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# Differential Requirement of the Transcription Factor Mcm1 for Activation of the *Candida albicans* Multidrug Efflux Pump *MDR1* by Its Regulators Mrr1 and Cap1[▽]

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**Overexpression of the multidrug efflux pump Mdr1 causes increased fluconazole resistance in the pathogenic yeast *Candida albicans*. The transcription factors Mrr1 and Cap1 mediate *MDR1* upregulation in response to inducing stimuli, and gain-of-function mutations in Mrr1 or Cap1, which render the transcription factors hyperactive, result in constitutive *MDR1* overexpression. The essential MADS box transcription factor Mcm1 also binds to the *MDR1* promoter, but its role in inducible or constitutive *MDR1* upregulation is unknown. Using a conditional mutant in which Mcm1 can be depleted from the cells, we investigated the importance of Mcm1 for *MDR1* expression. We found that Mcm1 was dispensable for *MDR1* upregulation by $H_2O_2$ but was required for full *MDR1* induction by benomyl. A C-terminally truncated, hyperactive Cap1 could upregulate *MDR1* expression both in the presence and in the absence of Mcm1. In contrast, a hyperactive Mrr1 containing a gain-of-function mutation depended on Mcm1 to cause *MDR1* overexpression. These results demonstrate a differential requirement for the coregulator Mcm1 for Cap1- and Mrr1-mediated *MDR1* upregulation. When activated by oxidative stress or a gain-of-function mutation, Cap1 can induce *MDR1* expression independently of Mcm1, whereas Mrr1 requires either Mcm1 or an active Cap1 to cause overexpression of the *MDR1* efflux pump. Our findings provide more detailed insight into the molecular mechanisms of drug resistance in this important human fungal pathogen.**

Overexpression of the multidrug efflux pump Mdr1 is one mechanism by which the pathogenic yeast *Candida albicans* can develop increased resistance to the antifungal drug fluconazole, which is widely used to treat *Candida* infections (reviewed in reference 13). The *MDR1* gene is not significantly expressed in drug-susceptible *C. albicans* strains under standard growth conditions, but its transcription can be induced by certain toxic compounds, like benomyl or $H_2O_2$ (6, 7, 9, 17). Many fluconazole-resistant clinical *C. albicans* isolates constitutively upregulate *MDR1* under noninducing conditions, and all such isolates studied to date contain gain-of-function mutations in the zinc cluster transcription factor Mrr1 (3, 14). *MRR1* plays a central role in *MDR1* expression, as its deletion abolishes both the inducible activation of the *MDR1* promoter in drug-susceptible strains and constitutive *MDR1* overexpression in fluconazole-resistant clinical isolates (14).

In addition to Mrr1, other transcription factors have also been implicated in the regulation of *MDR1* expression (1, 4, 16, 17, 20, 26, 27). The bZip transcription factor Cap1, which mediates oxidative-stress responses in *C. albicans*, is required for the induction of *MDR1* transcription by $H_2O_2$ and also contributes to benomyl-induced *MDR1* expression (17, 18a, 26). Therefore, Mrr1 and Cap1 upregulate *MDR1* in response to inducing chemicals in a cooperative fashion. However, Cap1 is dispensable for constitutive *MDR1* overexpression in strains containing gain-of-function mutations in Mrr1 (1, 17, 18a). Conversely, a C-terminally truncated, hyperactive Cap1 can also activate the *MDR1* promoter in the absence of *MRR1*, albeit less efficiently than in a wild-type background (18a). Hyperactive forms of either Mrr1 or Cap1 can therefore promote *MDR1* overexpression independently of each other.

Deletion analyses of the *MDR1* promoter identified a region that is important for benomyl-induced *MDR1* upregulation, as well as for constitutive *MDR1* overexpression (16, 17). This region, which was termed BRE (for benomyl response element) or MDRE (for MDR1 drug resistance element), contains a binding site for the MADS box transcription factor Mcm1. When inserted into a heterologous promoter, the BRE/MDRE rendered the promoter responsive to benomyl in a drug-susceptible strain and constitutively active in an *MDR1*-overexpressing strain background (16, 17). Mcm1 is involved in a variety of cellular processes and mediates both repressing and activating functions, presumably by recruiting coregulatory proteins to the respective promoters (18, 23). Mcm1 has been shown to bind to the *MDR1* promoter *in vivo* (11, 23), where it may act together with Mrr1 and/or Cap1 to control expression of the efflux pump in response to inducing stimuli and enable constitutive *MDR1* overexpression in strains containing hyperactive *MRR1* or *CAP1* alleles.

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

| Strain | Parent strain | Relevant characteristics or genotype[a] | Reference |
|---|---|---|---|
| SC5314 | | Wild-type reference strain | 5 |
| SCMPG2A and -B | SC5314 | *ACT1/act1*::$P_{MDR1}$-*GFP-caSAT1* | 14 |
| MRcan42 | SC5314 | *ade2*::*hisG/ade2*::*hisG ura3*::*imm434/ura3*::*imm434 ENO1/eno1*::*ENO1-tetR-ScHAP4AD-3×HA-ADE2 mcm1*::*URA3*::*97t*::*MCM1*::*myc/mcm1*::*FRT* | 18 |
| MRcan43 | SC5314 | *ade2*::*hisG/ade2*::*hisG ura3*::*imm434/ura3*::*imm434 ENO1/eno1*::*ENO1-tetR-ScHAP4AD-3×HA-ADE2 MCM1*::*myc-URA3*::*97t*::*MCM1/mcm1*::*FRT* | 18 |
| can42MPG2A and -B | MRcan42 | *ACT1/act1*::$P_{MDR1}$-*GFP-caSAT1* | This study |
| can43MPG2A and -B | MRcan43 | *ACT1/act1*::$P_{MDR1}$-*GFP-caSAT1* | This study |
| can42MRR1R31A and -B | MRcan42 | *MRR1/MRR1*$^{P683S}$-*SAT1-FLIP* | This study |
| can42MRR1R32A | can42MRR1R31A | *MRR1/MRR1*$^{P683S}$-*FRT* | This study |
| can42MRR1R32B | can42MRR1R31B | *MRR1/MRR1*$^{P683S}$-*FRT* | This study |
| can42MRR1R33A | can42MRR1R32A | *MRR1*$^{P683S}$-*SAT1-FLIP/MRR1*$^{P683S}$-*FRT* | This study |
| can42MRR1R33B | can42MRR1R32B | *MRR1*$^{P683S}$-*SAT1-FLIP/MRR1*$^{P683S}$-*FRT* | This study |
| can42MRR1R34A | can42MRR1R33A | *MRR1*$^{P683S}$-*FRT/MRR1*$^{P683S}$-*FRT* | This study |
| can42MRR1R34B | can42MRR1R33B | *MRR1*$^{P683S}$-*FRT/MRR1*$^{P683S}$-*FRT* | This study |
| can43MRR1R31A and -B | MRcan43 | *MRR1/MRR1*$^{P683S}$-*SAT1-FLIP* | This study |
| can43MRR1R32A | can43MRR1R31A | *MRR1/MRR1*$^{P683S}$-*FRT* | This study |
| can43MRR1R32B | can43MRR1R31B | *MRR1/MRR1*$^{P683S}$-*FRT* | This study |
| can43MRR1R33A | can43MRR1R32A | *MRR1*$^{P683S}$-*SAT1-FLIP/MRR1*$^{P683S}$-*FRT* | This study |
| can43MRR1R33B | can43MRR1R32B | *MRR1*$^{P683S}$-*SAT1-FLIP/MRR1*$^{P683S}$-*FRT* | This study |
| can43MRR1R34A | can43MRR1R33A | *MRR1*$^{P683S}$-*FRT/MRR1*$^{P683S}$-*FRT* | This study |
| can43MRR1R34B | can43MRR1R33B | *MRR1*$^{P683S}$-*FRT/MRR1*$^{P683S}$-*FRT* | This study |
| can42CAP1R11A | MRcan42 | *CAP1*$^{\Delta C333}$-*SAT1-FLIP/CAP1-2* | This study |
| can42CAP1R11B | MRcan42 | *CAP1-1/CAP1*$^{\Delta C333}$-*SAT1-FLIP* | This study |
| can42CAP1R12A | can42CAP1R11A | *CAP1*$^{\Delta C333}$-*FRT/CAP1-2* | This study |
| can42CAP1R12B | can42CAP1R11B | *CAP1-1/CAP1*$^{\Delta C333}$-*FRT* | This study |
| can42CAP1R13A | can42CAP1R12A | *CAP1*$^{\Delta C333}$-*FRT/CAP1*$^{\Delta C333}$-*SAT1-FLIP* | This study |
| can42CAP1R13B | can42CAP1R12B | *CAP1*$^{\Delta C333}$-*SAT1-FLIP/CAP1*$^{\Delta C333}$-*FRT* | This study |
| can42CAP1R14A | can42CAP1R13A | *CAP1*$^{\Delta C333}$-*FRT/CAP1*$^{\Delta C333}$-*FRT* | This study |
| can42CAP1R14B | can42CAP1R13B | *CAP1*$^{\Delta C333}$-*FRT/CAP1*$^{\Delta C333}$-*FRT* | This study |
| can43CAP1R11A and -B | MRcan43 | *CAP1-1/CAP1*$^{\Delta C333}$-*SAT1-FLIP* | This study |
| can43CAP1R12A | can43CAP1R11A | *CAP1-1/CAP1*$^{\Delta C333}$-*FRT* | This study |
| can43CAP1R12B | can43CAP1R11B | *CAP1-1/CAP1*$^{\Delta C333}$-*FRT* | This study |
| can43CAP1R13A | can43CAP1R12A | *CAP1*$^{\Delta C333}$-*SAT1-FLIP/CAP1*$^{\Delta C333}$-*FRT* | This study |
| can43CAP1R13B | can43CAP1R12B | *CAP1*$^{\Delta C333}$-*SAT1-FLIP/CAP1*$^{\Delta C333}$-*FRT* | This study |
| can43CAP1R14A | can43CAP1R13A | *CAP1*$^{\Delta C333}$-*FRT/CAP1*$^{\Delta C333}$-*FRT* | This study |
| can43CAP1R14B | can43CAP1R13B | *CAP1*$^{\Delta C333}$-*FRT/CAP1*$^{\Delta C333}$-*FRT* | This study |

[a] Apart from the indicated features, all strains are identical to their parents. *SAT1-FLIP* denotes the *SAT1* flipper cassette; *FRT* is the FLP recombination target sequence, one copy of which remains in the genome after recycling of the *SAT1* flipper cassette. The *CAP1* alleles in strain SC5314 were distinguished by a BglII restriction site polymorphism. The *CAP1* allele containing the polymorphic BglII site in the upstream region was designated *CAP1-2*. *caSAT1*, *Candida*-adapted *SAT1*.

As *MCM1* is essential in *C. albicans*, the role of this transcription factor in *MDR1* regulation cannot be addressed by deleting the gene. A conditional *mcm1* mutant that contains a single copy of *MCM1* under the control of a tetracycline-repressible promoter has therefore been used to analyze the function of Mcm1 in *C. albicans*. The addition of doxycycline to the conditional mutant results in depletion of Mcm1 within 3 h, but the cells remain viable for a prolonged period, allowing the study of their behavior in the absence of Mcm1 (18). It was shown that depletion of Mcm1 from the cells resulted in the loss of binding activity of protein extracts to an *MDR1* promoter fragment containing the MDRE (16). However, whether Mcm1 is indeed important for the regulation of *MDR1* expression and whether it acts as an activator or a repressor of the *MDR1* promoter were not directly tested in that study. To understand in more detail how the expression of this multidrug efflux pump is controlled in *C. albicans*, we investigated if Mcm1 is necessary for the upregulation of *MDR1* in response to inducing chemicals and if hyperactive forms of Mrr1 and Cap1 require Mcm1 as a coregulator to mediate *MDR1* overexpression.

## 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 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 *Candida*-adapted *FLP* (*caFLP*) gene in the *SAT1* flipper cassette. One hundred to 200 cells were then spread on YPD plates containing 10 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 them on YPD plates containing 200 μg/ml nourseothricin.

**Strain construction.** *C. albicans* strains were transformed by electroporation (10) with the following gel-purified linear DNA fragments. A KpnI-SacII frag-

ment from pMPG2S (14) was used to integrate a $P_{MDR1}$-*GFP* reporter fusion at the *ACT1* locus of strains MRcan42 and MRcan43, resulting in strains can42MPG2A and -B and can43MPG2A and -B, respectively. A SacI-ApaI fragment from pMRR1R3 (18a) was used to insert the hyperactive *MRR1*$^{P683S}$ allele in place of one of the *MRR1* wild-type alleles of strains MRcan42 and MRcan43 with the help of the *SAT1* flipper cassette, generating strains can42MRR1R31A and -B and can43MRR1R31A and -B, respectively. The *SAT1* flipper cassette was then recycled to obtain strains can42MRR1R32A and -B and can43MRR1R32A and -B. A second round of transformation and marker recycling resulted in strains can42MRR1R34A and -B and can43MRR1R34A and -B, in which both endogenous *MRR1* alleles had been replaced by the hyperactive *MRR1*$^{P683S}$ allele. In an analogous fashion, a SacI-ApaI fragment from pCAP1R1 (18a) was used to substitute the wild-type *CAP1* alleles of strains MRcan42 and MRcan43 for the hyperactive *CAP1*$^{\Delta C333}$ allele in two rounds of transformation and recycling of the *SAT1* flipper cassette, generating strains can42CAP1R14A and -B and can43CAP1R14A and -B, respectively. Nourseothricin-resistant transformants were selected as described previously (15), and correct integration of all constructs was confirmed by Southern hybridization with the upstream and downstream flanking sequences. The introduction of the P683S mutation into the first and second *MRR1* allele of the transformants was confirmed by reamplification and direct sequencing of the PCR products.

**Isolation of genomic DNA and Southern hybridization.** Genomic DNA from *C. albicans* strains was isolated as described previously (15). The DNA was 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.

**Analysis of *MDR1* promoter activity by FACS.** YPD overnight cultures of green fluorescent protein (*GFP*) reporter and parental control strains were each diluted $10^{-2}$ in six Erlenmeyer flasks containing 50 ml YPD medium (three without and three with 20 μg/ml doxycycline). After 3 h of growth at 30°C, 50 μg/ml benomyl or 0.005% $H_2O_2$ was added to two of the cultures (in both cases with and without doxycycline) to induce *MDR1* expression; the third culture in each case was left untreated. After 60 min of further incubation, the mean fluorescence of the cells was determined by flow cytometry. 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. Fluorescence data were collected by using logarithmic amplifiers. The mean fluorescence (arbitrary values) was determined with CellQuest Pro (Becton Dickinson) software.

**Analysis of doxycycline-induced *MCM1* repression by Western immunoblotting.** Strains can42MPG2A and -B were grown as described above for determination of *GFP* expression. Whole-cell protein extracts were prepared from two cultures grown for 3 h in the presence or absence of doxycycline and from the remaining cultures after further incubation for 60 min in the presence of benomyl or $H_2O_2$ (with and without doxycycline). Cells were collected by centrifugation, washed twice in water, and broken by vortexing them 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 stock solution [Roche Diagnostics GmbH, Mannheim, Germany], 0.1% β-mercaptoethanol). Samples were centrifuged at 13,000 rpm for 5 min at 4°C, the supernatant was collected, and the protein concentration was quantified with a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, MA). Extracts were heated at 65°C for 10 min, and 400 μg total protein from each sample was separated on an SDS-12% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane with a Trans-Blot SD Semi-Dry transfer apparatus (Bio-Rad, Munich, Germany). For detection of Mcm1-Myc, a monoclonal anti-c-Myc antibody (purified mouse immunoglobulin, clone 9E10, product number M 4439; Sigma-Aldrich Chemie GmbH) was used as the primary antibody at a dilution of 1:6,000, and goat anti-mouse IgG (Fab-specific) peroxidase conjugate (Sigma A 9917) was used as the second antibody at a dilution of 1:6,000. Blots were developed using a chemiluminescence detection system (GE Healthcare UK Limited, Chalfont, United Kingdom) under conditions recommended by the manufacturer.

**Analysis of *MDR1* expression by quantitative real-time reverse transcription (RT)-PCR.** YPD overnight cultures of strains SC5314, MRcan42, MRcan43, can42MRR1R34A and -B, can43MRR1R34A and -B, can42CAP1R14A and -B, and can43CAP1R14A and -B were diluted $10^{-2}$ in 50 ml fresh YPD medium with or without 20 μg/ml doxycycline and incubated for 4 h at 30°C. RNA was extracted by the hot-phenol method (2) combined with a purification step with an RNeasy kit (Qiagen, Hilden, Germany). Contaminating DNA was removed by treatment with the Ambion Turbo DNA-free kit (Applied Biosystems, Darmstadt, Germany), and cDNA was prepared from the RNA with the SuperScript III Reverse Transcriptase kit (Invitrogen, Karlsruhe, Germany). Generation of cDNA and absence of genomic DNA was controlled by PCR with the primers EFB1A (5′-ATTGAACGAATTCTTGGCTGAC-3′) and EFB1B (5′-CATCTTCTTCAACAGCAGCTTG-3′), which bind outside the *EFB1* intron and yield a PCR product of 0.55 kb from cDNA and a 0.8-kb PCR product from genomic DNA. Quantitative PCR (qPCR) was performed with the iQ SYBR green Supermix (Bio-Rad) and the primer pairs MDR5RT (5′-ATTTGTTCAGATCAGTCATTGCTTCAGTGT-3′) and MDR6RT (5′-GGTCCGTTCAAGTAAAACAAAACTGGAATA-3′) for *MDR1* and ACT1RT (5′-AGTGTGACATGGATGTTAGAAAAGAATTATACGG-3′) and ACT2RT (5′-ACAGAGTATTTTCTTTCTGGTGGAGCA-3′) for *ACT1*, which served as the reference, under the following conditions: initial denaturation for 3 min at 95°C; 40 cycles of 30 s at 95°C, 40 s at 50°C, and 10 s at 72°C; and 1 cycle of 30 s at 95°C and 30 s at 57°C. Melt curves were generated and threshold cycle ($C_t$) values were calculated by the Real Time program, Bio-Rad iQ5 V2.0 optical systems software. The $C_t$ values obtained with the software were then used to calculate the relative *MDR1* mRNA levels, adjusted to the *ACT1* mRNA levels, using *MDR1* expression in the wild-type strain SC5314 (set to 1) as a reference. Two independent RNA extractions, each with two technical replicates, were used to calculate means and standard deviations of the final relative expression values.

## RESULTS

**Mcm1 is required for benomyl-induced, but not $H_2O_2$-induced, *MDR1* expression.** To monitor activation of the *MDR1* promoter in response to inducing chemicals in the presence and absence of Mcm1, we introduced a $P_{MDR1}$-*GFP* reporter fusion into the conditional *mcm1* mutant MRcan42, which contains a single Myc-tagged *MCM1* allele under the control of a tetracycline-repressible promoter. In addition, the reporter fusion was introduced into the control strain MRcan43, which contains both a tetracycline-repressible *MCM1* copy and a Myc-tagged *MCM1* allele with its own promoter. Two independent transformants of each parental strain were used for further analysis.

We first confirmed that Mcm1 could be efficiently depleted by addition of doxycycline to the conditional mutants carrying the reporter fusion and that the presence of the *MDR1* inducers benomyl and $H_2O_2$ did not affect doxycycline-mediated repression. As can be seen in Fig. 1, Mcm1 was not detectably expressed after 3 h of growth in the presence of doxycycline, and the addition of benomyl or $H_2O_2$ did not alleviate this repression.

We then determined *MDR1* promoter activity by quantifying the fluorescence of the reporter strains. As previously reported (14), *MDR1* was not detectably expressed in the wild-type strain SC5314 in YPD medium but could be induced by $H_2O_2$ and, even more efficiently, by benomyl (Fig. 2). The presence of doxycycline did not affect the activity of the *MDR1* promoter under noninducing or inducing conditions in the wild-type background. $H_2O_2$ also induced the *MDR1* promoter in the conditional mutant MRcan42, albeit somewhat less efficiently than in strain SC5314, and this induction was also observed after depletion of Mcm1. In contrast, Mcm1 depletion resulted in reduced induction of the *MDR1* promoter by benomyl, as the fluorescence of the conditional mutants was decreased in the presence of doxycycline. Doxycycline had little effect on benomyl-induced *MDR1* expression in the control strain

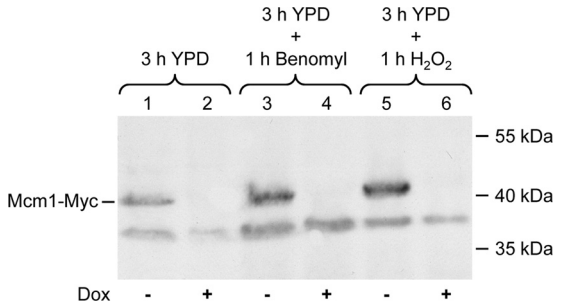


FIG. 1. Depletion of Mcm1 from reporter strains by treatment with doxycycline. Strains can42MPG2A and -B were grown in the absence (−) or presence (+) of doxycycline (Dox) and treated with benomyl or $H_2O_2$ as described in Materials and Methods. Whole-cell protein extracts were prepared and analyzed by Western immunoblotting with an anti-Myc antibody. The position of Myc-tagged Mcm1 is indicated; the lower band is a nonspecific cross-reacting protein. The positions of molecular mass markers are given on the right of the blot. Shown are the results for strain can42MPG2A; the same results were obtained with can42MPG2B.

MRcan43. These results demonstrate that Mcm1 is dispensable for the induction of *MDR1* expression by $H_2O_2$ but is required for full *MDR1* induction by benomyl.

**Mcm1 is required for *MDR1* overexpression by hyperactive Mrr1, but not by hyperactive Cap1.** The induction of the *MDR1* promoter by $H_2O_2$ requires the bZip transcription factor Cap1, which is activated by oxidative stress (17, 18a, 26). Cap1 also contributes to, but is not essential for, benomyl-induced *MDR1* expression (17, 18a, 26). In contrast, the zinc cluster transcription factor Mrr1 is indispensable for *MDR1* expression in the presence of either of these inducers (14). As Mcm1 was necessary for full benomyl-induced, but not for $H_2O_2$-induced, *MDR1* expression, we investigated whether

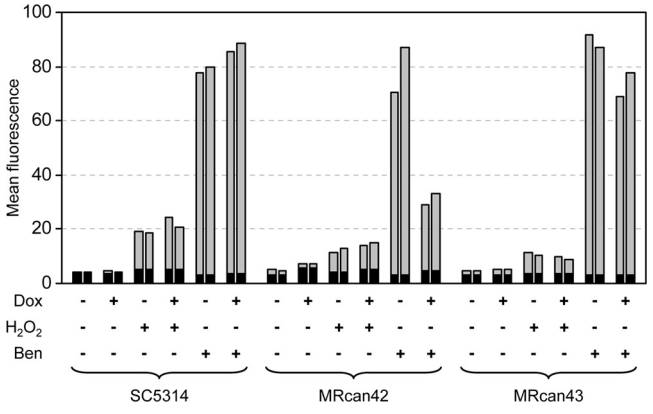


FIG. 2. Activation of the *MDR1* promoter by $H_2O_2$ and benomyl in the wild-type strain, SC5314; the conditional *mcm1* mutant, MRcan42; and the control strain, MRcan43. Parental strains and transformants carrying a $P_{MDR1}$-*GFP* reporter fusion were grown in the absence (−) or presence (+) of doxycycline (Dox) and treated with $H_2O_2$ or benomyl (Ben) as described in Materials and Methods. The mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated reporter strains (SCMPG2A and -B, can42MPG2A and -B, or can43MPG2A and -B) are shown in each case. The background fluorescence of the parental strains, which do not contain the *GFP* gene, is indicated by the black part of each bar.

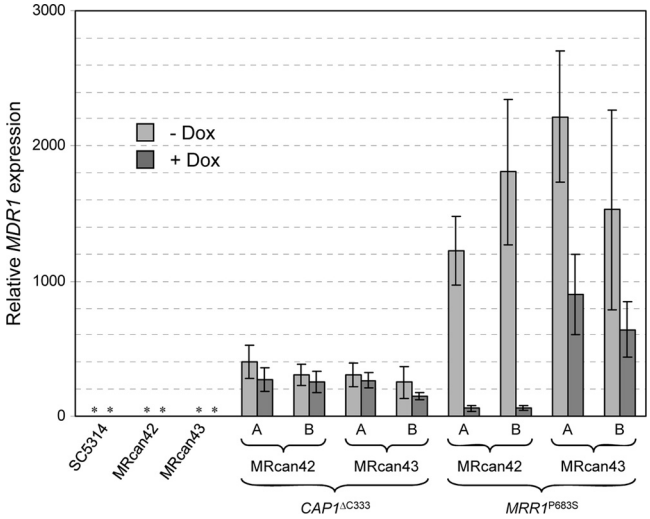


FIG. 3. Effect of Mcm1 depletion on *MDR1* overexpression mediated by hyperactive forms of Cap1 and Mrr1. The wild-type strain, SC5314; the conditional *mcm1* mutant, MRcan42; the control strain, MRcan43; and independent derivatives (A and B) of MRcan42 and MRcan43 that were rendered homozygous for the $CAP1^{\Delta C333}$ or the $MRR1^{P683S}$ allele were grown for 4 h in the absence (light-gray bars) or presence (dark-gray bars) of doxycycline as described in Materials and Methods. *MDR1* mRNA levels were determined by real-time RT-PCR and are presented as relative expression levels compared to those of the reference strain, SC5314, in the absence of doxycycline, which were set to 1. The graph shows the means and standard deviations of two independent experiments, with duplicate measurements performed with each strain. *, *MDR1* expression levels in SC5314 and the parental strains MRcan42 and MRcan43 were too low to be visible in the graph.

Mcm1 was required for the constitutive *MDR1* overexpression caused by hyperactive forms of Mrr1 and Cap1. To this end, we introduced the P683S gain-of-function mutation (14) into both resident *MRR1* alleles of the conditional *mcm1* mutant MRcan42 and the control strain MRcan43. In addition, both wild-type *CAP1* alleles of these strains were replaced by the C-terminally truncated, hyperactive $CAP1^{\Delta C333}$ allele. Homozygous strains were generated, because the activating mutations in *MRR1* and *CAP1* have a stronger effect on *MDR1* expression when they are present in both alleles (18a). Two independent series of mutants (A and B) were constructed in each case and used for further analysis.

As the *MDR1* promoter is constitutively activated in strains expressing hyperactive Mrr1 or Cap1, *GFP* was not useful as a reporter gene to measure the dependence of *MDR1* expression on Mcm1, because considerable amounts of the relatively stable GFP would remain in the cells after *MCM1* expression was shut off by the addition of doxycycline. We therefore measured *MDR1* mRNA levels in the presence and absence of doxycycline by quantitative RT-PCR. As can be seen in Fig. 3, *MDR1* was constitutively expressed at high levels in strains carrying the $CAP1^{\Delta C333}$ allele (between 250- and 400-fold higher than in the control strain SC5314), and Mcm1 depletion in the conditional *mcm1* mutants by doxycycline did not significantly reduce *MDR1* mRNA levels. *MDR1* expression was even higher in strains containing the hyperactive $MRR1^{P683S}$ allele (between 1,200- and 2,200-fold higher than in the control strain, SC5314). Mcm1 depletion by doxycycline resulted in a

drastic reduction of *MDR1* mRNA levels in the conditional mutants (between 20- and 30-fold). A moderate reduction of *MDR1* mRNA levels (ca. 2.5-fold) was also observed in the control strains, presumably because strain MRcan43 also strongly overexpresses *MCM1* from the Tet promoter and exhibits only low *MCM1* expression levels from the wild-type allele after the addition of doxycycline. These results indicate that a hyperactive Cap1 can mediate *MDR1* overexpression independently of Mcm1, whereas a hyperactive Mrr1 requires Mcm1 to promote *MDR1* expression.

## DISCUSSION

The transcription factors Mrr1 and Cap1 are both involved in the induction of *MDR1* expression in response to inducing chemicals, and activating mutations in either of the two transcription factors result in constitutive overexpression of the efflux pump (1, 3, 14, 17, 26). However, little is known about how Mrr1 and Cap1 activate *MDR1* transcription and which additional regulatory factors may be required. In this study, we have addressed the role of the MADS box transcription factor Mcm1, which has previously been implicated in the regulation of *MDR1* expression and was recently shown to bind to the *MDR1* promoter *in vivo* (11, 16, 17, 23).

Mcm1 can act as both a positive and a negative regulator of transcription (18, 23). We found that depletion of Mcm1 did not result in constitutive activity of the *MDR1* promoter, indicating that the role of Mcm1 is not that of a repressor of *MDR1* expression in the absence of inducing conditions (Fig. 2 and 3). Depletion of Mcm1 also did not affect the inducibility of the *MDR1* promoter by $H_2O_2$ or the constitutive *MDR1* overexpression caused by a hyperactive Cap1. On the other hand, benomyl-induced *MDR1* expression was reduced, but not abolished, after depletion of Mcm1, and a hyperactive Mrr1 could not upregulate *MDR1* in the absence of Mcm1. The *MDR1* transcripts that were still present after Mcm1 depletion in strains expressing the hyperactive Mrr1 may represent mRNAs that were produced before Mcm1 depletion and not yet degraded. Alternatively, the cells might have contained residual amounts of Mcm1 that were not detectable by Western blot analysis (Fig. 1). These results suggest that the induction of *MDR1* expression by $H_2O_2$ occurs mainly via activation of Cap1, which acts independently of Mcm1, and that induction by benomyl occurs partially via Mrr1, which depends on Mcm1 to activate the *MDR1* promoter, and partially via Cap1. Like $H_2O_2$, benomyl causes oxidative stress and activates Yap1, the homolog of Cap1, in *Saccharomyces cerevisiae* (12). In response to oxidative stress, conserved cysteine residues in the C terminus of Yap1 form disulfide bonds, resulting in conformational changes that interrupt the interaction of Yap1 with the nuclear export protein Crm1, so that Yap1 can accumulate in the nucleus and activate its target genes (24). In *C. albicans*, Cap1 activity is also regulated by nuclear-cytoplasmic shuttling, and mutation of one of the conserved cysteines in Cap1 or removal of the C terminus results in constitutive Cap1 activity (1, 25).

Based on the results of this and previous studies (1, 8, 11, 14, 16, 17, 18a, 23, 26), the model shown in Fig. 4 depicts the involvement of Mrr1, Cap1, and Mcm1 in four scenarios in which *MDR1* expression is upregulated. In the presence of $H_2O_2$ (Fig. 4A), Cap1 is activated, accumulates in the nucleus, and induces *MDR1* expression. Under these conditions, Cap1 requires Mrr1 to activate the *MDR1* promoter, but Mcm1 is dispensable. In the presence of benomyl (Fig. 4B), Cap1 is also activated by oxidative stress and can induce *MDR1* expression, together with Mrr1, independently of Mcm1 (a). However, *MDR1* induction by benomyl can also occur at a reduced level in the absence of Cap1 in an Mrr1-dependent fashion, and this requires the presence of Mcm1 (b). How benomyl activates Mrr1 is not known. It is possible that Mrr1 is activated by direct drug binding, similar to the zinc cluster transcription factors Pdr1 and Pdr3, which regulate efflux pump expression in *S. cerevisiae* and *Candida glabrata* (22), but benomyl may also activate Mrr1 in an indirect fashion. The C-terminally truncated, hyperactive Cap1 can upregulate *MDR1* expression, even in the absence of Mrr1, but not as efficiently as in a wild-type background (18a) (Fig. 4C). In this respect, the hyperactive Cap1 differs from activated wild-type Cap1, which requires Mrr1 to induce the *MDR1* promoter in response to

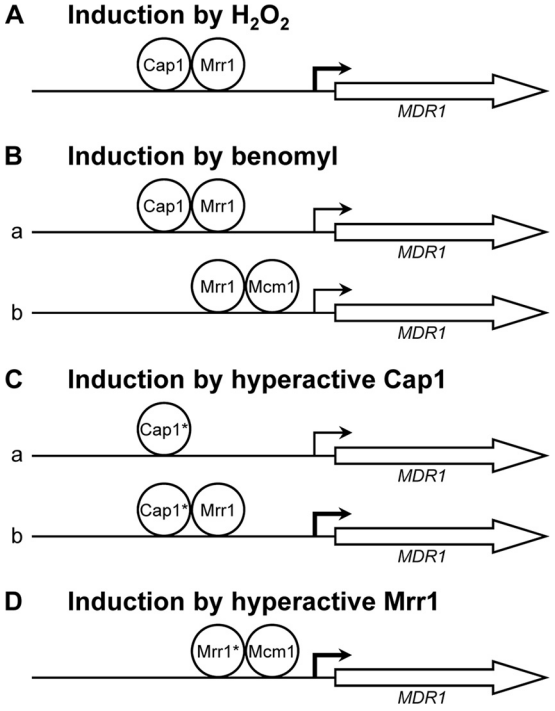


FIG. 4. Model of the roles of the transcription factors Cap1, Mrr1, and Mcm1 in *MDR1* upregulation by inducing chemicals or gain-of-function mutations in Cap1 and Mrr1. The thinner bent arrows indicate reduced *MDR1* promoter activity in the absence of the missing transcription factor. (A) $H_2O_2$ activates Cap1, resulting in accumulation of the transcription factor in the nucleus, where it can induce *MDR1* expression, together with Mrr1, in an Mcm1-independent fashion. (B) Benomyl activates Mrr1 in an unknown way and also, at least partially, Cap1. When Cap1 is available at the *MDR1* promoter, Mrr1 and Cap1 can induce *MDR1* expression independently of Mcm1 (a); in the absence of Cap1, Mrr1 requires Mcm1 to induce *MDR1* expression (b). (C) A hyperactive form of Cap1 (labeled Cap1*) can induce the *MDR1* promoter in the absence of inducing stimuli and independently of Mcm1 and Mrr1 (a), but full induction requires the presence of Mrr1 (b). (D) A hyperactive Mrr1 containing a gain-of-function mutation (labeled Mrr1*) can induce the *MDR1* promoter independently of Cap1 (which is localized in the cytoplasm in the absence of inducing stimuli) but requires the coregulator Mcm1.

$H_2O_2$ or benomyl. Similar to oxidative stress-activated Cap1, however, the hyperactive Cap1 does not depend on Mcm1 to induce *MDR1* expression. Finally, hyperactive Mrr1 can promote *MDR1* overexpression independently of Cap1, but in this case, it requires Mcm1 (Fig. 4D). Altogether, these results indicate that Mrr1 can upregulate *MDR1* expression in cooperation with either Cap1 or Mcm1. In response to oxidative stress, Mrr1 cooperates mainly with activated Cap1 to induce the *MDR1* promoter, and Mcm1 is largely dispensable. In the absence of inducers, Cap1 remains in the cytoplasm, and hyperactive Mrr1 depends on the presence of Mcm1 to cause *MDR1* overexpression. These observations are in accordance with previous findings that deletion of the putative Mcm1 binding sequence (the BRE/MDRE [see the introduction]) from a truncated *MDR1* promoter abolished benomyl-induced or constitutive *MDR1* overexpression (16, 17). However, deletion of the BRE/MDRE from the full-length *MDR1* promoter did not affect its constitutive activity in strains that contained gain-of-function mutations in Mrr1 (3, 8, 16). The result of our present study showing that hyperactive Mrr1 requires Mcm1 to mediate *MDR1* overexpression indicates that Mcm1 can contribute to *MDR1* expression in a manner that is independent of its binding site in the BRE/MDRE. This may occur either by binding of Mcm1 to an additional region in the *MDR1* promoter or by interacting with bound Mrr1 without itself binding to the DNA. Mcm1 has also been found to bind to the *MRR1* promoter, indicating that Mcm1 can also affect *MDR1* expression indirectly, by modulating the expression of *MRR1* (23). In contrast, transcriptional-profiling studies showed that the hyperactive Mrr1 did not affect the expression of *MCM1* or *CAP1*, and the activated Cap1 also did not affect *MCM1* or *MRR1* expression levels (14, 18a, 26).

An important goal for future research will be to unravel how Mrr1, Cap1, and Mcm1 activate *MDR1* transcription. Depending on the conditions, Pdr1 and Pdr3 of *S. cerevisiae* interact with different subunits of the mediator complex to recruit RNA polymerase II to the promoters of their target genes (21, 22). Mrr1 may act in a similar fashion, but it has also recently been shown that both Mrr1 and Cap1 recruit Ada2, a subunit of the SAGA-ADA coactivator complex, to induce transcription (19). It is likely that, depending on the inducing conditions and the combination of transcription factors involved, *C. albicans* uses different mechanisms to upregulate expression of the *MDR1* efflux pump.

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