

# Induction of *Candida albicans* Drug Resistance Genes by Hybrid Zinc Cluster Transcription Factors

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The pathogenic yeast *Candida albicans* can develop resistance to azole antifungal drugs by overexpressing *ERG11*, which encodes the drug target, or the multidrug efflux pumps *MDR1* and *CDR1/CDR2*. The constitutive upregulation of these genes is usually caused by gain-of-function mutations in the zinc cluster transcription factors Upc2, Mrr1, and Tac1, respectively. These transcription factors are also required for the induction of their target genes in drug-susceptible strains in the presence of specific stimuli. By swapping the DNA-binding domains of Mrr1, Tac1, and Upc2 we investigated if the hybrid transcription factors could activate their new target genes in response to the same signals. When Tac1 was targeted to the *MDR1* and *ERG11* promoters, the expression of these genes became inducible by fluphenazine. Similarly, *MDR1* and *CDR2* were strongly upregulated by fluconazole when Upc2 was fused to the DNA-binding domains of Mrr1 and Tac1, respectively. In contrast, Mrr1 was unable to promote gene expression in response to benomyl when it was targeted to the *CDR2* and *ERG11* promoters instead of the *MDR1* promoter. These results suggest that Tac1 and Upc2 themselves are activated by the inducers fluphenazine and fluconazole, respectively, whereas benomyl does not activate Mrr1 itself but a coregulatory factor that is present at the promoters of Mrr1 target genes. Strains in which the expression levels of Mrr1 and Tac1 target genes were controlled by Upc2 exhibited increased fluconazole resistance, demonstrating that the ability to efficiently upregulate the expression of efflux pumps in the presence of the drug results in enhanced intrinsic fluconazole resistance.

Infections by the pathogenic yeast *Candida albicans* are commonly treated with azole antifungal drugs, which inhibit ergosterol biosynthesis. *C. albicans* can develop resistance to azole drugs by various mechanisms. In addition to mutations in the target enzyme, encoded by *ERG11*, the overexpression of *ERG11* and overexpression of genes encoding multidrug efflux pumps are frequent causes of azole resistance in clinical *C. albicans* strains (1, 2). The constitutive upregulation of these genes is usually due to gain-of-function mutations in zinc cluster transcription factors, a family of transcriptional regulators that is specific for fungi. Activating mutations in Upc2 result in increased *ERG11* expression (3–6), while gain-of-function mutations in Mrr1 are responsible for the overexpression of the multidrug efflux pump *MDR1* (7–9) and hyperactive forms of Tac1 mediate the overexpression of the ABC transporters *CDR1* and *CDR2* (10–14).

The expression of *MDR1*, *CDR1/CDR2*, and *ERG11* is also upregulated in drug-susceptible *C. albicans* strains in the presence of certain chemicals. For example, benomyl is a strong inducer of *MDR1* expression (15, 16), fluphenazine efficiently stimulates the expression of *CDR1* and *CDR2* (16, 17), and *ERG11* expression is upregulated by ergosterol biosynthesis inhibitors, such as fluconazole (18, 19). In each case, the respective zinc cluster transcription factor is required for the induction of gene expression (8, 13, 20). How Mrr1, Tac1, and Upc2 are activated in the presence of inducing chemicals is not known. It is possible that the inducers directly bind to the transcription factors and effect a conformational change that enables the activation domains to recruit the transcriptional machinery, as has been reported for the drug resistance regulators Pdr1/Pdr3 of *Saccharomyces cerevisiae* and *Candida glabrata* (21). On the other hand, the transcription factors may also be activated by alterations within the cell that are caused by a drug. For example, the depletion of ergosterol and/or the accumulation of precursor molecules may be a signal that induces *ERG11* upregulation when ergosterol biosynthesis is inhibited by azoles

(22). It is also possible that Mrr1, Tac1, and Upc2 are not the direct sensors of inducing signals, which may act on upstream regulators or on coregulatory transcription factors. For instance, *MDR1* expression is also upregulated by oxidative stress, e.g., in the presence of H<sub>2</sub>O<sub>2</sub> (23). Oxidative stress activates the bZIP transcription factor Cap1 (24, 25), and loss of Cap1 abolishes *MDR1* induction by H<sub>2</sub>O<sub>2</sub> (26, 27). Although Mrr1 is also required for H<sub>2</sub>O<sub>2</sub>-induced *MDR1* upregulation (8, 27), it is likely that Cap1, and not Mrr1, is the sensor of this stimulus.

Most zinc cluster transcription factors contain an N-terminal DNA-binding domain, a C-terminal transcription activation domain, and a large central regulatory domain (28). This is also the case for Mrr1, Tac1, and Upc2, which contain the typical Zn<sub>2</sub>Cys<sub>6</sub> DNA-binding motif in the N terminus. Fusion proteins comprising the N-terminal 129 amino acids of Tac1 or the N-terminal 148 amino acids of Upc2 bound to Tac1 and Upc2 target genes, respectively (13, 29), and the N-terminal 128 amino acids of Mrr1 were sufficient for targeting the transcription factor to the *MDR1* promoter (30). Activating mutations are located in the (putative) regulatory or activation domains of the three transcription factors (1, 2). However, it is not known whether the regulatory/activation

Received 1 October 2014 Returned for modification 29 October 2014

Accepted 2 November 2014

Accepted manuscript posted online 10 November 2014

Citation Schneider S, Morschhäuser J. 2015. Induction of *Candida albicans* drug resistance genes by hybrid zinc cluster transcription factors. *Antimicrob Agents Chemother* 59:558–569. doi:10.1128/AAC.04448-14.

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doi:10.1128/AAC.04448-14

TABLE 1 *C. albicans* strains used in this study<sup>a</sup>

Strain(s)	Parent	Relevant characteristic or genotype	Reference or source
SC5314		Wild-type reference	50
SCMG3A and -B	SC5314	<i>MDR1/mdr1::P<sub>MDR1</sub>-GFP-caSAT1</i>	32
SCCG3A and -B	SC5314	<i>CDR2/cdr2::P<sub>CDR2</sub>-GFP-caSAT1</i>	32
SCEG2A and -B	SC5314	<i>ERG11/erg11::P<sub>ERG11</sub>-GFP-caSAT1</i>	5
SCMRR1M4A and -B	SC5314	<i>mrr1Δ::FRT/mrr1Δ::FRT</i>	8
SCTAC1M4A and -B	SC5314	<i>tac1Δ::FRT/tac1Δ::FRT</i>	32
UPC2M4A and -B	SC5314	<i>upc2Δ::FRT/upc2Δ::FRT</i>	3
SCΔ <i>mrr1</i> MDR1G42A	SCMRR1M4A	<i>mrr1Δ::FRT/mrr1Δ::FRT</i>	This study
		<i>MDR1/mdr1::P<sub>MDR1</sub>-GFP-FRT</i>	
SCΔ <i>mrr1</i> MDR1G42B	SCMRR1M4B	<i>mrr1Δ::FRT/mrr1Δ::FRT</i>	This study
		<i>MDR1/mdr1::P<sub>MDR1</sub>-GFP-FRT</i>	
SCΔ <i>tac1</i> CDR2G52A	SCTAC1M4A	<i>tac1Δ::FRT/tac1Δ::FRT</i>	This study
		<i>CDR2/cdr2::P<sub>CDR2</sub>-GFP-FRT</i>	
SCΔ <i>tac1</i> CDR2G52B	SCTAC1M4B	<i>tac1Δ::FRT/tac1Δ::FRT</i>	This study
		<i>CDR2/cdr2::P<sub>CDR2</sub>-GFP-FRT</i>	
SCΔ <i>upc2</i> ERG11G42A	UPC2M4A	<i>upc2Δ::FRT/upc2Δ::FRT</i>	This study
		<i>ERG11/erg11::P<sub>ERG11</sub>-GFP-FRT</i>	
SCΔ <i>upc2</i> ERG11G42B	UPC2M4B	<i>upc2Δ::FRT/upc2Δ::FRT</i>	This study
		<i>ERG11/erg11::P<sub>ERG11</sub>-GFP-FRT</i>	

<sup>a</sup> Derivatives containing the various hybrid transcription factors are not listed.

domains of Mrr, Tac1, and Upc2 contain the necessary information to respond to a specific stimulus. If this is the case, targeting the transcription factors to different promoters by exchanging their DNA-binding domains should result in the activation of the new target gene in response to the same signal. In the present study, we have addressed this question by swapping the DNA-binding domains of Mrr1, Tac1, and Upc2 and investigating the effect on the inducibility of drug resistance genes by specific stimuli.

## 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  $-80^{\circ}\text{C}$  and subcultured on yeast extract-peptone-dextrose (YPD) agar plates (10 g yeast extract, 20 g peptone, 20 g glucose, and 15 g agar per liter) at  $30^{\circ}\text{C}$ . The strains were routinely grown in YPD liquid medium at  $30^{\circ}\text{C}$  in a shaking incubator. The selection of nourseothricin-resistant transformants and recycling of the SAT1 flipper cassette were performed as described previously (31).

**Plasmid constructions.** Plasmids pMDR1G3 and pERG11G2, in which the GFP reporter gene is fused to the MDR1 and ERG11 promoters, respectively, have been described previously (5, 32). To allow recycling of the nourseothricin resistance marker after genomic insertion of the reporter fusions, the *caSAT1* marker in these plasmids was replaced by the SAT1 flipper cassette. An ApaI-SalI fragment from pMDR1G3 containing the MDR1 promoter was ligated together with a SalI-BamHI fragment from pOP4G4 (33) containing GFP, the TEF3 transcription termination sequence, and an FLP recombination target sequence (FRT) into the ApaI/BamHI-digested pMDR1M2 (27), which contains the remainder of the SAT1 flipper cassette and MDR1 downstream sequences. The resulting plasmid pMDR1G4 contains a  $P_{MDR1}$ -GFP reporter fusion that can be targeted to the endogenous MDR1 locus in the *C. albicans* genome, followed by recycling of the SAT1 flipper cassette. To obtain an analogous cassette with a  $P_{ERG11}$ -GFP reporter fusion, an ApaI-BglII fragment from pERG11G2 containing the  $P_{ERG11}$ -GFP fusion was first substituted for the  $P_{OP4}$ -GFP fragment in pOP4G4, generating pERG11G3. ERG11 downstream sequences were then amplified by PCR from genomic DNA of strain SC5314 with the primers ERG17 (5'-AATACCGCGCAA CTTCTTTCGATTTCAGTG-3') and ERG16 (5'-CTAAGAGCTCGAAT CCTGGTCTATATTAGC-3'), digested at the introduced SacII and SacI

sites (underlined), and substituted for the OP4 downstream sequences in pERG11G3 to yield pERG11G4. The analogous plasmid pCDR2G5 with a  $P_{CDR2}$ -GFP reporter fusion was described in a previous report (34).

Hybrids between MRR1, TAC1, and UPC2 were generated in the following way. A part of the MRR1 open reading frame (ORF) encoding amino acids 129 to 1108 was amplified from plasmid pZCF36K2 (8) with the primer ZCF36-95 (5'-ATATGGCGCCAATAATCAACAAA CAGCTTCTG-3') and a primer binding within the T<sub>ACT1</sub> sequence. The PCR product was digested at the introduced KasI site and at the BglII site behind the MRR1 stop codon and ligated together with an ApaI-KasI fragment from plasmid pZCF36DB1 (30) containing ADH1 upstream sequences and the 5' part of the MRR1 ORF (encoding amino acids 1 to 128) in the ApaI/BglII-digested pZCF36E2 (9). The resulting plasmid pMRR1DB1-MRR1 encodes a protein in which the DNA-binding domain and the remainder of Mrr1 are fused via a Gly-Ala linker and which is expressed from the ADH1 promoter after integration into the genomic ADH1 locus. To construct an MRR1DB-TAC1 fusion, the 3' part of the TAC1 ORF encoding amino acids 130 to 981 was amplified from plasmid pTAC1E2 (34) with the primers TAC1-16 (5'-ATATGGCGCCGAT CTAGAATCGAGATTGAGTCG-3') and TAC1-9 (5'-TTTTGGATCCT TAAATCCCCAAATTATTGTCAAAG-3'). The PCR product was digested at the introduced KasI and BamHI sites and ligated behind the MRR1 DNA-binding domain in the KasI/BglII-digested pMRR1DB1-MRR1, generating pMRR1DB1-TAC1. Similarly, the 3' part of the UPC2 ORF encoding amino acids 150 to 712 was amplified from plasmid pUPC2E2 (34) with the primers UPC2-9 (5'-ATATGGCGCCCAAC TAATCCACTTAGTGCTTTG-3') and UPC2-2 (5'-ATATAGATCTA TTTTCATATTCATAAACCATTATC-3'). The PCR product was digested at the introduced KasI and BglII sites and cloned in the KasI/BglII-digested pMRR1DB1-MRR1 to yield pMRR1DB1-UPC2. Fusions of the 3' parts of MRR1, TAC1, and UPC2 to the TAC1 DNA-binding domain (encoding amino acids 1 to 129) were obtained by ligating the KasI-SacI fragments from pMRR1DB1-MRR1, pMRR1DB1-TAC1, and pMRR1DB1-UPC2 (containing the respective MRR1, TAC1, and UPC2 sequences, the ACT1 transcription termination sequence, the *caSAT1* marker, and 3' ADH1 sequences) with the KasI/SacI-digested plasmid pTAC1DBH1 (34). Similarly, fusions of the 3' parts of MRR1, TAC1, and UPC2 to the UPC2 DNA-binding domain (encoding amino acids 1 to 148) were obtained by ligating the KasI-SacI fragments from

pMRR1DB1-MRR1, pMRR1DB1-TAC1, and pMRR1DB1-UPC2 with the *KasI/SacI*-digested plasmid pUPC2DBH1 (34).

Fusions of the *TAC1* and *UPC2* DNA-binding domains with hyperactive forms of *MRR1* were also generated. An *EcoRI*-*BglII* fragment from plasmid pZCF36E3 (9) containing the 3' part of the *MRR1*<sup>P683S</sup> allele was substituted for the corresponding wild-type *MRR1* sequences in pTAC1DB1-MRR1 and pUPC2DB1-MRR1 to produce plasmids pTAC1DB1-MRR1-4 and pUPC2DB1-MRR1-4, respectively. An *EcoRI*-*Sall* fragment from plasmid pZCF36EH2 (27) containing the 3' part of a hemagglutinin (HA)-tagged *MRR1* allele, the *ACT1* transcription termination sequence, and part of the *caSAT1* marker was substituted for the corresponding sequences in pTAC1DB1-MRR1 and pUPC2DB1-MRR1 to generate plasmids pTAC1DB1-MRR1-5 and pUPC2DB1-MRR1-5, respectively. To obtain hybrids of the *TAC1* or *UPC2* DNA-binding domains and *MRR1* with a C-terminal fusion to the *GAL4* activation domain, an *EcoRI*-*Sall* fragment from plasmid pMRR1-GAD1 (34) was inserted in the *EcoRI/Sall*-digested pTAC1DB1-MRR1, yielding pTAC1DB1-MRR1-6. Substitution of the *UPC2* DNA-binding domain for the *TAC1* DNA-binding domain in the latter plasmid resulted in pUPC2DB1-MRR1-6.

**Strain constructions.** *C. albicans* strains were transformed by electroporation (35) with the following gel-purified linear DNA fragments. The inserts from pMDR1G4, pCDR2G5, and pERG11G4 were used to integrate  $P_{MDR1}$ -*GFP*,  $P_{CDR2}$ -*GFP*, and  $P_{ERG11}$ -*GFP* reporter fusions at the corresponding endogenous loci in the *mrr1Δ* mutants SCMRR1M4A and -B, the *tac1Δ* mutants SCTAC1M4A and -B, and the *upc2Δ* mutants UPC2M4A and -B, respectively. Subsequent excision of the *SAT1* flipper cassette resulted in the reporter strains  $SCΔmrr1MDR1G42A$  and -B,  $SCΔtac1CDR2G52A$  and -B, and  $SCΔupc2ERG11G42A$  and -B, respectively. The inserts from pMRR1DB1-MRR1, pMRR1DB1-TAC1, pMRR1DB1-UPC2, pTAC1DB1-MRR1, pTAC1DB1-TAC1, pTAC1DB1-UPC2, pUPC2DB1-MRR1, pUPC2DB1-TAC1, pUPC2DB1-UPC2, pTAC1DB1-MRR1-4, pTAC1DB1-MRR1-5, pTAC1DB1-MRR1-6, pUPC2DB1-MRR1-4, pUPC2DB1-MRR1-5, and pUPC2DB1-MRR1-6 were used to integrate genes encoding hybrid transcription factors and control constructs under the control of the *ADH1* promoter at the *ADH1* locus of the reporter strains described above and of their parental *mrr1Δ*, *tac1Δ*, and *upc2Δ* mutants. The correct integration of each construct and the excision of the *SAT1* flipper cassette were confirmed by Southern hybridization using the flanking sequences as probes.

**Isolation of genomic DNA and Southern hybridization.** Genomic DNA from *C. albicans* strains was isolated as described previously (31). The DNA was digested with appropriate restriction enzymes, separated on a 1% agarose gel, 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 UK Limited, Little Chalfont, Buckinghamshire, United Kingdom) according to the instructions of the manufacturer.

**Flow cytometry.** Overnight cultures of the *GFP* reporter and control strains were diluted  $10^{-2}$  in fresh YPD medium and grown for 3 h at 30°C. After addition of 50 μg/ml benomyl, 10 μg/ml flufenazine, 50 μg/ml fluconazole, or no drug, the cultures were incubated for an additional hour. The cell suspensions were 10-fold diluted in 1 ml cold phosphate-buffered saline (PBS), and flow cytometry was performed using a MACS-QuantAnalyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) equipped with an argon laser emitting at 488 nm. Fluorescence was detected using the B1 fluorescence channel equipped with a 525-nm band-pass filter (bandwidth of 50 nm). A total of 20,000 cells were analyzed per sample and counted at a flow rate of approximately 500 cells/s. Fluorescence data were collected by using logarithmic amplifiers. The mean fluorescence (arbitrary values) was determined with MACSQuantify (version 2.4; Miltenyi Biotec) software.

**Fluconazole susceptibility testing.** To determine the fluconazole susceptibilities of the strains, a 2-fold dilution series of fluconazole (Sigma

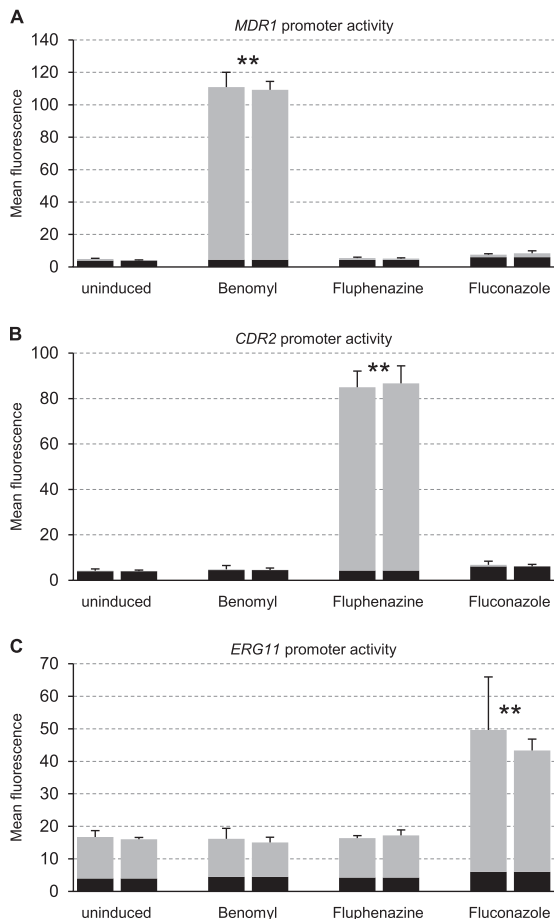
GmbH, Deisenhofen, Germany) was prepared in the assay medium, starting from an initial concentration of 256 μg/ml. Susceptibility tests were carried out using a previously described microdilution method (36), except that the assays were performed in SD medium (6.7 g of yeast nitrogen base without amino acids [YNB; BIO 101, Vista, CA], 20 g of glucose, and 0.77 g of complete supplement medium [CSM; BIO101]) instead of high-resolution (HR) medium (32). MICs of fluconazole were determined after 24 h of growth at 37°C, because the growth inhibition of the tested strains was more clearly observed at this time point than after longer incubation times.

## RESULTS

**Induction of *MDR1*, *CDR2*, and *ERG11* expression by specific stimuli.** To investigate the upregulation of *Mrr1*, *Tac1*, and *Upc2* target genes by specific inducers, we used reporter strains expressing *GFP* under the control of the *MDR1*, *CDR2*, or *ERG11* promoter in a wild-type background. *CDR2* was chosen as a *Tac1* target gene because it is not significantly expressed in the absence of inducers, whereas *CDR1* exhibits relatively high, *Tac1*-independent basal expression levels (13, 34). The reporter strains were grown in the presence or absence of the inducers benomyl, flufenazine, or fluconazole, and the induction of each promoter was assessed by quantifying the fluorescence of the cells (Fig. 1). In accordance with previous results (8, 32, 37–39), *MDR1* was not detectably expressed under uninduced conditions and was strongly induced by benomyl but not by flufenazine or fluconazole (Fig. 1A). *CDR2* was also not detectably expressed in the absence of inducers but was strongly upregulated by flufenazine, whereas benomyl and fluconazole had no effect (Fig. 1B). Basal *ERG11* expression levels were well detected with *GFP* as a reporter, and the *ERG11* promoter was further upregulated in the presence of fluconazole but not by benomyl and flufenazine (Fig. 1C). These results confirmed the specific induction of *MDR1*, *CDR2*, and *ERG11* expression by the inducers benomyl, flufenazine, and fluconazole, respectively, under the experimental conditions used. The experiments also demonstrated the suitability of the *GFP* reporter fusions for the analysis of the differential induction of the three genes.

**Construction of hybrid transcription factors.** To investigate whether the induction of the *MDR1*, *CDR2*, and *ERG11* promoters by benomyl, flufenazine, and fluconazole, respectively, could be transferred from one to the other genes by retargeting the corresponding transcription factor, we swapped the DNA-binding domains of *Mrr1*, *Tac1*, and *Upc2*. The resulting hybrid transcription factors, in which the two parts of each protein were fused via a Gly-Ala linker (encoded by a *KasI* site used for cloning), are illustrated in Fig. 2. As a control, the DNA-binding domain of each transcription factor was also fused in the same way to the remainder of its own protein to exclude a possible negative effect of the inserted Gly-Ala linker. The hybrid transcription factors and control constructs were introduced into reporter strains lacking the relevant wild-type transcription factor and expressed from the *ADH1* promoter.

**Inducibility of the *MDR1* promoter by hybrid transcription factors.** Figure 3 shows the activity of the *MDR1* promoter in strains from which the endogenous *MRR1* alleles were deleted and which contained hybrid transcription factors consisting of the *Mrr1* DNA-binding domain and the regulatory/activation domains of *Tac1* or *Upc2*. As previously reported (8, 27, 32), no induction of *MDR1* expression by benomyl was observed in the absence of *Mrr1*, and the inducibility by benomyl was restored



**FIG 1** Inducibility of the *MDR1*, *CDR2*, and *ERG11* promoters by benomyl, fluphenazine, and fluconazole. Transformants of the wild-type strain SC5314 expressing the *GFP* reporter gene from the *MDR1* (A), *CDR2* (B), or *ERG11* (C) promoter were grown in the absence or presence of inducers as indicated. 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: SCMG3A and -B (*MDR1*), SCCG3A and -B (*CDR2*), and SCEG2A and -B (*ERG11*). The background fluorescence of the parental strain SC5314 is indicated by the black part of each column. Fluorescence values that are at least 2-fold higher than those of the uninduced controls (combined data of the two independently generated strains) are marked by asterisks (\*\*,  $P < 0.01$ , two-tailed  $t$  test).

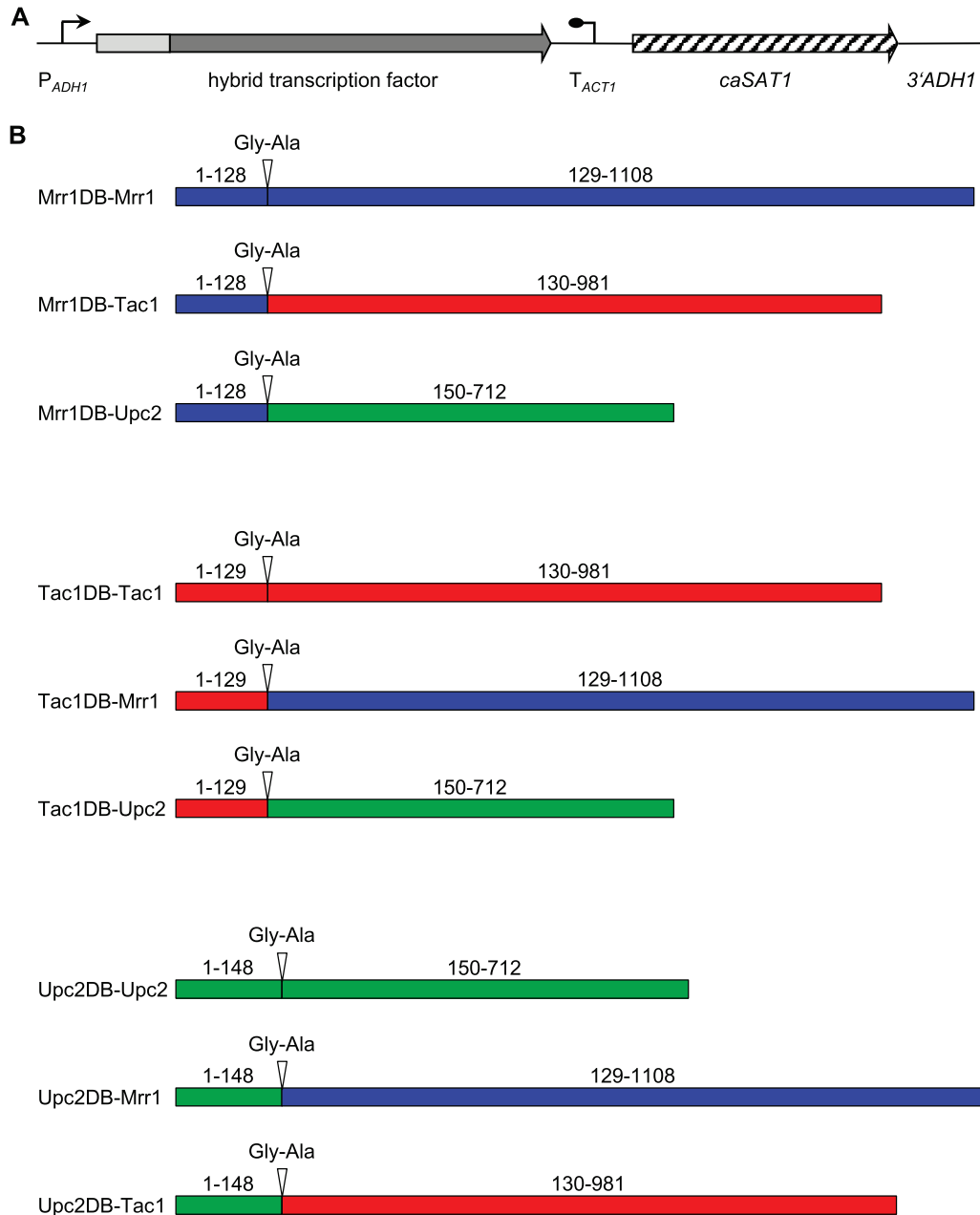
when the *MRR1DB-MRR1* control construct was expressed in the mutants (Fig. 3A). When the regulatory/activation domain of Mrr1 was replaced by that from Tac1, *MDR1* induction by benomyl was largely, but not completely, abolished (Fig. 3A). However, *MDR1* expression in these strains could be efficiently stimulated by fluphenazine (Fig. 3B), demonstrating that the presence of Tac1 at the *MDR1* promoter rendered it responsive to this inducer. A slight induction of the *MDR1* promoter by the hybrid Mrr1DB-Tac1 protein was also observed in the presence of fluconazole (Fig. 3C; see also Discussion). Interestingly, *MDR1* was constitutively expressed even in the absence of inducers when the Mrr1 DNA-binding domain was fused to the regulatory/activation domain of Upc2. *MDR1* promoter activity was further increased in the presence of fluconazole (Fig. 3C), but not by the original inducer benomyl (Fig. 3A) or by fluphenazine (Fig. 3B).

Therefore, Upc2 could upregulate *MDR1* expression in the presence of fluconazole when it was fused to the Mrr1 DNA-binding domain, similar to the fluphenazine-induced *MDR1* expression by Mrr1DB-Tac1 (see also Discussion). In additional control experiments, we tested *MDR1* expression in strains in which the Mrr1 DNA-binding domain was replaced by those of Tac1 or Upc2. No induction of the *MDR1* promoter was observed in these strains, except for a slight upregulation by the hybrid Upc2DB-Mrr1 protein in the presence of benomyl (Fig. 3A to C).

**Inducibility of the *CDR2* promoter by hybrid transcription factors.** Figure 4 shows the activity of the *CDR2* promoter in strains lacking the endogenous *TAC1* alleles and expressing *TAC1DB-MRR1* or *TAC1DB-UPC2* fusions. The inability of the *tac1* $\Delta$  mutants to upregulate *CDR2* expression in the presence of fluphenazine was complemented by the *TAC1DB-TAC1* control construct (Fig. 4A). When the Tac1 DNA-binding domain was fused to Upc2, *CDR2* expression could be induced by fluconazole instead of fluphenazine (Fig. 4A and C). Of note, no constitutive *CDR2* expression was observed when Upc2 was targeted to the *CDR2* promoter, which is in contrast to the activation of the *MDR1* promoter in the absence of inducers by the Mrr1DB-Upc2 hybrid transcription factor (see above). Therefore, Upc2 could promote *CDR2* expression when it was targeted to the *CDR2* promoter, but it had to be activated by the inducer fluconazole. When the Tac1 DNA-binding domain was fused to Mrr1, no *CDR2* expression was observed in the presence of any of the inducers, indicating that Mrr1 was unable to effect benomyl-induced gene expression when targeted to the *CDR2* promoter instead of the *MDR1* promoter (Fig. 4B). Not surprisingly, no induction of *CDR2* expression occurred when the DNA-binding domain of Tac1 was replaced by those of Mrr1 or Upc2, which do not bind to the *CDR2* promoter (27, 40).

**Inducibility of the *ERG11* promoter by hybrid transcription factors.** Figure 5 shows the activity of the *ERG11* promoter in *upc2* $\Delta$  mutants expressing *UPC2DB-MRR1* or *UPC2DB-TAC1* fusions. As previously reported (20, 29), no induction of the *ERG11* promoter by fluconazole occurred in the absence of Upc2; this deficiency was complemented after insertion of the *UPC2DB-UPC2* control construct (Fig. 5A). The fluconazole inducibility was lost when the Upc2 DNA-binding domain was fused to Tac1, but the hybrid Upc2DB-Tac1 transcription factor upregulated *ERG11* expression in response to fluphenazine (Fig. 5C), demonstrating that Tac1 could activate both *MDR1* and *ERG11* expression in response to this normally noninducing stimulus when targeted to the respective promoter. Interestingly, basal *ERG11* expression levels were reduced by Upc2DB-Tac1 (the fluorescence of the cells was 1.7-fold lower than that of the controls,  $P < 0.01$ ), indicating that Tac1 bound to the *ERG11* promoter acted as a repressor, unless it was activated by fluphenazine. When the Upc2 DNA-binding domain was fused to Mrr1, no upregulation of *ERG11* expression by any of the inducers was observed. Therefore, Mrr1 could not induce gene expression in response to benomyl when targeted to the *ERG11* (Fig. 5B) or *CDR2* promoter (Fig. 4B). When the DNA-binding domain of Upc2 was replaced by those of Mrr1 or Tac1, which do not bind to the *ERG11* promoter (27, 41), no upregulation of *ERG11* expression occurred in the presence of any of the inducers (Fig. 5A to C).

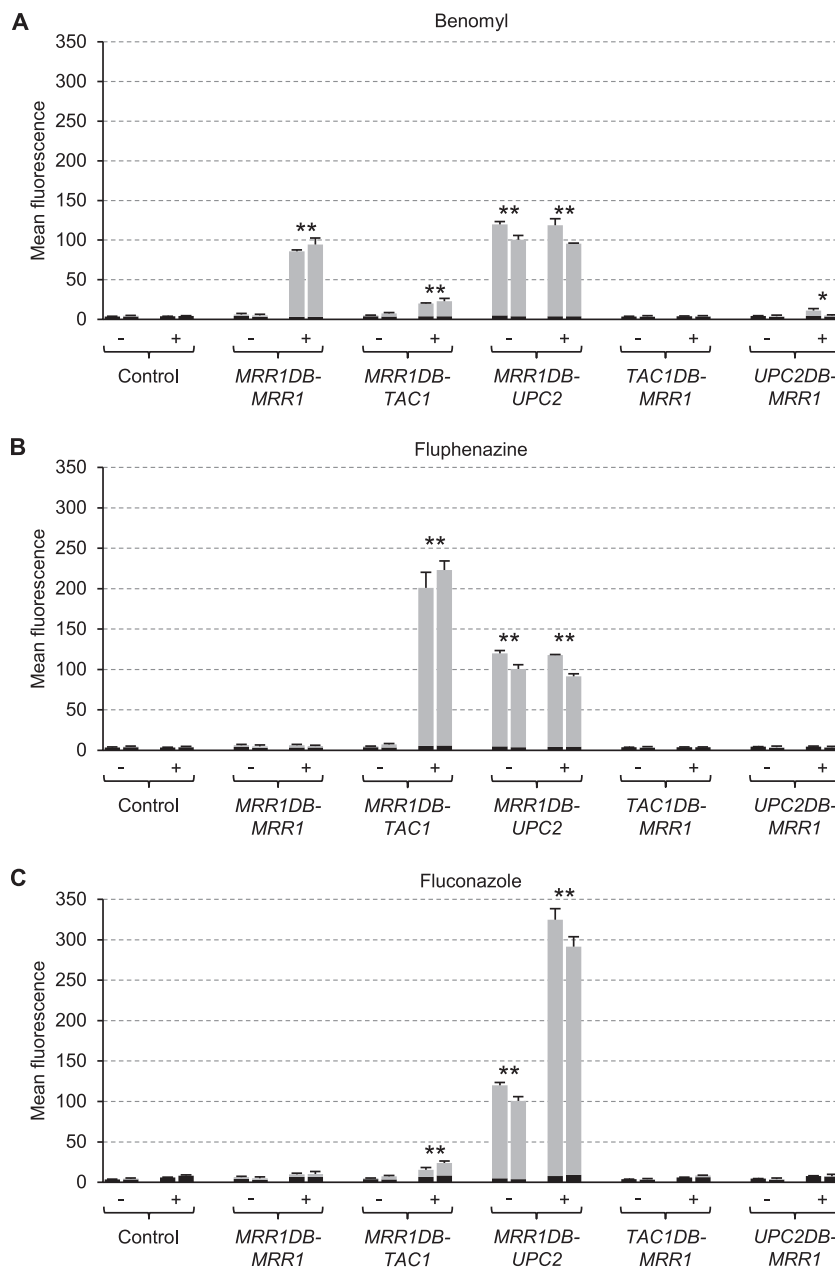
**Hyperactive forms of Mrr1 can activate gene expression when fused to heterologous DNA-binding domains.** The inability of Tac1DB-Mrr1 and Upc2DB-Mrr1 fusion proteins to acti-



**FIG 2** Expression of hybrid zinc cluster transcription factors in *C. albicans*. (A) General structure of the cassettes used for expression of hybrid transcription factors. The DNA-binding domains of the transcription factors are represented by the light gray box.  $P_{ADH1}$ , *ADH1* promoter;  $T_{ACT1}$ , transcription termination sequence of the *ACT1* gene; *caSAT1*, *Candida*-adapted nourseothricin resistance marker;  $3'ADH1$ , sequence from the 3' part of the *ADH1* gene. The flanking *ADH1* sequences ( $P_{ADH1}$  and  $3'ADH1$ ) served for integration into the *ADH1* locus by homologous recombination. (B) Schematic diagrams of the hybrid transcription factors constructed in this study. Mrr1, Tac1, and Upc2 sequences are represented in blue, red, and green, respectively. The extent of the DNA-binding domain (DB) and the remainder of the proteins are indicated (amino acid positions correspond to those in the wild-type transcription factors). The Gly-Ala linker that was inserted between the two parts of each protein is also shown.

vate the *CDR2* and *ERG11* promoters suggested that Mrr1 itself is unable to react to the presence of the inducer benomyl. However, it was also possible that these fusion proteins were simply unstable or incorrectly folded and therefore nonfunctional. To address this possibility, we constructed analogous fusions with constitutively active forms of Mrr1 that do not require the inducer benomyl. The tested fusion proteins contained the P683S gain-of-function mutation, which was previously found in a fluconazole-resistant clin-

ical *C. albicans* isolate (8), a C-terminal 3×HA tag, which acts like a gain-of-function mutation (27), or a C-terminal Gal4 activation domain (34). When expressed in *tac1Δ* and *upc2Δ* mutants carrying  $P_{CDR2}$ -GFP and  $P_{ERG11}$ -GFP reporter fusions, respectively, the target genes were constitutively activated by all three respective hybrid transcription factors, albeit with different efficiencies (Fig. 6). These results argued that the hybrid Tac1DB-Mrr1 and Upc2DB-Mrr1 transcription factors were not intrinsically unsta-

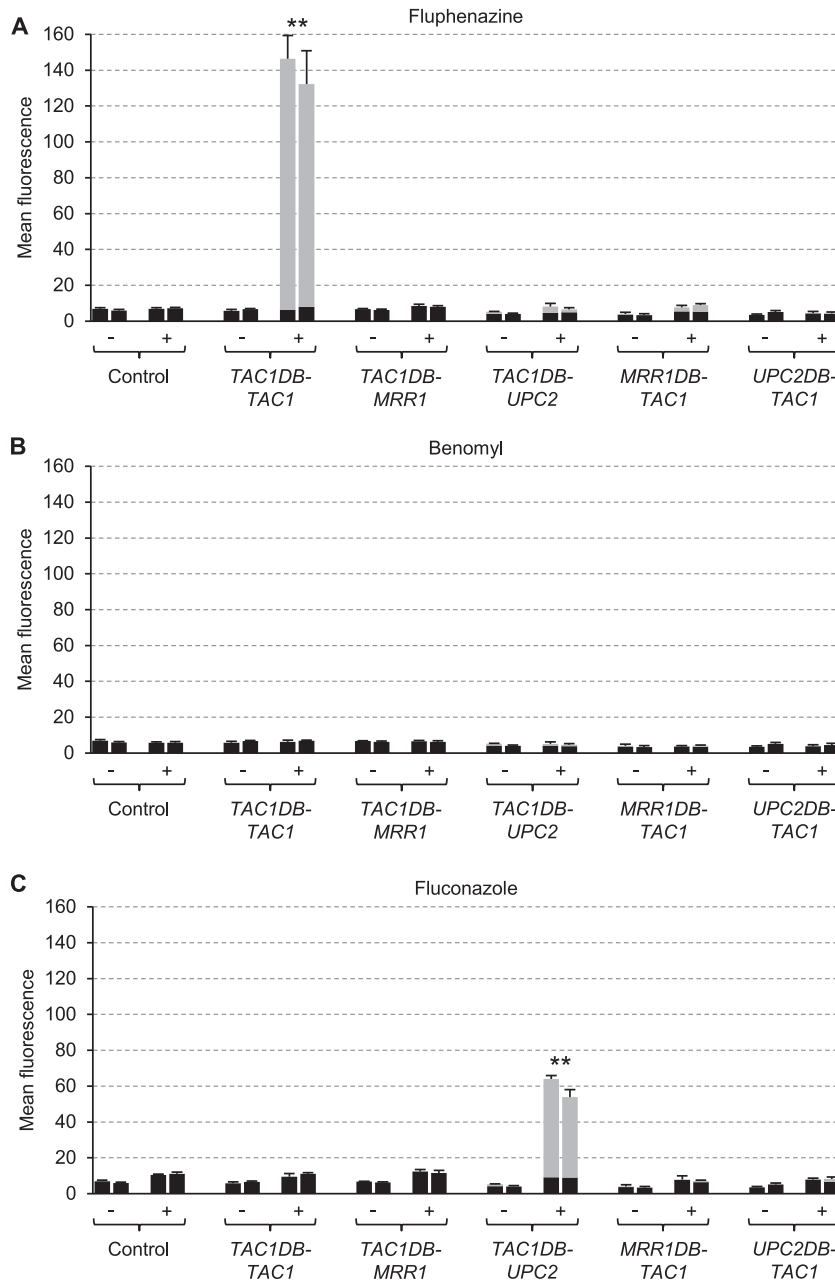


**FIG 3** Inducibility of the *MDR1* promoter in *mrr1* $\Delta$  mutants carrying a  $P_{MDR1}$ -GFP reporter fusion and the indicated hybrid transcription factors by benomyl (A), fluphenazine (B), and fluconazole (C). Strains were grown in the absence (-) or presence (+) of inducers, and the mean fluorescence 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 background fluorescence of otherwise identical strains without GFP is indicated by the black part of each column. Values of the uninduced cultures are from the same experiments in panels A to C and are included for comparison with each inducer. Fluorescence values that are at least 2-fold higher than those of the controls (combined data of the two independently generated strains) are marked by asterisks (\*\*,  $P < 0.01$ ; \*,  $P < 0.05$ , two-tailed *t* test).

ble. Their failure to induce gene expression in response to benomyl suggests that benomyl acts on a coregulatory factor, which is present at the *MDR1* but not the *CDR2* and *ERG11* promoters, rather than on Mrr1 itself.

**Fluconazole susceptibilities of strains containing hybrid transcription factors.** Mrr1 and Tac1 are not active under standard growth conditions and also do not induce their target genes in the presence of fluconazole. Deletion of *MRR1* and *TAC1* from a drug-susceptible wild-type strain, like SC5314, therefore does not result in

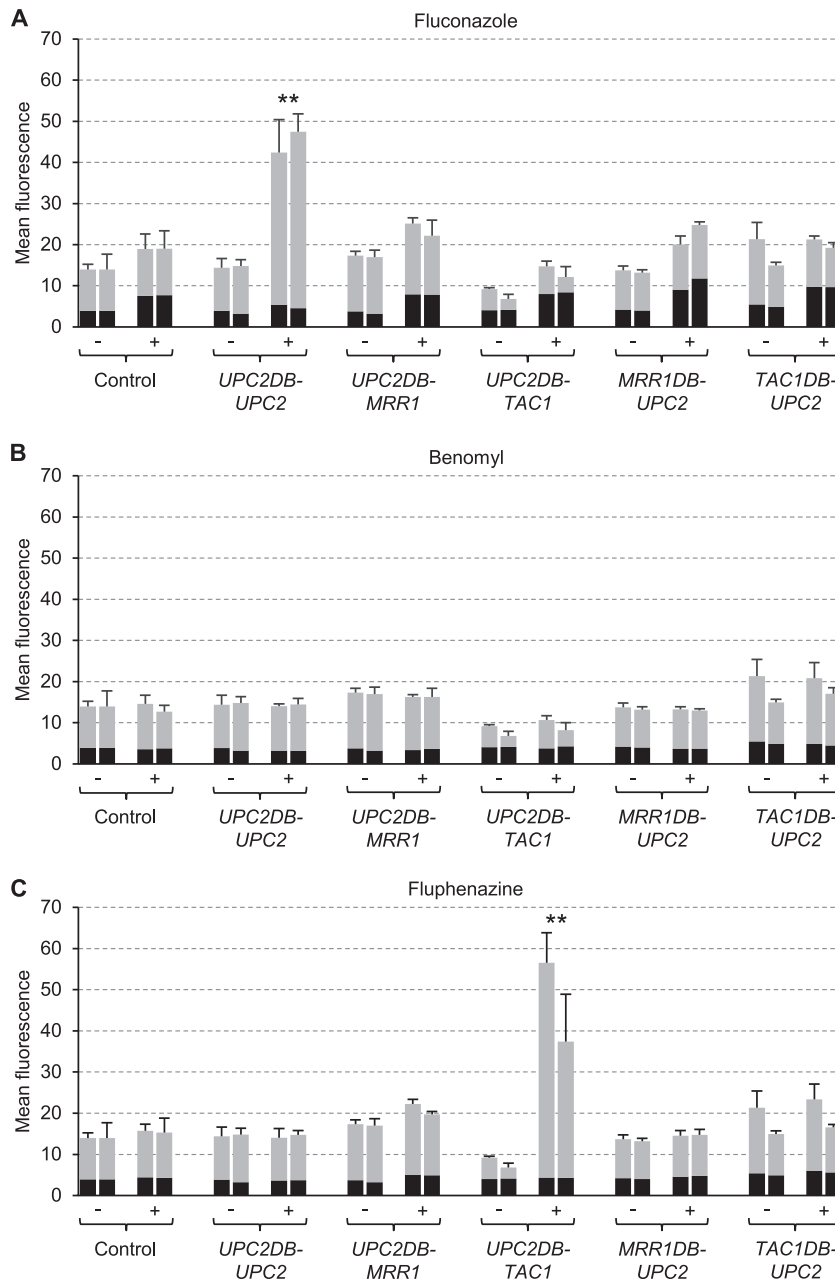
fluconazole hypersusceptibility (8, 34). Upc2, when targeted to the *MDR1* or *CDR2* promoters by fusion with the Mrr1 and Tac1 DNA-binding domains, respectively, constitutively activated *MDR1* expression (Fig. 3) and upregulated *MDR1* and *CDR2* expression in the presence of fluconazole (Fig. 3C and 4C). This observation suggested that strains containing Mrr1DB-Upc2 or Tac1DB-Upc2 proteins should exhibit increased fluconazole resistance. We therefore tested the drug susceptibilities of *mrr1* $\Delta$ , *tac1* $\Delta$ , and *upc2* $\Delta$  mutants expressing hybrid transcription factors or control constructs (Fig. 7). Unlike



**FIG 4** Inducibility of the *CDR2* promoter in *tac1Δ* mutants carrying a  $P_{CDR2}$ -*GFP* reporter fusion and the indicated hybrid transcription factors by fluphenazine (A), benomyl (B), and fluconazole (C). Strains were grown in the absence (–) or presence (+) of inducers, 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 background fluorescence of otherwise identical strains without *GFP* is indicated by the black part of each column. Values of the uninduced cultures are from the same experiments in panels A to C and are included for comparison with each inducer. Fluorescence values that are at least 2-fold higher than those of the controls (combined data of the two independently generated strains) are marked by asterisks (\*\*,  $P < 0.01$ , two-tailed *t* test).

Mrr1 itself, the Mrr1DB-Upc2 hybrid transcription factor conferred a 32-fold increased fluconazole resistance in the *mrr1Δ* mutants, reflecting the strong upregulation of *MDR1* (Fig. 3C) and presumably other Mrr1 target genes that contribute to fluconazole resistance (27) by this protein. Similarly, the Tac1DB-Upc2 hybrid protein caused a 4-fold increase in fluconazole resistance in the *tac1Δ* mutants, as expected from its ability to upregulate *CDR2* (Fig. 4C) and possibly other Tac1 target genes in the presence of fluconazole. The Mrr1DB-Tac1 hybrid also caused a minor (2-fold) increase in fluconazole re-

sistance in the *mrr1Δ* mutants, in accordance with its ability to slightly activate the *MDR1* promoter in the presence of fluconazole (Fig. 3C). However, this hybrid transcription factor did not confer increased resistance in *tac1Δ* mutants, presumably because it had to compete with (inactive) wild-type Mrr1 for binding to the *MDR1* promoter in this strain background. Overexpression of *UPC2* from the *ADH1* promoter rescued the fluconazole hypersusceptibility of the *upc2Δ* mutants, and the strains exhibited slightly higher resistance (2-fold) than the wild type. The



**FIG 5** Inducibility of the *ERG11* promoter in *upc2Δ* mutants carrying a  $P_{ERG11}$ -GFP reporter fusion and the indicated hybrid transcription factors by fluconazole (A), benomyl (B), and fluphenazine (C). Strains were grown in the absence (–) or presence (+) of inducers, 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 background fluorescence of otherwise identical strains without GFP is indicated by the black part of each column. Values of the uninduced cultures are from the same experiments in panels A to C and are included for comparison with each inducer. Fluorescence values that are at least 2-fold higher than those of the controls (combined data of the two independently generated strains) are marked by asterisks (\*\*,  $P < 0.01$ , two-tailed  $t$  test).

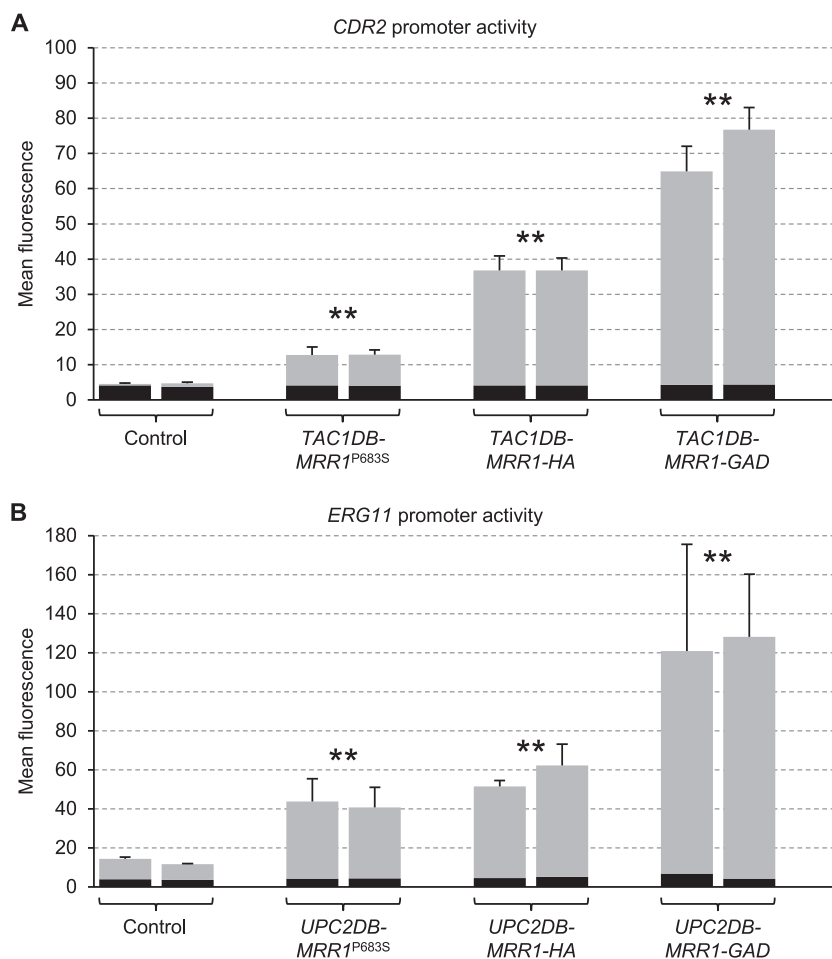
Mrr1DB-Upc2 and Tac1DB-Upc2 hybrid transcription factors also increased the resistance of the *upc2Δ* mutants, presumably because these strains now overexpressed Mrr1 and Tac1 target genes, respectively, in the presence of fluconazole.

## DISCUSSION

The zinc cluster proteins Mrr1, Tac1, and Upc2 mediate drug resistance in many azole-resistant, clinical *C. albicans* strains that have acquired gain-of-function mutations in these transcription

factors, which result in their constitutive activation. Mrr1, Tac1, and Upc2 are also required for the upregulation of their target genes in response to specific stimuli in drug-susceptible strains. However, it has not been established if the ability to respond to the presence of an inducer is intrinsic to these transcription factors or if the stimuli result in the activation of other transcription factors that bind to the same promoters and help Mrr1, Tac1, and Upc2 to promote gene expression. Here, we have addressed this question by exchanging the DNA-binding domains of Mrr1, Tac1, and





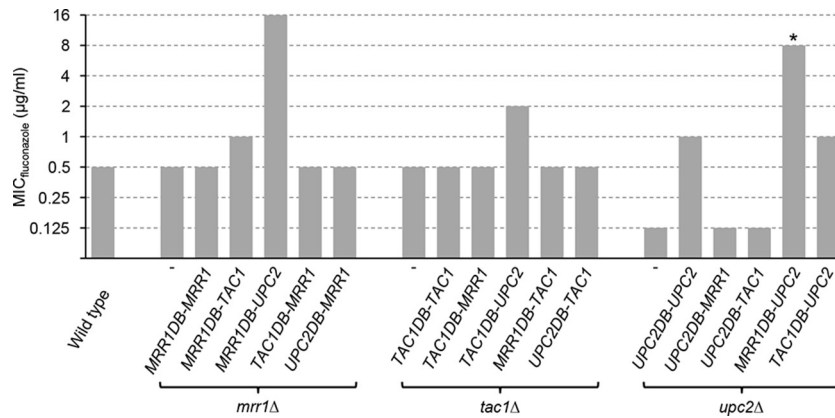
**FIG 6** Activation of the *CDR2* and *ERG11* promoters by hybrid transcription factors in which the Tac1 (A) or Upc2 (B) DNA-binding domains were fused to mutated forms of Mrr1. The Tac1DB-Mrr1 and Upc2DB-Mrr1 fusions correspond to those shown in Fig. 2 (amino acids 129 to 1108 of Mrr1) but contain the P683S gain-of-function mutation (*MRR1<sup>P683S</sup>*), a C-terminal 3×HA epitope tag (*MRR1-HA*), or a C-terminal Gal4 activation domain with a 3×HA tag (*MRR1-GAD*). The hybrid transcription factors were expressed in *tac1Δ* mutants carrying a *P<sub>CDR2</sub>-GFP* reporter fusion (A) or in *upc2Δ* mutants carrying a *P<sub>ERG11</sub>-GFP* reporter fusion (B). The mean fluorescence of cells grown in the absence of inducers 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 background fluorescence of otherwise identical strains without *GFP* is indicated by the black part of each column. Fluorescence values that are at least 2-fold higher than those of the controls (combined data of the two independently generated strains) are marked by asterisks (\*\*,  $P < 0.01$ , two-tailed *t* test).

Upc2 and assessing the inducibility of their target genes by specific stimuli.

Fluphenazine induces the expression of *CDR2* and other Tac1 target genes in a Tac1-dependent fashion. When Tac1 was targeted to the *MDR1* or *ERG11* promoters, which are not normally activated by this inducer, these promoters now became responsive to fluphenazine. Similarly, Upc2 could upregulate the expression of *MDR1* and *CDR2* in the presence of fluconazole when its DNA-binding domain was replaced by those of Mrr1 and Tac1, respectively. These results support the idea that Tac1 and Upc2 themselves are the targets for activation by the inducers fluphenazine and fluconazole. The drugs might directly bind to the transcription factors to promote a conformational transition to an activated state, but it is also possible that Tac1 and Upc2 are modified (e.g., by phosphorylation or some other protein modification) via a signaling pathway that is activated in the presence of the drugs.

In contrast, when the DNA-binding domain of Mrr1 was replaced by those of Tac1 and Upc2, the hybrid transcription factors

failed to induce the expression of the new target genes *CDR2* and *ERG11*, respectively, in response to benomyl. The fact that analogous fusion proteins with constitutively activated forms of Mrr1 mediated *CDR2* and *ERG11* overexpression argues that the Tac1DB-Mrr1 and Upc2DB-Mrr1 hybrid transcription factors were correctly targeted to the *CDR2* and *ERG11* promoters, respectively. It is therefore possible that another transcription factor that binds to the *MDR1* but not the *CDR2* and *ERG11* promoters is activated by benomyl and cooperates with Mrr1 to induce *MDR1* expression. Benomyl also causes oxidative stress (42), and benomyl-induced *MDR1* expression is strongly reduced in the absence of the oxidative stress response regulator Cap1 (27, 43). Cap1 does not bind to the *CDR2* and *ERG11* promoters (43) and can therefore facilitate *MDR1* but not *CDR2* and *ERG11* upregulation in the presence of benomyl. Nevertheless, a significant induction of *MDR1* expression by benomyl still occurs in the absence of Cap1 (26, 27, 43), demonstrating that benomyl or benomyl-induced effects on the cells are also sensed in a different



**FIG 7** Fluconazole susceptibilities of the wild-type strain SC5314 and of *mrr1Δ*, *tac1Δ*, and *upc2Δ* mutants expressing the indicated hybrid transcription factors. \*The MIC for one of the two independently constructed *upc2Δ* mutants expressing the *Mrr1DB-Upc2* fusion was slightly lower (4 µg/ml). In all other cases, the two independently generated strains behaved identically.

way. Like Cap1, the MADS box transcription factor Mcm1 binds to the *MDR1* but not to the *CDR2* and *ERG11* promoters and contributes to benomyl-induced *MDR1* expression (37, 44–46). Therefore, if Mrr1 is not itself activated by benomyl, the absence of its coregulators may prevent it from upregulating *CDR2* and *ERG11* expression when it is targeted to these promoters by fusion with the Tac1 and Upc2 DNA-binding domains. It should be noted that a weak but significant induction by benomyl was observed when Mrr1 was targeted to an artificial promoter by fusion with the Tet repressor TetR (30). Therefore, the possibility that some coactivator-independent activation of Mrr1 itself may occur in the presence of benomyl cannot be excluded. It is also worth mentioning that the hyperactive Mrr1 containing the P683S mutation can activate *MDR1* expression in the absence of Cap1 but requires Mcm1 to do so (27, 37). It is possible that this hyperactive form of Mrr1 may cooperate with other transcriptional regulators to promote the (comparatively weak) constitutive *CDR2* and *ERG11* expression that was observed when it was targeted to these promoters via the Tac1 and Upc2 DNA-binding domains (Fig. 6).

Interestingly, the Mrr1DB-Tac1 hybrid transcription factor induced *MDR1* expression not only in response to fluphenazine but also, albeit very weakly, in the presence of benomyl and fluconazole (Fig. 3). The induction by benomyl can be explained if activated Cap1 also interacts with Tac1 that is present instead of Mrr1 at the *MDR1* promoter, resulting in a slight *MDR1* upregulation. The weak but significant induction of the *MDR1* promoter by fluconazole in strains containing the Mrr1DB-Tac1 hybrid transcription factor instead of Mrr1 is somewhat surprising, as neither Mrr1 nor Tac1 is activated by fluconazole. It must be mentioned that the very low basal *MDR1* expression levels in drug-susceptible strains like SC5314 are indeed elevated in the presence of fluconazole (47). The increase in basal *MDR1* transcription can be detected by reverse transcription (RT)-PCR (47) but did not significantly enhance the fluorescence of cells containing a  $P_{MDR1}$ -GFP reporter fusion over the background fluorescence (Fig. 1A). Importantly, the level of *MDR1* upregulation in the presence of fluconazole is insufficient to confer increased drug resistance, because deletion of *MDR1* in fluconazole-susceptible *C. albicans* strains does not result in hypersusceptibility to the drug (27, 48, 49). The induction of *MDR1* expression by fluconazole in wild-type strains is most likely also mediated by Upc2 binding to the

*MDR1* promoter (40). Upc2 may upregulate *MDR1* expression in the presence of fluconazole more efficiently when Tac1 is present instead of Mrr1 at the *MDR1* promoter. This is also reflected by the slightly increased (2-fold) fluconazole resistance of the strains with the Mrr1DB-Tac1 hybrid protein compared with that of the wild-type strain (Fig. 7).

As Upc2 does bind to the *MDR1* promoter, replacement of the Mrr1 DNA-binding domain by the Upc2 DNA-binding domain would still allow the hybrid transcription factor to bind to the *MDR1* promoter. Nevertheless, *MDR1* expression was only slightly induced by benomyl in strains containing the Upc2DB-Mrr1 hybrid protein (Fig. 3A). Apparently, shifting the binding site of Mrr1 in the *MDR1* promoter to the Upc2 binding site prevented the hybrid transcription factor from productively interacting with coregulatory transcription factors, like Cap1 and Mcm1, resulting in a failure to efficiently induce *MDR1* expression in the presence of benomyl.

Remarkably, when fused to the Mrr1 DNA-binding domain, Upc2 caused a constitutive activation of the *MDR1* promoter (Fig. 3). This observation is compatible with a model in which Mrr1 is constitutively localized to the *MDR1* promoter but needs to be activated in order to induce gene expression. Upc2, in contrast, might bind to its target promoters only under inducing conditions. In that case, constitutive binding of the Mrr1DB-Upc2 hybrid protein to the *MDR1* promoter could result in *MDR1* expression even in the absence of an inducer and further upregulation in the presence of fluconazole. However, Tac1 also binds to its target genes in the absence of inducers (41), but *CDR2* expression was induced by the Tac1DB-Upc2 fusion protein only in the presence of fluconazole (Fig. 4C). Upc2 may therefore be in a partially activated state when fused to the Mrr1 DNA-binding domain but not when fused to the Tac1 DNA-binding domain. Yet, both hybrid transcription factors could be stimulated in the presence of fluconazole.

Mrr1 and Tac1 do not induce the expression of efflux pumps in the presence of fluconazole, which may explain the high susceptibility of the majority of *C. albicans* strains to this drug. If Mrr1 and Tac1 acquired the ability to be activated by azole drugs, like the artificially created Mrr1DB-Upc2 and Tac1DB-Upc2 hybrid proteins, this would result in efflux pump overexpression in the presence of azoles and, consequently, increased drug resistance

(Fig. 7). In *C. glabrata*, the zinc cluster transcription factor Pdr1, which regulates the expression of several drug efflux pumps, has this ability and is activated by azoles and other toxic compounds, resulting in the high intrinsic azole resistance of this pathogenic yeast (21).

## ACKNOWLEDGMENTS

This study was supported by the Deutsche Forschungsgemeinschaft (DFG grant MO 846/7 and SFB 630).

Nico Dunkel, Dagmar Sedding, and Anna-Lena Geiselhöringer are acknowledged for their help in the construction of some plasmids and strains.

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