate a *ura3Δ* mutant of fluconazole-resistant isolate F5, which would allow introduction of the reporter fusions with the help of the *URA3* marker. For this purpose, we transformed strain F5 with a *URA3* deletion construct in which the MPA^R flipper cassette (32) was inserted between *URA3* flanking sequences. This construct had previously been used to create *uraΔ* mutants of the *C. albicans* model strain WO-1 (28). Two rounds of integration of the MPA^R flipper cassette and subsequent FLP-mediated excision of the cassette resulted in strain F5U4, in which both *URA3* alleles were specifically deleted from the genome (Table 1). *uraΔ* mutant F5U4 retained the drug-resistant phenotype of its parental strain, strain F5 (data not shown), and the constitutive activation of the *MDR1* promoter (see below), demonstrating that it could be used to identify the sequences in the *MDR1* promoter that mediated its upregulation by the unknown regulatory factor(s).

Analysis of *MDR1* promoter activity in strain F5U4. To verify the constitutive activation of the *MDR1* promoter in strain F5U4, we placed the *GFP* reporter gene under the control of the same 1.1-kb *MDR1* promoter fragment used in our previous studies (31), but in a cassette containing the *URA3* selection marker instead of the MPA^R marker. In addition, we used flanking sequences from the *ACT1* gene instead of the inconveniently long *CDR4* flanking sequences, such that the P_{MDR1} -*GFP* fusions would be integrated into the *ACT1* locus in an orientation opposite that of the *ACT1* gene, and introduced unique restriction sites upstream and downstream of the *MDR1* promoter fragment to facilitate the generation of deletion derivatives (see Materials and Methods) (Fig. 1).

Integration of the P_{MDR1} -*GFP* fusion from plasmid pMPG2 into strain F5U4 resulted in fluorescence of the cells, whereas no fluorescence was observed when the same reporter fusion

was integrated in an identical fashion into fluconazole-sensitive laboratory strain CAI4, which does not detectably express the *MDR1* gene (Fig. 1 and data not shown). This result confirmed that the 1.1-kb *MDR1* promoter fragment (for the purpose of this study referred to as the full-length *MDR1* promoter) integrated at the *ACT1* locus was constitutively active in the genetic background of clinical *C. albicans* isolate F5 and did not exhibit detectable activity in a laboratory strain.

Multiple *cis*-acting sequences mediate constitutive *MDR1* upregulation in clinical *C. albicans* isolate F5. We then constructed a series of reporter fusions with shorter *MDR1* promoter regions, which were integrated in an analogous fashion in strain F5U4, to identify the sequences in the *MDR1* promoter that mediated its constitutive activation in clinical isolate F5. As can be seen in Fig. 1, deletion of *MDR1* promoter sequences distal to position -495 (plasmids pMPG7, pMPG9, and pMPG10) had no effect on its activity, and a further deletion to position -397 (pMPG13) affected only the level of expression but not the constitutive activity of the promoter. However, removal of the next 100 bp to position -300 (pMPG12) or -286 (pMPG8) abolished the activity of the *MDR1* promoter, suggesting that sequences located between positions -397 and -301 (hereafter termed activating region 1) are required for constitutive *MDR1* expression in isolate F5.

We also constructed a set of internal deletions within the full-length *MDR1* promoter (Fig. 2). As expected from the results described above, deletion of sequences from positions -916 to -593 (pMPG3) or from positions -599 to -497 (pMPG4) did not affect the activity of the *MDR1* promoter, whereas a deletion comprising the region from positions -599 to -288 (pMPG11) abolished *MDR1* promoter activity. Surprisingly, however, deletion of the region from positions -499 to -288 (pMPG5) had only a minor effect on *MDR1* promoter activity; i.e., activating region 1 was dispensable for constitutive *MDR1* expression when the sequences upstream of position -499 were present. Small deletions ranging from positions -355 to -309 , -312 to -272 , -275 to -240 , or -242 to -209 (plasmids pMPG14 to pMPG17, respectively) had only a minor effect or no effect on *MDR1* promoter activity in strain F5U4, whereas deletion of sequences from positions -272 to -101 (pMPG6) or from positions -205 to -101 (pMPG19) abolished *MDR1* expression. The *MDR1* transcriptional start site has been mapped to position -65 (12); therefore, it is likely that the latter deletions affected the *MDR1* core promoter.

To delimit the region that could mediate *MDR1* upregulation in the absence of activating region 1, additional *MDR1* promoter truncation-deletion constructs were tested (Fig. 3). A fragment ranging from positions -588 to -500 (hereafter termed activating region 2) was sufficient to mediate *MDR1* expression in strain F5U4 when *MDR1* promoter sequences starting from position -287 were also present (pMPG20). Neither of two overlapping fragments within activating region 2, either that from positions -588 to -547 (plasmid pMPG27) or that from positions -552 to -500 (plasmid pMPG23), was sufficient to mediate *MDR1* expression in this context. The latter fragment contained a Yap1p response element (YRE), located between positions -532 and -526 , indicating that YRE alone was not sufficient to mediate the constitutive *MDR1* activation conferred by the whole activating region 2. Activating region 2 was unable to mediate *MDR1* expression

FIG. 1. *MDR1* promoter analysis. The structure of the insert of plasmid pMPG2 containing the P_{MDR1} -GFP reporter fusion is shown on top. The *MDR1* promoter is symbolized by the bent arrow; the *GFP* gene, which is fused to the transcription termination sequence of the *ACT1* gene (T_{ACT1} ; filled circle), is symbolized by the gray arrow; and the *URA3* selection marker is symbolized by the hatched arrow. The *ACT1* flanking sequences (position numbers are with respect to the position of the *ACT1* start codon) used for ectopic integration of the P_{MDR1} -GFP fusion into the *ACT1* locus are represented by the white arrows, which also indicate the orientation of the *ACT1* gene at the integration site. The relevant restriction sites used for the plasmid constructions are shown. Enlarged representations of the *MDR1* regulatory region and deletion derivatives are shown below, and the names of the corresponding plasmids are indicated to the left. The extent of the *MDR1* promoter sequences contained in the various plasmids is given. The transcriptional start site mapped at position -65 by Harry et al. (12) is indicated by the small bent arrow, and activating regions 1 to 3 identified in the present study are indicated by the black bars in pMPG2. The YRE is indicated by the small gray box. The fluorescence micrographs to the right of each construct show cells of strain F5U4 carrying the corresponding reporter fusion for illustration, and the mean fluorescence of the cells measured by flow cytometry is given. The inducibilities of the various *MDR1* promoter derivatives by benomyl were also determined by measuring the fluorescence of transformants of strain CAI4 carrying the same reporter fusions in the presence of benomyl. All values are the means of results obtained for two independently constructed reporter strains.

when the remaining *MDR1* promoter sequences were shortened to position -208 (pMPG21), indicating that the region located between positions -287 and -209 contains an additional activating region, activating region 3.

In an attempt to define essential sequences in the proximal *MDR1* promoter region, we generated another series of *MDR1* promoter truncation-deletion derivatives (Fig. 4). Small deletions were introduced into the *MDR1* promoter fragment starting from position -397 (pMPG13). All these overlapping deletions, comprising the sequences from positions -312 to

-288 (pMPG24), positions -312 to -272 (pMPG22), or positions -275 to -240 (pMPG26), abolished *MDR1* expression in strain F5U4. Interestingly, the same deletions, which presumably affected activating region 1 (pMPG24 and pMPG22) or activating region 3 (pMPG26), had almost no effect when they were introduced into the full-length promoter (compare pMPG22 and pMPG26 in Fig. 4 with pMPG14 and pMPG16 in Fig. 2).

Altogether, these results demonstrate that multiple *cis*-acting sequences within the *MDR1* promoter mediate its consti-

FIG. 2. Structures of *MDR1* promoter derivatives carrying internal deletions (indicated by the dashed lines). The extent of the *MDR1* promoter sequences in each derivative is indicated, and the names of the plasmids containing the corresponding P_{MDR1} -*GFP* reporter fusions are given to the left. The constitutive and benomyl-inducible activities of the *MDR1* promoter derivatives in strains F5U4 and CAI4, respectively, were determined as explained in the legend to Fig. 1. The results obtained with strains carrying the P_{MDR1} -*GFP* fusion contained in pMPG2 are taken from Fig. 1 and are included for comparison.

tutive activation in clinical *C. albicans* isolate F5. These *cis*-acting sequences include activating region 1, located between positions -397 and -301 (and possibly beyond), and activating region 2, located between positions -588 and -500. Together

with proximal *MDR1* promoter sequences starting from position -300, either of these regions is sufficient to mediate constitutive *MDR1* upregulation. Sequences between positions -287 and -209 contain a third activating region that is re-

FIG. 3. Structures and activities of truncated *MDR1* promoter derivatives with internal deletions. For explanations, see the legend to Fig. 1. The results obtained with strains carrying the P_{MDR1} -GFP fusions contained in pMPG2 and pMPG5 are taken from Fig. 2 and are included for comparison.

quired for the activity of activating region 2 (compare pMPG20 and pMPG21 in Fig. 3) as well as activating region 1 (compare pMPG13 and pMPG26 in Fig. 4) but that is dispensable in the full-length *MDR1* promoter (compare pMPG2 with pMPG16 and pMPG17 in Fig. 2). Other sequences, e.g., the region between positions -495 and -397 (compare pMPG9 and pMPG13 in Fig. 1), are not required for constitutive *MDR1* expression in isolate F5 but influence *MDR1* expression levels.

Activating region 2 mediates benomyl-induced *MDR1* expression in drug-susceptible *C. albicans* laboratory strain CAI4. In drug-susceptible *C. albicans* isolates, *MDR1* is expressed only at low, basal levels (usually below the detection limit in Northern hybridization experiments), but *MDR1* expression can be induced in these strains by certain chemicals, like benomyl (10, 13, 27). To investigate whether the same sequences that are required for constitutive *MDR1* upregulation in clinical isolate F5 are also involved in inducible *MDR1* expression in a drug-susceptible strain, we introduced all our reporter constructs into laboratory strain CAI4. None of the promoter deletion derivatives exhibited detectable activity in the absence of an inducing substance, suggesting that *MDR1* expression is not under negative control, which might have been relieved after deletion of a binding site for a repressor. Benomyl efficiently induced the wild-type *MDR1* promoter in strain CAI4 (Fig. 1, pMPG2); however, in contrast to the constitutive *MDR1* expression in strain F5U4, benomyl-induced *MDR1* expression depended on the presence of activat-

ing region 2. Whereas the truncated *MDR1* promoter fragment starting at position -495 retained full activity in strain F5U4, the same promoter fragment was inactive and could not be induced by benomyl in strain CAI4 (Fig. 1, pMPG9). Furthermore, while deletion of sequences from positions -599 to -495, which contain activating region 2, from the full-length *MDR1* promoter did not affect its activity in strain F5U4, the same deletion abolished benomyl-induced *MDR1* expression in strain CAI4 (Fig. 2, pMPG4). Of note, activating region 2 was not sufficient for benomyl induction when the region from positions -499 to -287 was deleted (Fig. 3, pMPG20). These results indicate that the constitutive activation of the *MDR1* promoter observed in clinical isolate F5 involves mechanisms different from those used to induce the promoter in the presence of benomyl.

DISCUSSION

In this work we tried to define the *cis*-acting sequences in the *MDR1* promoter that mediate the constitutive overexpression of this efflux pump in fluconazole-resistant, clinical *C. albicans* isolates. In a simple model, such drug-resistant isolates may contain a mutation in a transcription factor regulating *MDR1* expression which rendered the transcription factor constitutively active. Deletion of the binding site for this transcription factor from the *MDR1* promoter should therefore abolish its constitutive upregulation in a drug-resistant *C. albicans* strain.

FIG. 4. Structures and activities of truncated *MDR1* promoter derivatives with internal deletions in the region between positions -313 and -239 . For explanations, see the legend to Fig. 1. The results obtained with strains carrying the P_{MDR1} -GFP fusions contained in pMPG2 and pMPG13 are taken from Fig. 1 and are included for comparison.

Such a situation has recently been described for the overexpression of the ABC transporter *CDR2*, whose expression is controlled by the transcription factor Tac1p. Gain-of-function mutations in *TAC1* result in *CDR2* (and *CDR1*) upregulation in drug-resistant *C. albicans* strains, and this upregulation depends on a single Tac1p binding site in the *CDR2* promoter (3–5). The results of our promoter analysis indicate that the constitutive upregulation of *MDR1* is more complex. We identified multiple *cis*-activating regions in the *MDR1* promoter which in different combinations could mediate its activation in a drug-resistant *C. albicans* isolate. None of these regions was essential for constitutive *MDR1* expression in drug-resistant isolate F5 since each of them could be deleted in the full-length *MDR1* promoter without a loss of its activity, but the presence of at least two of them was required. A comparison of the three activating regions identified did not reveal sequences with striking similarity, suggesting that different transcription factors bind to these regions and activate the *MDR1* promoter. Therefore, the constitutive *MDR1* upregulation in clinical isolate F5 can be explained by different models. One possibility is that a gain-of-function mutation has occurred in a regulatory protein that controls the activities of several transcription factors that bind to the different activating regions in the *MDR1* promoter. Such a mutation would result in the simultaneous activation of these transcription factors, which, in turn, can act together in different combinations to mediate *MDR1* expression. Alternatively, several transcription factors might bind to each of the activating regions, including one that is common to all activating complexes and may be recruited by different factors to their binding sites. A mutation in such a transcription factor would also result in the simultaneous activation of sev-

eral different activating regions. It is indeed likely that more than one transcription factor binds to the activating regions identified in the *MDR1* promoter. For example, sequences from positions -552 to -500 containing the Yap1p response element could not substitute for the complete activating region 2 located between positions -588 and -500 . Therefore, the *C. albicans* homologue of Yap1p, Cap1p, which has been shown to induce expression of the *MDR1* homologue *FLR1* in *Saccharomyces cerevisiae* and which is also involved in the regulation of *MDR1* expression in *C. albicans* (1, 2), may bind to activating region 2 and mediate *MDR1* upregulation, but only in combination with a second transcription factor binding within this region. *MDR1* upregulation in other drug-resistant *C. albicans* strains may be caused by a similar mechanism, since strikingly similar alterations in the protein expression pattern were recently found in isolate F5 and an unrelated *MDR1*-overexpressing clinical *C. albicans* isolate compared with the patterns in their matched drug-susceptible isolates (15). In contrast, the inducible *MDR1* expression that is also seen in drug-susceptible *C. albicans* strains exposed to certain chemicals seems to occur by a different mechanism. We found that activating region 2 was essential for benomyl-induced *MDR1* expression in laboratory strain CAI4, whereas this region was dispensable for constitutive *MDR1* upregulation in clinical isolate F5 when activating regions 1 and 3 were present. Therefore, *MDR1* induction by benomyl may involve one but not all of the regulatory factors that mediate *MDR1* overexpression in the clinical isolate. Karababa et al. recently compared the alterations in the gene expression profile of an *MDR1*-overexpressing, clinical *C. albicans* isolate and those seen upon induction of *MDR1* expression by benomyl in a laboratory strain

and found that the drug-induced gene expression only partially mimicked the alterations observed in the clinical isolate in which *MDR1* was constitutively overexpressed (13). These findings also indicate that the mechanisms resulting in constitutive *MDR1* overexpression in drug-resistant isolates are different from those leading to benomyl-induced *MDR1* expression and involve additional regulatory factors.

Harry et al. recently also performed an *MDR1* promoter deletion analysis with a *C. albicans* laboratory strain (11). Interestingly, they also identified two activating regions in the *MDR1* promoter that mediate its induction by various chemicals, and these regions correspond to activating regions 1 and 2 identified in our study. Those authors did not identify activating region 3 described by us because they used only truncated *MDR1* promoter constructs and no derivatives containing internal deletions, which were necessary to detect this third activating region. Similar to our results, activating region 2 (positions –600 to –500) was found in the study performed by Harry et al. (11) to mediate *MDR1* induction in response to the presence of different toxic chemicals. In striking contrast, however, in the study by Harry et al. (11) induction by benomyl did not require activating region 2 but depended on activating region 1 (positions –400 to –300). While it is possible that the contrasting results with respect to the *MDR1* promoter region involved in benomyl induction were caused by the differences in the origin of the *MDR1* promoter or the integration site of the reporter fusions in the two studies, we favor a different explanation. It is not known how toxic compounds like benomyl induce gene expression, which may be a secondary response of the cells to stress conditions caused by the presence of the chemical and may not involve direct binding of benomyl to a transcription factor. Differences in the protocols used to expose the cells to benomyl in the two studies may have affected the cells in different ways, resulting in the use of alternative pathways to induce *MDR1* expression.

The regulatory factors controlling expression of the *MDR1* multidrug efflux pump in *C. albicans* and the mutations that cause constitutive *MDR1* overexpression in drug-resistant clinical isolates still remain unknown. Our results demonstrating the involvement of several *cis*-activating regions in constitutive *MDR1* upregulation and the existence of different mechanisms of *MDR1* induction will help to explain how these regulatory factors control *MDR1* expression once they have been identified.

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REFERENCES

- Alarco, A. M., I. Balan, D. Talibi, N. Mainville, and M. Raymond. 1997. AP1-mediated multidrug resistance in *Saccharomyces cerevisiae* requires *FLR1* encoding a transporter of the major facilitator superfamily. *J. Biol. Chem.* **272**:19304–19313.
- Alarco, A. M., and M. Raymond. 1999. The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. *J. Bacteriol.* **181**:700–708.
- Coste, A. T., M. Karababa, F. Ischer, J. A. 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.
- Coste, A. T., 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.
- de Micheli, M., J. Bille, C. Schueller, and D. Sanglard. 2002. A common drug-responsive 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.
- 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 benomyl and methotrexate. *Mol. Gen. Genet.* **227**:318–329.
- Fonzi, W. A., and M. Y. Irwin. 1993. Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134**:717–728.
- Franz, R., S. L. Kelly, D. C. Lamb, D. E. Kelly, M. Ruhnke, and J. Morschhäuser. 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. *Antimicrob. Agents Chemother.* **42**:3065–3072.
- Franz, R., M. Ruhnke, and J. Morschhäuser. 1999. Molecular aspects of fluconazole resistance development in *Candida albicans*. *Mycoses* **42**:453–458.
- Gupta, V., A. Kohli, S. Krishnamurthy, N. Puri, S. A. Aalamgeer, S. Panwar, and R. Prasad. 1998. Identification of polymorphic mutant alleles of *CaMDR1*, a major facilitator of *Candida albicans* which confers multidrug resistance, and its in vitro transcriptional activation. *Curr. Genet.* **34**:192–199.
- Harry, J. B., B. G. Oliver, J. L. Song, P. M. Silver, J. T. Little, J. Choiniere, and T. C. White. 2005. Drug-induced regulation of the *MDR1* promoter in *Candida albicans*. *Antimicrob. Agents Chemother.* **49**:2785–2792.
- Harry, J. B., J. L. Song, C. N. Lyons, and T. C. White. 2002. Transcription initiation of genes associated with azole resistance in *Candida albicans*. *Med. Mycol.* **40**:73–81.
- Karababa, M., A. T. Coste, B. Rognon, J. Bille, and D. Sanglard. 2004. Comparison of gene expression profiles of *Candida albicans* azole-resistant clinical isolates and laboratory strains exposed to drugs inducing multidrug transporters. *Antimicrob. Agents Chemother.* **48**:3064–3079.
- Köhler, G. A., T. C. White, and N. Agabian. 1997. Overexpression of a cloned IMP dehydrogenase gene of *Candida albicans* confers resistance to the specific inhibitor mycophenolic acid. *J. Bacteriol.* **179**:2331–2338.
- Kusch, H., K. Biswas, S. Schwanfelder, S. Engelmann, P. D. Rogers, M. Hecker, and J. Morschhäuser. 2004. A proteomic approach to understanding the development of multidrug-resistant *Candida albicans* strains. *Mol. Genet. Genomics* **271**:554–565.
- Lopez-Ribot, J. L., R. K. McAtee, L. N. Lee, W. R. Kirkpatrick, T. C. White, D. Sanglard, and T. F. Patterson. 1998. Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. *Antimicrob. Agents Chemother.* **42**:2932–2937.
- Millon, L., A. Manteaux, G. Reboux, C. Drobacheff, M. Monod, T. Barale, and Y. Michel-Briand. 1994. Fluconazole-resistant recurrent oral candidiasis in human immunodeficiency virus-positive patients: persistence of *Candida albicans* strains with the same genotype. *J. Clin. Microbiol.* **32**:1115–1118.
- Morschhäuser, J. 2002. The genetic basis of fluconazole resistance development in *Candida albicans*. *Biochim. Biophys. Acta* **1587**:240–248.
- Morschhäuser, J., S. Michel, and J. Hacker. 1998. Expression of a chromosomally integrated, single-copy *GFP* gene in *Candida albicans*, and its use as a reporter of gene regulation. *Mol. Gen. Genet.* **257**:412–420.
- Morschhäuser, J., P. Staib, and G. Köhler. 2005. Targeted gene deletion in *Candida albicans* wild-type strains by *MPA^R* flipping. *Methods Mol. Med.* **118**:35–44.
- Odds, F. C. 1988. *Candida* and candidosis: a review and bibliography. Baillière Tindall, London, United Kingdom.
- Perea, S., J. L. Lopez-Ribot, W. R. Kirkpatrick, R. K. McAtee, R. A. Santillan, 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.* **45**:2676–2684.
- Prasad, R., P. De Wergifosse, A. Goffeau, and E. Balzi. 1995. Molecular cloning and characterization of a novel gene of *Candida albicans*, *CDR1*, conferring multiple resistance to drugs and antifungals. *Curr. Genet.* **27**:320–329.
- Sanglard, 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 ABC transporter gene. *Microbiology* **143**:405–416.
- Sanglard, 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.
26. Sanglard, D., K. Kuchler, F. Ischer, J. L. Pagani, M. Monod, and J. Bille. 1995. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob. Agents Chemother.* **39**:2378–2386.
 27. Staib, P., G. P. Moran, D. J. Sullivan, D. C. Coleman, and J. Morschhäuser. 2001. Isogenic strain construction and gene targeting in *Candida dubliniensis*. *J. Bacteriol.* **183**:2859–2865.
 28. Strauß, A., S. Michel, and J. Morschhäuser. 2001. Analysis of phase-specific gene expression at the single-cell level in the white-opaque switching system of *Candida albicans*. *J. Bacteriol.* **183**:3761–3769.
 29. White, T. C. 1997. Increased mRNA levels of *ERG16*, *CDR*, and *MDR1* correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. *Antimicrob. Agents Chemother.* **41**:1482–1487.
 30. White, T. C., K. A. Marr, and R. A. Bowden. 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin. Microbiol. Rev.* **11**:382–402.
 31. Wirsching, S., S. Michel, G. Köhler, and J. Morschhäuser. 2000. Activation of the multiple drug resistance gene *MDR1* in fluconazole-resistant, clinical *Candida albicans* strains is caused by mutations in a *trans*-regulatory factor. *J. Bacteriol.* **182**:400–404.
 32. Wirsching, S., S. Michel, and J. Morschhäuser. 2000. Targeted gene disruption in *Candida albicans* wild-type strains: the role of the *MDR1* gene in fluconazole resistance of clinical *Candida albicans* isolates. *Mol. Microbiol.* **36**:856–865.