ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2011, p. 2212–2223 Vol. 55, No. 5
0066-4804/11/$12.00 doi:10.1128/AAC.01343-10

Regulation of Efflux Pump Expression and Drug Resistance by the Transcription Factors Mrr1, Upc2, and Cap1 in *Candida albicans*▽†

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Received 1 October 2010/Returned for modification 16 December 2010/Accepted 1 March 2011

Constitutive overexpression of the Mdr1 efflux pump is an important mechanism of acquired drug resistance in the yeast *Candida albicans*. The zinc cluster transcription factor Mrr1 is a central regulator of *MDR1* expression, but other transcription factors have also been implicated in *MDR1* regulation. To better understand how *MDR1*-mediated drug resistance is achieved in this fungal pathogen, we studied the interdependence of Mrr1 and two other *MDR1* regulators, Upc2 and Cap1, in the control of *MDR1* expression. A mutated, constitutively active Mrr1 could upregulate *MDR1* and confer drug resistance in the absence of Upc2 or Cap1. On the other hand, Upc2 containing a gain-of-function mutation only slightly activated the *MDR1* promoter, and this activation depended on the presence of a functional *MRR1* gene. In contrast, a C-terminally truncated, activated form of Cap1 could upregulate *MDR1* in a partially Mrr1-independent fashion. The induction of *MDR1* expression by toxic chemicals occurred independently of Upc2 but required the presence of Mrr1 and also partially depended on Cap1. Transcriptional profiling and *in vivo* DNA binding studies showed that a constitutively active Mrr1 binds to and upregulates most of its direct target genes in the presence or absence of Cap1. Therefore, Mrr1 and Cap1 cooperate in the environmental induction of *MDR1* expression in wild-type *C. albicans*, but gain-of-function mutations in either of the two transcription factors can independently mediate efflux pump overexpression and drug resistance.

The overexpression of efflux pumps that transport endogenous metabolites as well as xenobiotics out of the cell is a common mechanism of resistance to drugs and other toxic compounds in organisms from bacteria to humans. Fungi possess two types of efflux pumps, ABC transporters and major facilitators, which use ATP or the proton gradient across the cytoplasmic membrane, respectively, to drive active transport of their substrates (9). In the pathogenic yeast *Candida albicans*, multidrug resistance is mediated mainly by the ABC transporters Cdr1 and Cdr2 and the major facilitator Mdr1 (22). These efflux pumps are usually expressed at low or nondetectable levels and are upregulated in the presence of certain chemicals. Constitutive overexpression of Cdr1 and Cdr2 or Mdr1 is frequently observed in *C. albicans* strains that have become resistant to the antifungal drug fluconazole, which inhibits ergosterol biosynthesis, especially after long-term therapy of oropharyngeal candidiasis in AIDS patients (35). The analysis of deletion mutants lacking these transporters has confirmed that their overexpression contributes to the multidrug-resistant phenotype of such strains (34, 36).

The transcription factors controlling the expression of multidrug efflux pumps in *C. albicans* have recently been identified. The zinc cluster transcription factor Tac1 mediates the upregulation of the *CDR1* and *CDR2* genes in response to inducing chemicals, and the constitutive overexpression of these efflux pumps in drug-resistant *C. albicans* strains is caused by gain-of-function mutations in Tac1 (5–8, 40). Another zinc cluster transcription factor, Mrr1, controls *MDR1* expression, and gain-of-function mutations in Mrr1 that render the transcription factor constitutively active are responsible for *MDR1* overexpression in all fluconazole-resistant strains investigated so far (10, 23, 30). Most drug-resistant strains that overexpress *CDR1* and *CDR2* or *MDR1* have become homozygous for hyperactive *TAC1* and *MRR1* alleles, respectively, because the loss of heterozygosity further increases drug resistance once a gain-of-function mutation in these transcription factors has been acquired (5, 6, 10, 23).

Mrr1 is essential for both the environmentally inducible expression of *MDR1* and the constitutive overexpression of the efflux pump in drug-resistant strains. However, other transcription factors have also been implicated in the regulation of *MDR1* expression. Upc2, another member of the zinc cluster transcription factor family, controls the expression of ergosterol biosynthesis (*ERG*) genes and mediates their upregulation in response to ergosterol depletion (20, 32). Upc2 also binds to the *MDR1* promoter and, depending on the growth conditions, activates or represses *MDR1* expression (41). A gain-of-function mutation in Upc2 that was found in a flucona-

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† Supplemental material for this article may be found at http://aac.asm.org/.

▽ Published ahead of print on 14 March 2011.

zole-resistant, clinical *C. albicans* isolate and caused constitutive upregulation of *ERG* genes also resulted in moderately elevated *MDR1* mRNA levels (11). The bZip transcription factor Cap1, which mediates the oxidative stress response in *C. albicans* (1, 37), was recently shown to bind to the *MDR1* promoter *in vivo* (39). Although deletion of *CAP1* in an *MDR1*-overexpressing strain did not reduce *MDR1* transcript levels, expression of a C-terminally truncated, hyperactive *CAP1* allele resulted in *MDR1* upregulation and multidrug resistance (1). These findings suggest that, in addition to Mrr1, Upc2 and Cap1 might also be important regulators of *MDR1* expression, and activating mutations in these transcription factors could be a cause of Mdr1-mediated drug resistance in clinical *C. albicans* strains. To gain insight into the regulatory network controlling *MDR1* expression and drug resistance in *C. albicans*, we investigated if Mrr1 requires Upc2 and Cap1 for upregulation of *MDR1* and other Mrr1 target genes and if Upc2 and Cap1 can mediate *MDR1* overexpression and drug resistance independently from Mrr1.

MATERIALS AND METHODS

Strains and growth conditions. The *C. albicans* strains used in this study are listed in Table S1 of the supplemental material. All strains were stored as frozen stocks with 15% glycerol at −80°C and subcultured on YPD agar plates (10 g yeast extract, 20 g peptone, 20 g glucose, 15 g agar per liter) at 30°C. Strains were routinely grown in YPD liquid medium at 30°C in a shaking incubator. For selection of nourseothricin-resistant transformants, 200 μg/ml nourseothricin (Werner Bioagents, Jena, Germany) was added to YPD agar plates. To obtain nourseothricin-sensitive derivatives in which the *SAT1* flipper cassette was excised by FLP-mediated recombination, transformants were grown overnight in YPM medium (10 g yeast extract, 20 g peptone, 20 g maltose per liter) without selective pressure to induce the *MAL2* promoter that controls expression of the *caFLP* gene in the *SAT1* flipper cassette. A total of 100 to 200 cells were then spread on YPD plates containing 20 μg/ml nourseothricin and grown for 2 days at 30°C. Nourseothricin-sensitive clones were identified by their small colony size and confirmed by restreaking on YPD plates containing 100 μg/ml nourseothricin as described previously (25).

Plasmid constructions. To obtain a *CAP1* deletion cassette, the *CAP1* upstream region was amplified from genomic DNA of strain SC5314 by PCR with the primers CAP1-5 (5′-TATTGAGCTCAGGATTGTAAACGGTGTGTC-3′) and CAP1-6 (5′-AGTTCCGCGGTATCATCTAGATTTGCTGGTG-3′). The PCR product was digested at the introduced SacI and SacII sites (underlined) and cloned into plasmid pSFS4, a derivative of pSFS2 (25) in which a SalI site in the *caSAT1* marker had been destroyed, to generate pCAP1M1. A fragment containing the *CAP1* downstream region was then amplified with the primers CAP1-7 (5′-TTCTCTCGAGGGAGTAGTGATAAATACTGC-3′) and CAP1-8 (5′-CATTGGGCCCTGAAGACAAGAGGGAAGGG-3′), digested at the introduced XhoI and ApaI sites (underlined), and cloned between the same sites in pCAP1M1 to produce pCAP1M2.

An *MDR1* deletion construct was obtained by amplifying the *MDR1* upstream and downstream regions with the primer pair MDR1M1 (5′-GCTCGTTTAGTTGGGCCCATTCGCATC-3′) and MDR1M2 (5′-GCATTCTCGAGTTCTATGTAAGTAGATGTATTGC-3′) and the primer pair MDR1M3 (5′-GATCTAAGTATGCTACCGCGGAGGTGTCATTG-3′) and MDR1M4 (5′-ATGAGAGCTCTACACCGTCTCACGCGTAAAGCC-3′), respectively. The PCR products were digested with ApaI/XhoI and SacII/SacI, respectively (restriction sites are underlined), and cloned on both sides of the *SAT1* flipper cassette in plasmid pCZF1M2 (24) to generate pMDR1M2.

Plasmid pUPC2R1, which was used to sequentially replace the *UPC2* wild-type alleles with the *UPC2*^{G648D} allele with the help of the *SAT1* flipper cassette, was described previously (12). Apart from the G648D gain-of-function mutation, which was originally identified in a fluconazole-resistant, clinical *C. albicans* isolate, the *UPC2* allele contained in pUPC2R1 is identical to one of the resident *UPC2* alleles (*UPC2-1*) of strain SC5314 (11). To obtain an analogous cassette for introduction of the *MRR1*^{P683S} allele, an XhoI-ApaI fragment from pZCF36M2 (23) containing the *MRR1* downstream region was first substituted for the *UPC2* downstream region in pUPC2R1, yielding pMRR1R1. A SacI-BglII fragment from pZCF36K3 (23) containing the *MRR1*^{P683S} allele was then inserted instead of the *UPC2*^{G648D} allele to obtain pMRR1R3. Apart from the P683S mutation, which was originally identified in a fluconazole-resistant, clinical *C. albicans* isolate, the *MRR1* allele contained in pMRR1R3 is identical to the *MRR1* alleles of strain SC5314 (23). Similarly, for introduction of the *CAP1*$^{\Delta C333}$ allele, a C-terminally truncated *CAP1* gene was first amplified from genomic DNA of strain SC5314 with the primers CAP1-1 (5′-ATATGTCGACAATGACAGATATTAAAAGAAATTTTTC-3′) and CAP1-4 (5′-ATATAGATC**TTA**GATCTTGAATGGAACCATTCTTGC-3′), digested at the introduced SalI and BglII sites (underlined), and cloned into pNIM6 (24). The stop codon (reverse sequence) introduced at codon 334 of *CAP1* is highlighted in bold. A SacI-BglII fragment containing the C-terminal part of the *CAP1*$^{\Delta C333}$ allele was then cloned together with a BglII-XhoI fragment from pMRR1R3 containing the *ACT1* transcription termination sequence (T_{ACT1}) and the *SAT1*-flipper cassette into SacI/XhoI-digested pCAP1M2 to generate pCAP1R1.

For C-terminal tagging of Mrr1 with a 3×HA epitope, the *ACT1* transcription termination sequence from plasmid pMPG2 (13) was amplified with the primers HAT6 (5′-TGCTAGGATCC*TACCCATACGATGTTCCGGATTACGCTTACCCATACGATGTTCCGGATTACGCTTACCCATACGATGTTCCGGATTACGCT***TAA**GAGTGAAATTCTGGA-3′) and URA16 (5′-TGTTTCCGCGGATACCATCCAAATCAATTCC-3′). The 3×HA epitope encoded in primer HAT6 is highlighted in italics, and the stop codon is shown in bold; the BamHI site, which creates a Gly-Ser linker, is underlined. The PCR product was digested with BamHI/SalI and cloned together with a SacI-BamHI *MRR1* fragment from pZCF36TF7-1 (23) and a SalI-ClaI *caSAT1* fragment from pSAT1 (25) in SacI/ClaI-digested pZCF36K2 (23) to obtain pZCF36H2, in which the 3×HA-tagged *MRR1* gene is placed under the control of its own promoter. An EcoRI-ClaI fragment from this plasmid was then substituted for the corresponding fragment in pZCF36E2 (30) to produce pZCF36EH2, which allows expression of the 3×HA-tagged *MRR1* gene from the *ADH1* promoter. Substitution of the *MRR1*^{P683S} allele for the wild-type *MRR1* allele in pZCF36H2 and pZCF36EH2 generated plasmids pZCF36H3 and pZCF36EH3, respectively.

***C. albicans* transformation.** *C. albicans* strains were transformed by electroporation (17) with the following gel-purified linear DNA fragments: the SacI-ApaI fragments from pCAP1M2 and pMDR1M2 were used to delete the *CAP1* and *MDR1* genes, respectively, in strain SC5314. The SacI-ApaI fragments from pMRR1R3, pUPC2R1, and pCAP1R1 were used to substitute the hyperactive *MRR1*^{P683S}, *UPC2*^{G648D}, and *CAP1*$^{\Delta C333}$ alleles, respectively, for the corresponding wild-type alleles in various strain backgrounds. The KpnI-SacII fragment from pMPG2S (23) was used to integrate a P_{MDR1}-*GFP* reporter fusion at the *ACT1* locus of different strains (see Table S1 of the supplemental material). The SacI-ApaI fragments from pZCF36H2 and pZCF36H3 were used for expression of 3×HA-tagged wild-type *MRR1* and *MRR1*^{P683S} alleles, respectively, from the *MRR1* promoter in *mrr1*Δ mutants of strain SC5314 and in the reporter strain CAG48MRR1M4B. The ApaI-SacII fragments from pZCF36EH2 and pZCF36EH3 served to express these hemagglutinin (HA)-tagged *MRR1* alleles from the *ADH1* promoter in the same strains. The ApaI-SacII fragment from pZCF36EH3 was also used to express the 3×HA-tagged *MRR1*^{P683S} allele under the control of the *ADH1* promoter in *mrr1*Δ *cap1*Δ mutants of strain SC5314. Selection of nourseothricin-resistant transformants was performed as described previously (25), and the correct integration of each construct was confirmed by Southern hybridization with gene-specific probes.

Isolation of genomic DNA and Southern hybridization. Genomic DNA from *C. albicans* was isolated as described previously (25), digested with appropriate restriction enzymes, separated on a 1% agarose gel and, after ethidium bromide staining, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. Southern hybridization with enhanced chemiluminescence-labeled probes was performed with the Amersham ECL direct nucleic acid labeling and detection system (GE Healthcare, Braunschweig, Germany) according to the instructions of the manufacturer.

FACS analysis. 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. Twenty thousand cells were analyzed per sample and were counted at a flow rate of 500 cells per second. Fluorescence data were collected by using logarithmic amplifiers. The mean fluorescence values were determined with CellQuest Pro software (Becton Dickinson).

Drug susceptibility tests. Stock solutions of the drugs were prepared as follows. Fluconazole (5 mg/ml) was dissolved in water, and cerulenin (5 mg/ml) was dissolved in dimethyl sulfoxide (DMSO). In the assays, serial 2-fold dilutions in the assay medium were prepared from an initial concentration of 100 μg/ml of each drug. Susceptibility tests were carried out in high-resolution medium (14.67

g HR medium [Oxoid GmbH, Wesel, Germany], 1 g $NaHCO_3$, 0.2 M phosphate buffer [pH 7.2]), using a previously described microdilution method (28).

Transcription profiling. Gene expression profiles were obtained by hybridizing labeled cRNAs generated from *C. albicans* total RNA onto Affymetrix *C. albicans* custom expression arrays (CAN07; 49-5241 array format), which have been described elsewhere (11). Total RNA was isolated using the hot sodium dodecyl sulfate-phenol method, and subsequent cRNA synthesis and labeling was performed as previously described (11).

The cRNA pellet was suspended in 10 μl of RNase-free water, and 10 μg was fragmented by ion-mediated hydrolysis at 95°C for 35 min in 200 mM Tris-acetate (pH 8.1), 500 mM potassium acetate, 150 mM magnesium acetate. The fragmented cRNA was hybridized for 16 h at 45°C to the *C. albicans* Affymetrix expression arrays. Arrays were washed at 25°C with 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 0.01% Tween 20, followed by a stringent wash at 50°C with 100 mM MES [2-(*N*-morpholino)ethanesulfonic acid], 0.1 M NaCl, 0.01% Tween 20. Hybridizations and washes employed the Affymetrix Fluidics station 450 using their standard EukGE-WS2v5 protocol. The arrays were then stained with phycoerythrin-conjugated streptavidin (Molecular Probes), and the fluorescence intensities were determined using the GCS 3000 high-resolution confocal laser scanner (Affymetrix). The scanned images were analyzed using software resident in the GeneChip operating system v2.0 (Affymetrix). Sample loading and variations in staining were standardized by scaling the average of the fluorescent intensities of all genes on an array to a constant target intensity (250). The signal intensity for each gene was calculated as the average intensity difference, represented by [$\sum$(PM − MM)/(number of probe pairs)], where PM and MM denote perfect match and mismatch probes.

The scaled gene expression values from the GeneChip operating system v2.0 software were generated using the MAS5.0 algorithm. The value of each probe set was generated using Tukey's biweight computation. The algorithm considers the contribution of each match and mismatch probe corrected for the background of the average of the probe set. A weighted mean is then calculated for each probe pair. The raw signal values are then $\log_2$ transformed, and the array is normalized to this value. Probe sets were deleted from subsequent analysis if they were determined to be absent by the Affymetrix criterion. Pairwise comparison of gene expression levels was performed for each matched experiment. Among direct comparisons between strains, genes were considered to be differentially expressed if their change in expression was 2-fold or greater (for upregulated genes) or 0.5-fold or less (for downregulated genes) in both independent experiments of each comparison. For the purpose of direct comparison, the SCMRR1R34A versus SC5314 and SCMRR1R34B versus SC5314 comparisons are displayed in the same table (see Table S2 in the supplemental material) as the Δ*cap1*MRR1R34A versus SCCAP1M4A and Δ*cap1*MRR1R34B versus SCCAP1M4B comparisons. Cells highlighted in green are genes that were differentially expressed by the above criteria for the corresponding comparison/experiment. Cells that are not highlighted but for which there is a value are genes whose values did not meet the differential expression criterion for the corresponding comparison/experiment. Cells that are empty are genes which were called "absent" by the Affymetrix criteria for the corresponding comparison/experiment and thus the expression value was not valid. In addition, genes whose promoter regions were bound by Mrr1 as measured by chromatin immunoprecipitation (ChIP)-chip analysis are indicated by yellow highlighting. Data files for each scanned chip were submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/.)

Western immunoblot analysis. YPD overnight cultures of the strains were diluted to an optical density at 600 nm (OD_{600}) of 0.2 and grown at 30°C to an OD_{600} of 1.0. Cells were collected by centrifugation, washed twice in water, and broken by vortexing for 10 min at 4°C with 300 μl 0.5-mm glass beads in 300 μl breaking buffer (100 mM Tris-Cl [pH 7.5], 200 mM NaCl, 20% glycerol, 5 mM EDTA, 4% Complete EDTA-free protease inhibitor cocktail [Roche Diagnostics GmbH, Mannheim, Germany], 0.1% β-mercaptoethanol). Samples were centrifuged at 13,000 rpm for 5 min at 4°C, the supernatant collected, and the protein concentration quantified with a NanoDrop 1000 apparatus (Thermo Fisher Scientific, Wilmington, MA). Extracts were boiled for 5 min, and 400 μg protein of each sample was separated on an SDS–12% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane with a Trans-Blot SD semidry transfer apparatus (Bio-Rad, Munich, Germany). $Mrr1^{P683S}$-HA was detected with a rat anti-HA monoclonal antibody (anti-HA-peroxidase, high affinity [3F10]; Roche Diagnostics GmbH, Mannheim, Germany) at a dilution of 1:500 using a chemiluminescence detection system (GE Healthcare UK Limited, Chalfont, United Kingdom) under conditions recommended by the manufacturer.

ChIP-chip experiments. Three independent cultures of strains SCMRR1M4E3A (untagged control strain; *mrr1*Δ P_{ADH1}-$MRR1^{P683S}$), SCMRR1M4EH3A (tagged strain; *mrr1*Δ P_{ADH1}-$MRR1^{P683S}$-HA), and SCΔ*mrr1*Δ*cap1*MEH3A (tagged strain; *mrr1*Δ *cap1*Δ P_{ADH1}-$MRR1^{P683S}$-HA) were grown in YPD at 30°C to an OD_{600} of 1.0. The subsequent steps of DNA cross-linking, DNA shearing, ChIP, DNA labeling with Cy dyes, hybridization to DNA microarrays, and data analysis were performed as described previously (18). Both pools of labeled DNA from the tagged strain (SCMRR1M4EH3A or SCΔ*mrr1*Δ*cap1*MEH3A, Cy5 labeled) and the untagged control strain (SCMRR1M4E3A, Cy3 labeled) were mixed and hybridized to a *C. albicans* whole-genome tiled oligonucleotide DNA microarray (NimbleGen Systems, Inc.) (33) according to the manufacturer's instructions. Scanning of the slides (n = 3) was performed used a GenePix 4000B scanner (Molecular Devices). Scanned images were preprocessed using NimbleScan software (version 2.4; NimbleGen Systems, Inc.). General feature format reports were created for the Cy5 (tagged strain) and Cy3 (untagged control strain) intensity signals from each independent replicate and were then imported into the Tilescope program (http://tilescope.gersteinlab.org/8080/mosaic/pipeline.html) (38). Quantile normalization was applied to the data. The parameters used were as follows: a window size of 400 bp, a maximum genomic distance of 60 bp, and a minimum length of 120 bp. The replicate data were combined, and peak finding (i.e., determination of the Mrr1 binding sites) was done using a pseudomedian signal threshold of at least 2-fold and a P value cutoff of 0.01.

Microarray sequence accession number. The microarray data obtained were deposited with the Gene Expression Omnibus database; the accession number for the series is GSE23532.

RESULTS

Role of Upc2 in *MDR1* expression and Mdr1-mediated drug resistance. To investigate the capacity of a constitutively active Upc2 to induce *MDR1* expression, we integrated a P_{MDR1}-*GFP* reporter fusion into derivatives of strain SC5314 in which one or both endogenous *UPC2* alleles had been replaced by the hyperactive $UPC2^{G648D}$ allele. The fluorescence of the cells was quantified by FACS analysis. *MDR1* is not detectably expressed in wild-type strain SC5314 under standard growth conditions, and transformants of this strain exhibited only background fluorescence (Fig. 1, bar pair 1). The fluorescence of reporter strains that were homozygous for the $UPC2^{G648D}$ allele was increased about 2.5-fold over background, confirming that this hyperactive *UPC2* allele can activate *MDR1* expression (Fig. 1, bar pair 5). However, the Upc2-mediated *MDR1* induction was negligible compared to the strong activation of *MDR1* expression by the hyperactive $MRR1^{P683S}$ allele, especially when the P683S gain-of-function mutation was present in both *MRR1* alleles (Fig. 1, bar pairs 8 and 9). In addition, while induction of the *MDR1* promoter by the hyperactive *UPC2* allele required the presence of *MRR1* (Fig. 1, bar pairs 4 to 7), the $MRR1^{P683S}$ allele activated the *MDR1* promoter with equal efficiency in the presence or absence of a functional *UPC2* gene (Fig. 1, bar pairs 8 to 11). These results demonstrate that Upc2 plays only a minor role in *MDR1* expression and that Mrr1 is a more important regulator of this efflux pump.

To evaluate the effect of the hyperactive $UPC2^{G648D}$ allele on drug resistance in the presence and absence of *MRR1*, we tested the susceptibilities of the strains to fluconazole and cerulenin (Table 1). As previously reported (12), strains that carried the G648D mutation in both *UPC2* alleles exhibited an 8-fold-increased resistance to fluconazole compared to the wild-type parental strain (MIC increased from 0.2 μg/ml to 1.56 μg/ml), and the same increase was also observed in an *mrr1*Δ background. In contrast, the $UPC2^{G648D}$ allele did not confer increased resistance to cerulenin, which is a substrate of the Mdr1 efflux pump, even in the presence of *MRR1*. These results indicate that the increased fluconazole resistance of

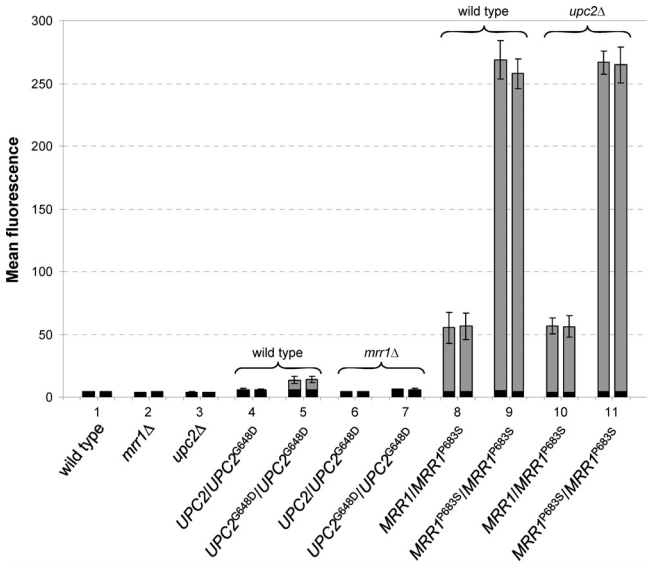

FIG. 1. Activation of the *MDR1* promoter by hyperactive *UPC2* and *MRR1* alleles. *C. albicans* strains carrying a P_{MDR1}-*GFP* reporter fusion in the indicated genetic backgrounds were grown to log phase in YPD medium, and the mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated reporter strains are shown in each case (with means and standard deviations from three experiments). The following strains were used (see also Table S1 in the supplemental material): SCMPG2A and -B (wild type), SCMRR1M4MPG2A and -B (*mrr1*Δ), UPC2M4MPG2A and -B (*upc2*Δ), SCUPC2R12MPG2A and -B (*UPC2*/*UPC2*^{G648D}, wild type), SCUPC2R14MPG2A and -B (*UPC2*^{G648D}/*UPC2*^{G648D}, wild type), Δ*mrr1*UPC2R12MPG2A and -B (*UPC2*/*UPC2*^{G648D}, *mrr1*Δ), Δ*mrr1*UPC2R14MPG2A and -B (*UPC2*^{G648D}/*UPC2*^{G648D}, *mrr1*Δ), SCMRR1R32MPG2A and -B (*MRR1*/*MRR1*^{P683S}, wild type), SCMRR1R34MPG2A and -B (*MRR1*^{P683S}/*MRR1*^{P683S}, wild type), Δ*upc2*MRR1R32MPG2A and -B (*MRR1*/*MRR1*^{P683S}, *upc2*Δ), Δ*upc2*MRR1R34MPG2A and -B (*MRR1*^{P683S}/*MRR1*^{P683S}, *upc2*Δ). The background fluorescence of the parental strains, which do not contain the *GFP* gene, is indicated by the black part in each column (one measurement).

strains containing the *UPC2*^{G648D} allele was caused by the upregulation of *ERG11* and other Upc2 target genes, but not by increased *MDR1* expression. On the other hand, the hyperactive *MRR1*^{P683S} allele caused a 16-fold increase in resistance to cerulenin (MIC increased from 0.39 μg/ml to 6.25 μg/ml) in the presence or absence of *UPC2*, in line with its ability to activate the *MDR1* promoter in both wild-type and *upc2*Δ strains. As previously reported (11), the *upc2*Δ mutants were hypersusceptible to fluconazole. Nevertheless, the P683S mutation in *MRR1* caused a 16-fold increase in fluconazole resistance also in the *upc2*Δ mutants when present in both *MRR1* alleles (MIC increased from 0.05 μg/ml to 0.78 μg/ml), similar to its effect in a wild-type background. These results demonstrate that a hyperactive Mrr1 confers increased drug resistance independently of Upc2.

Role of Cap1 in *MDR1* expression and Mdr1-mediated drug resistance. A C-terminally truncated *CAP1* allele has been shown to cause constitutive *MDR1* upregulation and increased fluconazole resistance (1). To investigate if *MDR1* activation by a hyperactive *CAP1* allele requires *MRR1*, we replaced one or both wild-type *CAP1* alleles with a C-terminally truncated *CAP1* allele (*CAP1*$^{\Delta C333}$) in the wild-type strain SC5314 as well as in mutants lacking *MRR1*. The activity of the *MDR1* promoter was monitored using a P_{MDR1}-*GFP* reporter fusion. *MDR1* expression was especially pronounced when the C-terminal truncation was present in both *CAP1* alleles (Fig. 2, bar pair 5). *MDR1* activation by the *CAP1*$^{\Delta C333}$ allele was also observed in the *mrr1*Δ mutants, albeit at a reduced level compared to that in the wild-type background, demonstrating that the induction of the *MDR1* promoter by the hyperactive *CAP1*$^{\Delta C333}$ allele was partially Mrr1 independent (Fig. 2, bar pairs 4 to 7). However, the *MRR1*^{P683S} allele caused a stronger *MDR1* activation than the *CAP1*$^{\Delta C333}$ allele. This activation was largely *CAP1* independent, because similar *MDR1* expression levels were achieved by the hyperactive Mrr1 in the wild-type strain SC5314 and two independently constructed *cap1*Δ derivatives (Fig. 2, bar pairs 8 to 11).

We also compared the effects of hyperactive *CAP1* and *MRR1* alleles on drug resistance in the different strain backgrounds (Table 2). An increased resistance to the Mdr1 substrate cerulenin was only observed when the hyperactive *CAP1*$^{\Delta C333}$ allele was substituted for both endogenous *CAP1* wild-type alleles. In contrast, a reproducibly increased resistance to fluconazole was seen already in the heterozygous strains. Fluconazole resistance was further elevated in strains that were homozygous for the *CAP1*$^{\Delta C333}$ allele, but only in the presence of *MRR1*. Similar to its ability to activate the *MDR1* promoter in a *CAP1*-independent fashion, the hyperactive

TABLE 1. Susceptibilities to fluconazole and cerulenin of the wild-type parental strain SC5314 and mutant derivatives in which one or both resident *UPC2* and *MRR1* alleles were replaced by the hyperactive *UPC2*^{G648D} and *MRR1*^{P683S} alleles, respectively

Strain(s)	Relevant genotype	MIC (μg/ml)	
		Fluconazole	Cerulenin
SC5314	Wild type	0.2	0.39
SCMRR1M4A and -B	*mrr1*Δ/*mrr1*Δ	0.2	0.39
UPC2M4A and -B	*upc2*Δ/*upc2*Δ	0.05	0.39
SCUPC2R12A and -B	*UPC2*/*UPC2*^{G648D}	0.78	0.39
SCUPC2R14A and -B	*UPC2*^{G648D}/*UPC2*^{G648D}	1.56	0.39
Δ*mrr1*SCUPC2R12A and -B	*mrr1*Δ/*mrr1*Δ *UPC2*/*UPC2*^{G648D}	0.78	0.39
Δ*mrr1*SCUPC2R14A and -B	*mrr1*Δ/*mrr1*Δ *UPC2*^{G648D}/*UPC2*^{G648D}	1.56	0.39
SCMRR1R32A and -B	*MRR1*/*MRR1*^{P683S}	0.39	0.78
SCMRR1R34A and -B	*MRR1*^{P683S}/*MRR1*^{P683S}	3.13	6.25
Δ*upc2*SCMRR1R32A and -B	*upc2*Δ/*upc2*Δ *MRR1*/*MRR1*^{P683S}	0.1	0.78
Δ*upc2*SCMRR1R34A and -B	*upc2*Δ/*upc2*Δ *MRR1*^{P683S}/*MRR1*^{P683S}	0.78	6.25

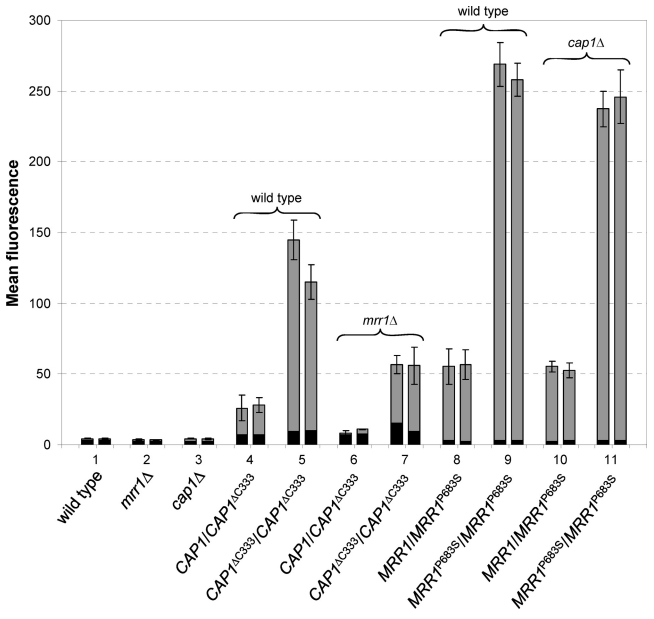

FIG. 2. Activation of the *MDR1* promoter by hyperactive *CAP1* and *MRR1* alleles. *C. albicans* strains carrying a P_{MDR1}-*GFP* reporter fusion in the indicated genetic backgrounds were grown to log phase in YPD medium, and the mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated reporter strains are shown in each case (means and standard deviations from three experiments). The following strains were used (see also Table S1 in the supplemental material): SCMPG2A and -B (wild type), SCMRR1M4MPG2A and -B (*mrr1*Δ), SCCAP1M4MPG2A and -B (*cap1*Δ), SCCAP1R12MPG2A and -B (*CAP1*/*CAP1*$^{\Delta C333}$, wild type), SCCAP1R14MPG2A and -B (*CAP1*$^{\Delta C333}$/*CAP1*$^{\Delta C333}$, wild type), Δ*mrr1*CAP1R12MPG2A and -B (*CAP1*/*CAP1*$^{\Delta C333}$, *mrr1*Δ), Δ*mrr1*CAP1R14MPG2A and -B (*CAP1*$^{\Delta C333}$/*CAP1*$^{\Delta C333}$, *mrr1*Δ), SCMRR1R32MPG2A and -B (*MRR1*/*MRR1*^{P683S}, wild type), SCMRR1R34MPG2A and -B (*MRR1*^{P683S}/*MRR1*^{P683S}, wild type), Δ*cap1*MRR1R32MPG2A and -B (*MRR1*/*MRR1*^{P683S}, *cap1*Δ), Δ*cap1*MRR1R34MPG2A and -B (*MRR1*^{P683S}/*MRR1*^{P683S}, *cap1*Δ). The background fluorescence of the parental strains, which do not contain the *GFP* gene, is indicated by the black portion of each column (one measurement). Data are from the same experiments as in Fig. 1, and the values of the control strains are included for comparison.

MRR1^{P683S} allele did not require the presence of an intact *CAP1* gene to mediate resistance to fluconazole and cerulenin. These data show that hyperactive *CAP1* and *MRR1* alleles can independently confer increased drug resistance.

Cooperation between Mrr1 and Cap1 in *MDR1* expression. As hyperactive *MRR1* and *CAP1* alleles are each able to activate the *MDR1* promoter, we investigated whether the presence of both types of activated transcription factors would have an additive or even synergistic effect on the expression of the efflux pump. For this purpose, the two wild-type *CAP1* alleles were replaced by the *CAP1*$^{\Delta C333}$ allele in strains that were homozygous for the *MRR1*^{P683S} allele. The activity of the *MDR1* promoter in these strains was compared with that in strains containing only the *MRR1*^{P683S} or *CAP1*$^{\Delta C333}$ alleles by using the P_{MDR1}-*GFP* reporter fusion. As can be seen in Fig. 3A, strains carrying activated forms of both transcription factors displayed higher *MDR1* expression levels than strains expressing only hyperactive Mrr1 or Cap1. Similarly, the resistance to fluconazole and cerulenin was further increased when both Mrr1 and Cap1 were constitutively active (Fig. 3B). These results demonstrate that activating mutations in Mrr1 and Cap1 have additive effects on *MDR1* expression and drug resistance.

Inducibility of the *MDR1* promoter in *mrr1*Δ, *upc2*Δ, and *cap1*Δ mutants. *MDR1* expression can be induced in *C. albicans* wild-type strains by certain toxic compounds, like benomyl and H_2O_2 (23, 27). To compare the requirement of *MRR1*, *UPC2*, and *CAP1* for *MDR1* upregulation in response to these inducers, we quantified the activity of the *MDR1* promoter in the presence or absence of benomyl and H_2O_2 in wild-type and mutant strains carrying a P_{MDR1}-*GFP* reporter fusion (Fig. 4). As reported previously (23), the induction of the *MDR1* promoter was abolished in *mrr1*Δ mutants. Deletion of *UPC2* had no effect on the inducibility of the *MDR1* promoter, whereas *MDR1* expression in response to benomyl was reduced and no *MDR1* induction by H_2O_2 was observed in the *cap1*Δ mutants under the experimental conditions used. These results demonstrate that Upc2 is not required for *MDR1* induction in response to these chemicals, whereas Cap1 is required for H_2O_2-induced *MDR1* expression and contributes to its induction by benomyl, and Mrr1 is essential for *MDR1* upregulation by both inducers.

Contribution of the Mdr1 efflux pump to Mrr1-, Upc2-, and Cap1-mediated drug resistance. In order to evaluate the importance of the Mdr1 efflux pump for drug resistance conferred by hyperactive Mrr1, Upc2, and Cap1, we introduced the *MRR1*^{P683S}, *UPC2*^{G648D}, and *CAP1*$^{\Delta C333}$ alleles into

TABLE 2. Susceptibilities to fluconazole and cerulenin of the wild-type parental strain SC5314 and mutant derivatives in which one or both resident *CAP1* and *MRR1* alleles were replaced by the hyperactive *CAP1*$^{\Delta C333}$ and *MRR1*^{P683S} alleles, respectively

Strain(s)	Relevant genotype	MIC (μg/ml)	
		Fluconazole	Cerulenin
SC5314	Wild type	0.2	0.39
SCMRR1M4A and -B	*mrr1*Δ/*mrr1*Δ	0.2	0.39
CAP1M4A and -B	*cap1*Δ/*cap1*Δ	0.2	0.39
SCCAP1R12A and -B	*CAP1*/*CAP1*$^{\Delta C333}$	0.39	0.39
SCCAP1R14A and -B	*CAP1*$^{\Delta C333}$/*CAP1*$^{\Delta C333}$	0.78	0.78
Δ*mrr1*CAP1R12A and -B	*mrr1*Δ/*mrr1*Δ *CAP1*/*CAP1*$^{\Delta C333}$	0.39	0.39
Δ*mrr1*CAP1R14A and -B	*mrr1*Δ/*mrr1*Δ *CAP1*$^{\Delta C333}$/*CAP1*$^{\Delta C333}$	0.39	0.78
SCMRR1R32A and -B	*MRR1*/*MRR1*^{P683S}	0.39	0.78
SCMRR1R34A and -B	*MRR1*^{P683S}/*MRR1*^{P683S}	3.13	6.25
Δ*cap1*MRR1R32A and -B	*cap1*Δ/*cap1*Δ *MRR1*/*MRR1*^{P683S}	0.39	0.78
Δ*cap1*MRR1R34A and -B	*cap1*Δ/*cap1*Δ *MRR1*^{P683S}/*MRR1*^{P683S}	3.13	6.25

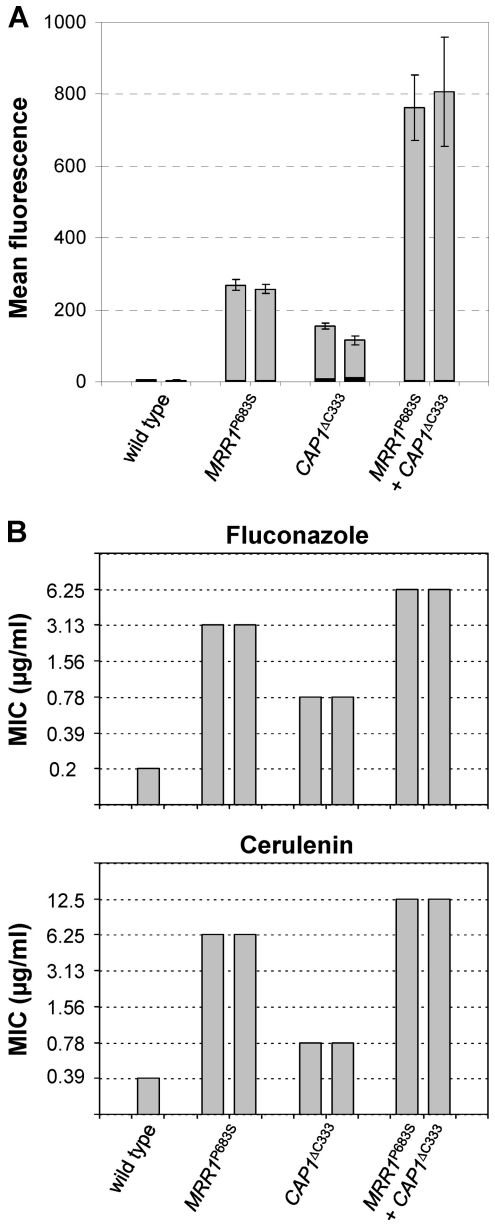

FIG. 3. Effects of combining hyperactive *MRR1* and *CAP1* alleles on *MDR1* promoter activity and drug resistance. (A) *C. albicans* strains that are homozygous for the indicated hyperactive *MRR1* and *CAP1* alleles and contain a P_{MDR1}-*GFP* reporter fusion were grown to log phase in YPD medium, and the mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated reporter strains are shown in each case (means and standard deviations from three experiments). The following strains were used (see also Table S1 in the supplemental material): SCMPG2A and -B (wild type), SCMRR1R34MPG2A and -B (*MRR1*^{P683S}), SCCAP1R14MPG2A and -B (*CAP1*$^{\Delta C333}$), SCMRR1R34CAP1R14MPG2A and -B (*MRR1*^{P683S} + *CAP1*$^{\Delta C333}$). Data are from the same experiments as in Fig. 1 and 2, and the values of the control strains are included for comparison. (B) Susceptibilities to fluconazole and cerulenin of the wild-type parental strain SC5314 and mutant derivatives in which both resident *MRR1* and/or *CAP1* alleles were replaced by the hyperactive *MRR1*^{P683S} and *CAP1*$^{\Delta C333}$ alleles, respectively. The results obtained with two independently generated strains are shown in each case. The following strains were used: SC5314 (wild type), SCMRR1R34A and -B (*MRR1*^{P683S}), SCCAP1R14A and -B (*CAP1*$^{\Delta C333}$), SCMRR1R34CAP1R14A and -B (*MRR1*^{P683S} + *CAP1*$^{\Delta C333}$).

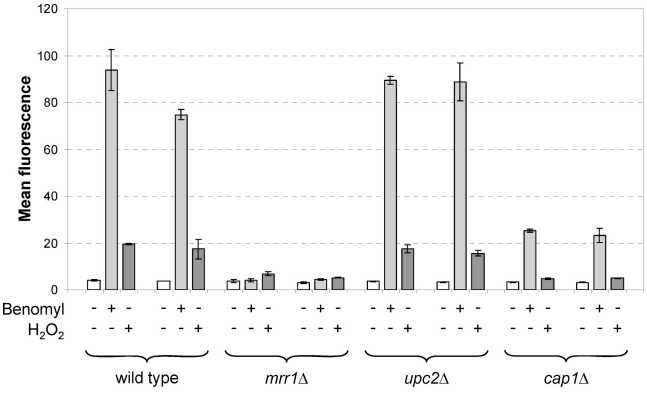

FIG. 4. Activation of the *MDR1* promoter by benomyl and H_2O_2 in wild-type, *mrr1*Δ, *cap1*Δ, and *upc2*Δ strains. Overnight cultures of *C. albicans* strains carrying a P_{MDR1}-*GFP* reporter fusion in the indicated genetic backgrounds were diluted 10^{-2} in three tubes with fresh YPD medium and grown to log phase. One culture was left untreated, and 50 μg/ml benomyl or 0.005% H_2O_2 was added to the other cultures to induce *MDR1* expression. The cultures were incubated for 80 min, and the mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated reporter strains are shown in each case (means and standard deviations from three experiments). The following strains were used (see also Table S1 in the supplemental material): SCMPG2A and -B (wild type), SCMRR1M4MPG2A and -B (*mrr1*Δ), SCCAP1M4MPG2A and -B (*cap1*Δ), UPC2M4MPG2A and -B (*upc2*Δ).

*mdr1*Δ mutants generated from strain SC5314 (see Materials and Methods; see also Table S1 in the supplemental material). As can be seen in Table 3, the increased fluconazole resistance conferred by the hyperactive Mrr1 was reduced, but not abolished, in cells lacking the Mdr1 efflux pump (from 16-fold in the wild type to 4- to 8-fold in *mdr1*Δ mutants), demonstrating that other Mrr1 target genes contribute to the fluconazole resistance phenotype of strains containing gain-of-function mutations in this transcription factor. On the other hand, the Mrr1-mediated cerulenin resistance seen in a wild-type background was largely abrogated in *mdr1*Δ mutants (from 16-fold to 2-fold), indicating that cerulenin resistance is mediated mostly, but not exclusively, by the Mdr1 efflux pump. The increased fluconazole resistance caused by the hyperactive *CAP1*$^{\Delta C333}$ allele was reduced from 4-fold to 2-fold in the absence of *MDR1*, whereas the moderate cerulenin resistance conferred by this allele depended on a functional Mdr1 efflux pump. In contrast, the increased fluconazole resistance conferred by the hyperactive Upc2 did not depend on the presence of a functional *MDR1* gene, confirming that Upc2-mediated azole resistance is caused by upregulation of the *ERG* and possibly other Upc2 target genes.

Comparison of the gene expression profiles of the wild type and *cap1*Δ mutants expressing a hyperactive *MRR1* allele. The results shown above demonstrate that overexpression of the Mdr1 efflux pump contributes only partially to the fluconazole resistance conferred by activated *MRR1* and *CAP1* alleles. Mrr1 and Cap1 share some of their target genes (23, 39). In order to evaluate whether a hyperactive Mrr1 can activate all its target genes independently of Cap1, we compared the global gene expression patterns of strains containing the P683S gain-of-function mutation in both *MRR1* alleles in wild-type and *cap1*Δ backgrounds. In a previous study, we found 19

TABLE 3. Contribution of the Mdr1 efflux pump to drug resistance conferred by hyperactive *MRR1*, *CAP1*, and *UPC2* alleles[a]

Strain(s)	Relevant genotype	MIC (μg/ml)	
		Fluconazole	Cerulenin
SC5314	Wild type	0.2	0.39
SCMDR1M4A and -B	*mdr1*Δ/*mdr1*Δ	0.2	0.39
SCMRR1R34A and -B	$MRR1^{P683S}$/$MRR1^{P683S}$	3.13	6.25
Δ*mdr1*MRR1R34A and -B	*mdr1*Δ/*mdr1*Δ $MRR1^{P683S}$/$MRR1^{P683S}$	0.78–1.56	0.78
SCCAP1R14A and -B	$CAP1^{\Delta C333}$/$CAP1^{\Delta C333}$	0.78	0.78
Δ*mdr1*CAP1R14A and -B	*mdr1*Δ/*mdr1*Δ $CAP1^{\Delta C333}$/$CAP1^{\Delta C333}$	0.39	0.39
SCUPC2R14A and -B	$UPC2^{G648D}$/$UPC2^{G648D}$	1.56	0.39
Δ*mdr1*UPC2R14A and -B	*mdr1*Δ/*mdr1*Δ $UPC2^{G648D}$/$UPC2^{G648D}$	1.56	0.39

[a] Shown are the susceptibilities to fluconazole and cerulenin of wild-type strains and *mdr1*Δ mutants in which both resident *MRR1*, *CAP1*, and *UPC2* alleles were replaced by the $MRR1^{P683S}$, $CAP1^{\Delta C333}$, and $UPC2^{G648D}$ alleles, respectively.

genes to be significantly upregulated in strains expressing one copy of the $MRR1^{P683S}$ allele (23). In line with the stronger effect of two copies of the $MRR1^{P683S}$ allele on *MDR1* expression (Fig. 1 and 2), a higher number of genes (83) were reproducibly upregulated in two independently constructed strains that were homozygous for the P683S mutation (see Table S2 in the supplemental material). For 67 of these 83 genes we obtained reliable hybridization signals in both experiments with the *cap1*Δ mutants. Twenty-five of the 67 genes were also reproducibly upregulated, while 27 were not significantly upregulated (<2-fold) in the *cap1*Δ mutants. For 15 genes the results of the two experiments were inconsistent (>2-fold upregulation in one experiment and <2-fold in the other experiment). However, almost all genes that were upregulated <2-fold in the *cap1*Δ mutants (21/27) were also not strongly upregulated (<3-fold) in the wild type. In contrast, among the 31 genes that were upregulated >4-fold in the wild type, only one gene (*OYE23*) was reproducibly not upregulated in the *cap1*Δ mutants.

Identification of the *in vivo* DNA binding sites of Mrr1. To find out whether genes that are differentially expressed in strains carrying a hyperactive *MRR1* allele are direct targets of Mrr1, we determined the *in vivo* DNA binding pattern of Mrr1 by chromatin immunoprecipitation followed by hybridization on whole-genome oligonucleotide tiling microarrays (ChIP-chip). Mrr1 was tagged at its C terminus with a 3×HA epitope to enable immunoprecipitation with an anti-HA antibody, an approach that has been previously used to identify the binding sites of the transcription factors Cap1, Upc2, and Tac1 in the *C. albicans* genome (18, 39, 41). In pilot experiments, we evaluated the effect of the HA tag on the activity of wild-type and hyperactive Mrr1 proteins. Untagged and HA-tagged *MRR1* and $MRR1^{P683S}$ alleles were first expressed from the endogenous *MRR1* promoter in *mrr1*Δ mutants carrying a P_{MDR1}-*GFP* reporter fusion. Figure 5A shows that the presence of the HA tag resulted in activation of Mrr1, as the *MDR1* promoter was constitutively upregulated in strains expressing HA-tagged wild-type *MRR1*, similar to strains expressing the hyperactive $MRR1^{P683S}$ allele. Such an activating effect of a C-terminal HA tag has also been observed for Upc2 (41) and is likely caused by unmasking of an activation domain. When the same proteins were expressed in *mrr1*Δ mutants of strain SC5314, the presence of the HA tag also resulted in increased fluconazole resistance, confirming the activation of wild-type Mrr1 by the HA tag (Table 4).

In order to achieve a similar Mrr1 activity level as in strains that are homozygous for a hyperactive *MRR1* allele (Fig. 1 and 2 and Tables 1 and 2), we also expressed the same set of tagged and untagged *MRR1* alleles from the strong *ADH1* promoter in *mrr1*Δ mutants. While overexpression of the untagged wild-type *MRR1* gene had no detectable effect on *MDR1* promoter activity and drug susceptibility, expression of the HA-tagged *MRR1* alleles from the *ADH1* promoter resulted in a further increase in *MDR1* expression and fluconazole resistance compared to strains expressing the same alleles from the endogenous *MRR1* promoter (Fig. 5A and Table 4). We chose to use the strains overexpressing the HA-tagged $MRR1^{P683S}$ allele for the ChIP-chip experiments. To compare binding of Mrr1 in the presence and absence of Cap1, the HA-tagged $MRR1^{P683S}$ allele was also expressed from the *ADH1* promoter in *mrr1*Δ *cap1*Δ double mutants. Western blot analysis showed that the HA-tagged $Mrr1^{P683S}$ protein was expressed at comparable levels in independent transformants of the *mrr1*Δ single and *mrr1*Δ *cap1*Δ double mutants (Fig. 5B). Therefore, one clone was used for the ChIP-chip experiments in each case, and a strain that expressed the untagged $MRR1^{P683S}$ allele in the *mrr1*Δ background served as the reference.

Using the criteria described in Materials and Methods, we identified 710 Mrr1 binding sites in the wild-type strain, 608 of which were also found in the *cap1*Δ mutant (see Table S3 in the supplemental material). Therefore, Mrr1 binds to most of its target sequences independently of Cap1. A total of 149 of the Mrr1 binding sites could not be assigned to an open reading frame (ORF), and 71 binding sites could be associated with more than one ORF. The remaining 490 binding sites were assigned to specific ORFs, 453 of which had one Mrr1 binding peak and 31 of which had more than one peak, i.e., 484 defined ORFs (and additional ORFs that shared a binding site) were bound by Mrr1. Of the Mrr1-bound genes, 40 were upregulated in cells expressing the hyperactive $Mrr1^{P683S}$ and 9 were downregulated (see Table S3). Therefore, 40 of the 83 upregulated genes seem to be direct targets of Mrr1, including the *MDR1* efflux pump and *MRR1* itself, whereas the downregulation of genes seems to be an indirect effect (see Table S2). Table 5 lists all genes that were both upregulated and bound by the hyperactive $Mrr1^{P683S}$. Almost all of these (37/40) were also bound by Mrr1 in the absence of Cap1, and the majority of them (22/40) were upregulated in a Cap1-independent fashion, while only 7 of these 40 genes were reproducibly not upregulated (<2-fold in both experiments) in the *cap1*Δ mutants, despite being bound by Mrr1.

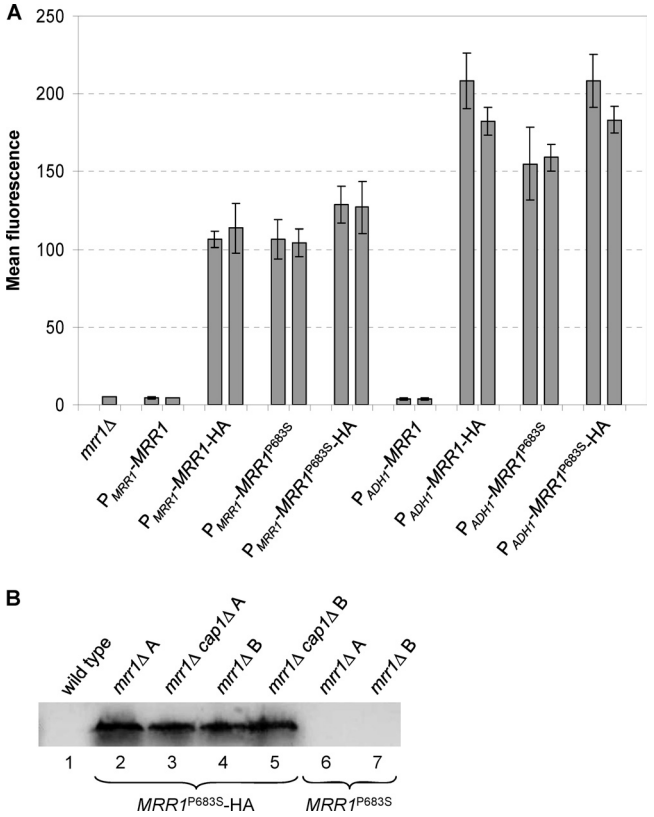

FIG. 5. (A) *MDR1* promoter activity in *C. albicans mrr1*Δ mutants expressing a wild-type *MRR1* allele or the *MRR1*^{P683S} allele, without or with a C-terminal 3×HA tag, from the endogenous *MRR1* promoter or the strong *ADH1* promoter. The strains were grown to log phase in YPD medium, and the mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independent transformants are shown in each case (means and standard deviations from three experiments). The following strains were used: CAG48MRR1M4B (*mrr1*Δ), CAG48MRR1M4K2B1 and -2 (P$_{MRR1}$-*MRR1*), CAG48MRR1M4H2B1 and -2 (P$_{MRR1}$-*MRR1*-HA), CAG48MRR1M4K3B1 and -2 (P$_{MRR1}$-*MRR1*^{P683S}), CAG48MRR1M4H3B1 and -2 (P$_{MRR1}$-*MRR1*^{P683S}-HA), CAG48MRR1M4E2B1 and -2 (P$_{ADH1}$-*MRR1*), CAG48MRR1M4EH2B1 and -2 (P$_{ADH1}$-*MRR1*-HA), CAG48MRR1M4E3B1 and -2 (P$_{ADH1}$-*MRR1*^{P683S}), CAG48MRR1M4EH3B1 and -2 (P$_{ADH1}$-*MRR1*^{P683S}-HA). (B) Expression of the HA-tagged Mrr1^{P683S} protein in *mrr1*Δ single and *mrr1*Δ *cap1*Δ double mutants. Whole-cell protein extracts of the strains were analyzed by Western immunoblotting with an anti-HA antibody. The following strains were used: 1, SC5314; 2, SCΔ*mrr1*MEH3A; 3, SCΔ*mrr1*Δ*cap1*MEH3A; 4, SCΔ*mrr1*MEH3B; 5, SCΔ*mrr1*Δ*cap1*MEH3B; 6, SCΔ*mrr1*ME3A; 7, SCΔ*mrr1*ME3B. The HA-tagged Mrr1^{P683S} was expressed at similar levels in independent transformants of the *mrr1*Δ (lanes 2 and 4) and *mrr1*Δ *cap1*Δ (lanes 3 and 5) mutants. No cross-reacting proteins were detected in the parental strain SC5314 (lane 1) or in *mrr1*Δ mutants expressing untagged Mrr1^{P683S} (lanes 6 and 7).

Identification of potential Mrr1 binding motifs. To search for a putative Mrr1 DNA binding site, a set of DNA sequences corresponding to 710 hits identified in the HA-tagged *MRR1*^{P683S} binding data was generated. It has been shown that DNA motifs bound by transcription factors in ChIP-chip fragments are distributed close to the center of the peaks (4). Therefore, for each of the 710 hits, the position with the highest signal intensity in the tiling arrays was identified and a region of 1 kb centered around the maximum peak (±500 bp) was retrieved from the *C. albicans* Assembly 19 genomic sequences. The top scoring 100 sequences (displaying an enrichment of ≥4-fold) were analyzed using the Suite for Computational Identification of Promoter Elements (SCOPE) program, which allows determination of potential transcription factor binding sites in a set of sequences by using three different motif discovery algorithms (http://genie.dartmouth.edu/scope/) (38). The following three highest-scoring motifs were obtained: DCSGHD (where D designates A, T, or G, S designates C or G, and H designates A, T or C; significance value of 668, 100% coverage) (Fig. 6A), AAAN$_{4-5}$AAT (significance value of 208, 99 to 100% coverage), and TCCGA (significance value of 201, 84% coverage; a variant of the DCSGHD motif). Compared to sets of random sequences, the DCSGHD motif was significantly enriched in the center of the 710 sequences (Fig. 6B) as well as in the sequences corresponding to the 40 genes bound and upregulated by Mrr1 (Fig. 6C and Table 5). Unlike the two other motifs, the AAAN$_{4-5}$AAT sequence was found to be randomly distributed (data not shown). Many zinc cluster factors have been shown to bind as dimers to CGG triplets with different orientations and spacing (21). Our analysis using the SCOPE program did not identify specific CSG repeated motifs in the Mrr1-bound fragments. Taken together, these results suggest that Mrr1 can bind to single CGG triplets (CCG on the opposite strand) flanked by A/T-rich sequences, potentially as a monomer, as already reported for some members of the zinc cluster factor family (2). However, the presence of multiple occurrences of the DCSGHD motif in the Mrr1 promoter targets (data not shown; see Fig. S1 in the supplemental material) suggests that Mrr1 has to bind to more than one site to activate transcription.

DISCUSSION

The zinc cluster transcription factor Mrr1 is a central regulator of *MDR1* expression in *C. albicans*, because *MRR1* is required for the induction of *MDR1* by toxic chemicals and gain-of-function mutations in *MRR1* are responsible for the constitutive *MDR1* overexpression in fluconazole-resistant strains (10, 23). Nevertheless, additional transcription factors have been implicated in the control of *MDR1* expression, because they bind to the *MDR1* promoter (1, 11, 19, 26, 27, 31, 39, 41). Here, we studied the role of two of these transcription factors,

TABLE 4. Fluconazole susceptibilities of *mrr1*Δ mutants expressing a wild-type *MRR1* allele or the *MRR1*^{P683S} allele, without or with a C-terminal 3×HA tag, from the endogenous *MRR1* promoter or the strong *ADH1* promoter

Strains	Description	Fluconazole MIC (μg/ml)
SCMRR1M4A and -B	*mrr1*Δ	0.2
SCMRR1M4K2A and -B	P$_{MRR1}$-*MRR1*	0.2
SCΔ*mrr1*MH2A and -B	P$_{MRR1}$-*MRR1-HA*	0.39
SCMRR1M4K3A and -B	P$_{MRR1}$-*MRR1*^{P683S}	0.78
SCΔ*mrr1*MH3A and -B	P$_{MRR1}$-*MRR1*^{P683S}-HA	0.78
SCΔ*mrr1*ME2A and -B	P$_{ADH1}$-*MRR1*	0.2
SCΔ*mrr1*MEH2A and -B	P$_{ADH1}$-*MRR1-HA*	3.13
SCΔ*mrr1*ME3A and -B	P$_{ADH1}$-*MRR1*^{P683S}	3.13
SCΔ*mrr1*MEH3A and -B	P$_{ADH1}$-*MRR1*^{P683S}-HA	3.13

TABLE 5. Genes upregulated and bound by activated Mrr1

orf19 no.	Gene(s)	Molecular function	Upregulation[a]		Binding[b]	
			WT	*cap1*Δ	WT	*cap1*Δ
orf19.4476		Aryl-alcohol dehydrogenase activity	15,424.4	2,019.7	4.5	4.0
orf19.1048	*IFD6*	Aryl-alcohol dehydrogenase activity	778.0	422.9	4.1	3.9
orf19.271	*ADH4*	2,4-Dienoyl coenzyme A reductase (NADPH) activity	269.6	90.4	4.6	4.9
orf19.5604	*MDR1*	Multidrug efflux pump activity	263.1	829.5	5.5	4.6
orf19.629	*IFD7*	Aryl-alcohol dehydrogenase activity	222.0	260.3	3.6	
orf19.4477	*CSH1*	Aryl-alcohol dehydrogenase activity	203.3	202.1	3.7	4.0
orf19.7306		Unknown	81.8	83.9	2.7	4.3
orf19.4309	*GRP2*	Oxidoreductase activity	34.5	32.8	5.1	4.1
orf19.251		Unknown	27.1	51.8	5.0	4.3
orf19.3131	*OYE32*	NADPH dehydrogenase activity	16.6	9.2	3.7	3.7
orf19.7166		Unknown	12.7	17.8	4.6	3.8
orf19.7042		Unknown	11.5	16.1	5.8	7.2
orf19.4505	*ADH3*	Alcohol dehydrogenase (NAD) activity	8.1		4.7	4.8
orf19.5862		Arginase activity	7.7		2.7	3.1
orf19.1449		Unknown	6.5	15.4	3.3	4.0
orf19.3433	*OYE23*	NADPH dehydrogenase activity	6.1		5.4	3.7
orf19.6993	*GAP2*	Polyamine transmembrane transporter activity	5.9		3.0	3.1
orf19.7204		Unknown	5.4	29.9	3.9	5.6
orf19.3668	*HGT2*	Glucose transmembrane transporter activity	5.1		2.1	
orf19.780	*DUR1,2*	Allophanate hydrolase activity, urea carboxylase activity	5.0	4.3	2.7	2.8
orf19.6311		Unknown	4.6	3.6	3.6	3.3
orf19.1523	*FMO1*	Unknown	4.4		2.3	2.1
orf19.86		Glutathione peroxidase activity	4.3	6.0	3.4	3.2
orf19.5741	*ALS1*	Cell adhesion molecule binding	4.2		2.8	3.0
orf19.111	*CAN2*	Arginine transmembrane transporter activity	4.0		2.5	2.7
orf19.1240		Unknown	3.0	4.6	5.3	6.0
orf19.742	*ALD6*	Aldehyde dehydrogenase (NAD) activity	2.9		2.7	2.4
orf19.2726		Unknown	2.8	2.7	3.5	3.5
orf19.918	*CDR11*	Xenobiotic-transporting ATPase activity	2.7		4.3	4.1
orf19.789	*PYC2*	Pyruvate carboxylase activity	2.7		2.5	
orf19.5911	*CMK1*	Calmodulin-dependent protein kinase activity	2.6		3.0	3.0
orf19.7148	*TPO2*	Drug transmembrane transporter activity	2.6		5.8	5.1
orf19.4056	*GAT2*	Transcription factor activity	2.5		2.7	2.8
orf19.6586		Unknown	2.4	2.8	4.2	3.5
orf19.4527	*HGT1*	Glucose transmembrane transporter activity	2.4		3.0	2.1
orf19.5713	*YMX6*	NADH dehydrogenase activity	2.4	2.8	4.2	4.7
orf19.3364		Unknown	2.4		2.9	3.7
orf19.7372	*MRR1*	Specific RNA polymerase II transcription factor activity	2.3	2.7	3.7	4.0
orf19.4778	*LYS142*	Transcription factor activity	2.3		3.7	4.0
orf19.5282		Unknown	2.2		3.6	4.0

[a] Average values from two experiments with independently constructed strains. Values for the *cap1*Δ mutants are omitted when the gene was not upregulated by at least 2-fold or no reliable hybridization signals were obtained in one or both experiments (see Table S2 of the supplemental material). WT, wild type.
[b] Pseudomedian binding ratio in *MRR1*^{P683S}-HA ChIP-chip data (see Table S3 in the supplemental material).

Upc2 and Cap1, for which gain-of-function mutations have been described that render them hyperactive (1, 11, 12).

Upc2 binds to the *MDR1* promoter and modulates *MDR1* expression in response to environmental conditions in *C. albicans* wild-type strains (41). We found that Upc2 is not required for upregulation of *MDR1* by benomyl and hydrogen peroxide, two chemicals that are commonly used to induce expression of this efflux pump (16, 23, 27). To date, only three gain-of-function mutations that render Upc2 hyperactive have been found in fluconazole-resistant, clinical *C. albicans* isolates. Of these, the G648D mutation has a stronger effect on *ERG11* overexpression and fluconazole resistance than the A643T and A643V mutations (11, 12, 14). However, even when present in both *UPC2* alleles, the G648D mutation caused only a moderate *MDR1* upregulation that did not contribute to drug resistance. This result suggests that gain-of-function mutations in Upc2 are unlikely to be a cause of Mdr1-mediated drug resistance in *C. albicans*.

In contrast to Upc2, Cap1 was essential for *MDR1* upregulation in the presence of hydrogen peroxide and also contributed to *MDR1* induction by benomyl. The former result confirms earlier observations showing the requirement of Cap1 for hydrogen peroxide-induced *MDR1* expression (27). Conflicting results were reported with respect to the involvement of Cap1 in *MDR1* induction by benomyl. One study found that Cap1 was largely dispensable for benomyl-induced *MDR1* expression (27), while in another study *MDR1* transcript levels in response to benomyl were found to be 4-fold reduced in a *cap1*Δ mutant compared to a wild-type control strain (39). In line with the latter results, we found that full activation of the *MDR1* promoter by benomyl depended on Cap1 (Fig. 4). As Mrr1 is essential for *MDR1* upregulation in response to both inducing chemicals, Mrr1 cooperates with Cap1, but not Upc2, to mediate *MDR1* induction by these environmental stimuli.

While Cap1 requires Mrr1 to induce *MDR1* expression in response to benomyl and hydrogen peroxide, the C-terminally

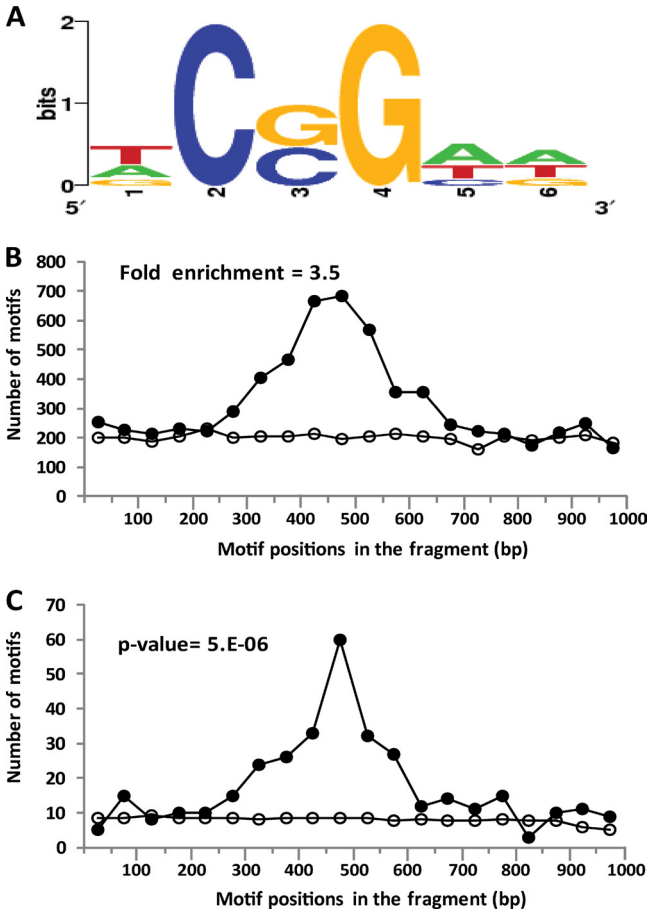

FIG. 6. Identification of putative Mrr1 DNA binding motifs. (A) Weblogo representation of the DCSGHD motif identified by using SCOPE. (B) Enrichment of the DCSGHD motif in the 710 sequence data set (filled circles), compared to what would be expected randomly in the whole genome (open circles). Each of the 710 1-kb sequences was divided into 20 intervals of 50 bp, and the number of DCSGHD motif occurrences was compiled and plotted for each interval. The maximum fold enrichment value (3.5) is observed in the center of the analyzed 1-kb sequences. (C) Enrichment of the DCSGHD motif in the sequences corresponding to 40 genes bound and regulated by Mrr1 (filled circles) compared to all genes (open circles). The *P* value (3.E−06) represents the probability of observing this motif distribution in random data sets.

truncated, hyperactive *CAP1*$^{\Delta C333}$ allele caused a constitutive *MDR1* overexpression also in the absence of Mrr1, demonstrating that it could activate the *MDR1* promoter in an Mrr1-independent fashion. Similar gain-of-function mutations in Cap1 could also be a cause of *MDR1* overexpression and drug resistance in clinical *C. albicans* isolates. However, no such mutations have been found so far in fluconazole-resistant isolates from patients. Instead, all *MDR1*-overexpressing strains contain gain-of-function mutations in Mrr1 (10, 23, 30). One reason for this could be that hyperactive *MRR1* alleles cause a stronger increase in fluconazole resistance than the C-terminally truncated Cap1 used in our study. Another possible explanation is that the C-terminal truncation in Cap1 also has a deleterious effect on some Cap1 functions, because the mutated Cap1 failed to complement the hydrogen peroxide hypersusceptibility of a *cap1*Δ mutant (1). As resistance to oxidative stress is likely to be important for the ability of *C. albicans* to persist within the host, the environmental conditions *in vivo* may select against Cap1 mutations as a cause of fluconazole resistance. It remains to be seen if other activating mutations in Cap1 exist that do not negatively affect its ability to induce expression of all its target genes.

The fluconazole resistance of strains expressing hyperactive Mrr1 was only reduced, but not abolished, after deletion of *MDR1*, indicating that Mrr1 can mediate increased fluconazole resistance in an Mdr1-independent fashion. This is in line with earlier findings that deletion of *MRR1* from *MDR1*-overexpressing clinical isolates increased fluconazole susceptibility more than did deletion of *MDR1* (23). Therefore, some of the other genes that are upregulated by hyperactive Mrr1 are likely to contribute to drug resistance. The contribution of other Mrr1 target genes to fluconazole resistance was even higher than that of *MDR1*, as was evident from the still-4- to 8-fold increase in the MIC of strains expressing the hyperactive Mrr1 in an *mdr1*Δ background compared to the 16-fold increase in the parental wild-type background (Table 3). It is currently not known which of these genes are involved in Mrr1-mediated fluconazole resistance. As the drug resistance conferred by the hyperactive Mrr1 did not depend on the presence of *CAP1* (Table 2), the most likely candidates are those genes that are upregulated by Mrr1 in a Cap1-independent fashion. As noted previously (23), many Mrr1 target genes encode putative oxidoreductases which may act to remedy fluconazole-induced cell damage (Table 5).

Interestingly, the increased fluconazole resistance of strains expressing the hyperactive Cap1 was also not completely abolished after deletion of *MDR1*, indicating that Cap1, like Mrr1, can mediate fluconazole resistance in an Mdr1-independent (and Mrr1-independent) fashion (Tables 2 and 3). Some known Cap1 target genes that were not found to be upregulated by Mrr1, like *PDR16*, *FLU1*, and *RTA2*, have previously been associated with fluconazole resistance and may therefore be responsible for this phenotype (3, 15, 29, 39).

The *in vivo* localization studies showed that Mrr1 bound to about half of the 83 genes that were reproducibly upregulated by the hyperactive Mrr1^{P683S}. Therefore, these genes are probably direct targets of Mrr1. Similar to *MDR1*, most other direct Mrr1 target genes were also bound and upregulated by the hyperactive Mrr1 independently of Cap1, including the known Cap1 targets orf19.251, orf19.3131, orf19.7042, and orf19.6586 (39). Even if Mrr1 cooperates with Cap1 to induce common target genes in response to environmental signals, gain-of-function mutations in Mrr1 enable the transcription factor to act independently of Cap1. However, some genes (e.g., *OYE23*) were bound but not upregulated by the hyperactive Mrr1 in a *cap1*Δ mutant background (Table 5; see also Table S2 in the supplemental material), indicating that a hyperactive Mrr1 requires Cap1 for the transcriptional upregulation of these genes. We found a surprisingly high number of additional Mrr1 binding sites in the genome that were not associated with genes whose expression was affected by the presence of the activated Mrr1. It is possible that some of the peaks are artifacts caused by nonspecific binding of the overexpressed, HA-tagged Mrr1 and do not represent biologically relevant Mrr1 binding sites. But it is also conceivable that even a hyperactive Mrr1 can induce the expression of the corresponding genes

only in cooperation with other regulators, which may not be active under the growth conditions used in our assays. In addition, Mrr1 may also be involved in the regulation of noncoding transcripts, as many Mrr1 binding sites were not in the vicinity of known ORFs.

The identification of the *in vivo* DNA binding sites of a hyperactive Mrr1 also allowed us to deduce a potential Mrr1 binding motif. Interestingly, the DCSGHD motif occurs repeatedly in the *MDR1* upstream region (see Fig. S1 in the supplemental material). It remains to be established, by *in vitro* DNA binding studies with purified Mrr1 and by *in vivo* transactivation assays with mutated versions of the *MDR1* promoter fused to a reporter, whether the transcription factor indeed binds to these sites. However, the presence of multiple Mrr1 binding motifs explains the previous observation that no single region in the *MDR1* promoter is absolutely required for the constitutive *MDR1* upregulation in strains carrying gain-of-function mutations in Mrr1, whereas Mrr1 itself is essential for *MDR1* overexpression (10, 13, 23, 26).

The physiological function of the Mdr1 efflux pump, which is not significantly expressed under standard growth conditions, is not known. Our results demonstrate that two transcription factors that bind to the *MDR1* promoter, Mrr1 and Cap1, cooperate to induce *MDR1* expression under certain environmental conditions. Mutations that activate these transcription factors enable each of them to independently upregulate *MDR1*. This effect is especially pronounced for Mrr1, which also results in the upregulation of additional genes that contribute to fluconazole resistance, explaining why gain-of-function mutations in Mrr1 are the cause of *MDR1* overexpression in fluconazole-resistant clinical *C. albicans* isolates. In future studies it will be interesting to determine the importance of other transcription factors that bind to the *MDR1* promoter (Mcm1, Ndt80, and Wor1) for Mrr1-mediated upregulation of this multidrug efflux pump.

ACKNOWLEDGMENTS

This study was supported by the Deutsche Forschungsgemeinschaft (DFG grant MO 846/3 and SFB 630), the National Institutes of Health (NIH grant AI058145), and the Canadian Institutes of Health Research (CIHR grant MOP-84341).

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