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TAC1, Transcriptional Activator of *CDR* Genes, Is a New Transcription Factor Involved in the Regulation of *Candida albicans* ABC Transporters *CDR1* and *CDR2*†

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The ABC transporter genes *CDR1* and *CDR2* can be upregulated in *Candida albicans* developing resistance to azoles or can be upregulated by exposing cells transiently to drugs such as fluphenazine. The *cis*-acting drug-responsive element (DRE) present in the promoters of both genes and necessary for their upregulation contains 5′-CGG-3′ triplets that are often recognized by transcriptional activators with Zn(2)-Cys(6) fingers. In order to isolate regulators of *CDR1* and *CDR2*, the *C. albicans* genome was searched for genes encoding proteins with Zn(2)-Cys(6) fingers. Interestingly, three of these genes were tandemly arranged near the mating locus. Their involvement in *CDR1* and *CDR2* upregulation was addressed because a previous study demonstrated a link between mating locus homozygosity and azole resistance. The deletion of only one of these genes (orf19.3188) was sufficient to result in a loss of transient *CDR1* and *CDR2* upregulation by fluphenazine and was therefore named *TAC1* (transcriptional activator of *CDR* genes). Tac1p has a nuclear localization, and a fusion of Tac1p with glutathione *S*-transferase could bind the *cis*-acting regulatory DRE in both the *CDR1* and the *CDR2* promoters. *TAC1* is also relevant for azole resistance, since a *TAC1* allele (*TAC1-2*) recovered from an azole-resistant strain could trigger constitutive upregulation of *CDR1* and *CDR2* in an azole-susceptible laboratory strain. Transcript profiling experiments performed with a *TAC1* mutant and a revertant containing *TAC1-2* revealed not only *CDR1* and *CDR2* as targets of *TAC1* regulation but also other genes (*RTA3*, *IFU5*, and *HSP12*) that interestingly contained a DRE-like element in their promoters. In conclusion, *TAC1* appears to be the first *C. albicans* transcription factor involved in the control of genes mediating antifungal resistance.

Several antifungal agents are available to treat infections due to *Candida albicans*. The success of antifungal treatments depends on the patient's condition, the type of antifungal agent given, and the biological response of the fungal pathogen. It has been demonstrated on many occasions that *C. albicans* can respond by developing specific resistance mechanisms upon exposure to antifungal drugs. Resistance to the class of azole antifungal agents used for the treatment of oropharyngeal candidiasis in human immunodeficiency virus-positive patients is a classical example.

In *C. albicans*, one of the well-documented mechanisms of resistance to azole antifungal agents is the upregulation of multidrug transporter genes (29, 33, 35, 39). The upregulation of multidrug transporter genes leads to the enhanced efflux of azoles and therefore results in decreased drug accumulation and reduced inhibition of their target encoded by the *ERG11* gene. At least two families of multidrug transporters, the ABC (ATP-binding cassette) transporter family and the major facilitator superfamily (MFS), have been shown to be involved in resistance to azole antifungal agents. Cdr1p and Cdr2p (*Candida* drug resistance) from the family of ABC transporters and CaMdr1p (*C. albicans* multidrug resistance 1) from the family of MFS transporters are the principal mediators of resistance to azoles due to transport phenomena (33, 35). Each of the genes encoding these proteins can be upregulated in distinct clinical azole-resistant strains. The transcription of *CaMDR1*, the gene encoding CaMdr1p, is almost not detectable in azole-susceptible isolates but is measurable in some azole-resistant isolates. In contrast, the transcription of *CDR1*, the gene encoding Cdr1p, is detectable in azole-susceptible isolates but is increased to higher levels in some azole-resistant isolates. *CDR1* is usually upregulated in these isolates together with *CDR2*, a gene with no detectable transcriptional activity in azole-susceptible isolates. Interestingly, *CDR1* and *CDR2* can also be induced transiently by treating cells with various drugs, such as estradiol and fluphenazine (8, 15), thus mimicking their expression in azole-resistant cells.

The upregulation of multidrug transporters in the development of antifungal resistance is well described in the baker's yeast *Saccharomyces cerevisiae*. Like *C. albicans*, *S. cerevisiae* possesses ABC transporters (for example, *PDR5* [3, 4]) or MFS transporters (for example, *FLR1* [1]) that are able to confer resistance to antifungal drugs. The regulation of ABC transporters and, more specifically, of *PDR5*, has been extensively documented. Two transcription factors, *PDR1* and *PDR3*, encoding proteins with Zn(2)-Cys(6) finger motifs, have been described to regulate the transcription of *PDR5* (7, 20). *cis*-Acting elements, so-called PDRE (pleiotropic drug response

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

TABLE 1. Strains used in this study

Strain	Genotype	Parent strain	Reference or source
C. albicans			
CAF2-1	*ura3*Δ::*imm434*/*URA3*	SC5314	11
CAF4-2	*ura3*Δ::*imm434*/*ura3*Δ::*imm434*	CAF2-1	11
CHY439	*ura3*/*ura3 MTL***a**/*mtlα1*::*hisG mtlα2*::*hisG*	CAI4	26
CHY477	*ura3*/*ura3 mtla1*::*hisG*/*MTLα ade2*::*hisG-URA3-hisG*/*ade2*::*hisG*	CAI4	26
DSY294	Azole-susceptible clinical strain (*MTL***a**/*MTLα*)		35
DSY296	Azole-resistant clinical strain (*MTLα*/*MTLα*)	DSY294	35
DSY296-3	*gal1*	DSY296	This study
DSY2781	*GAL1*/*gal1 ura3*/*URA3 MTL***a**/*mtlα1*::*hisG mtlα2*::*hisG*/*MTLα*	DSY296-3, CHY439	This study
DSY2875	*TAC1*/*tac1*Δ::*hisG-URA3-hisG*	CAF4-2	This study
DSY2883	*TAC1*/*tac1*Δ::*hisG*	DSY2875	This study
DSY2903	*tac1*Δ::*hisG*/*tac1*Δ::*hisG-URA3-hisG*	DSY2883	This study
DSY2906	*tac1*Δ::*hisG*/*tac1*Δ::*hisG*	DSY2903	This study
DSY2937	*tac1*Δ::*hisG*/*tac1*Δ::*hisG LEU2*::*TAC1*/*URA3*	DSY2906	This study
DSY2925	*tac1*Δ::*hisG*/*tac1*Δ::*hisG LEU2*::*TAC1-1*/*URA3*	DSY2096	This study
DSY2926	*tac1*Δ::*hisG*/*tac1*Δ::*hisG LEU2*::*TAC1-2*/*URA3*	DSY2096	This study
DSY2884	*ZNC3*/*znc3*Δ::*hisG-URA3-hisG*	CAF4-2	This study
DSY2869	*ZNC1*/*znc1*Δ::*hisG-URA3-hisG*	CAF4-2	This study
DSY654	*cdr1*Δ::*hisG*/*cdr1*Δ::*hisG cdr2*Δ::*hisG*/*cdr2*Δ::*hisG-URA3-hisG*	CAF4-2	33
S. cerevisiae DSY669	*MAT***a** *pdr1*::*TRP1 pdr3*::*HIS3 PDR5 ura3-52 leu2-*Δ*1 GAL2*	FY1679-28C	7

element), have been localized in the promoter of *PDR5* and are the targets of *PDR1* and *PDR3* (21). PDRE have also been found in the promoters of several other genes in the *S. cerevisiae* genome and to be coregulated with *PDR5* (9). The regulation of *FLR1* is mediated by another transcriptional circuit that involves the leucine zipper transcription factor Yap1 (1).

In *C. albicans*, the molecular mechanisms governing the regulation of multidrug transporter genes have not been examined in great detail. No *cis*-acting element capable of regulating the expression of *CaMDR1* has yet been determined. Up to now, elements permitting the basal activity of *CDR1* and the upregulation of *CDR1* and *CDR2* have been determined. A 22-bp drug-responsive element (DRE) containing two 6-bp repetitive elements with 5′-CGG-3′ triplets is known to be required for the induction of both *CDR1* and *CDR2*. Band shift assays revealed the presence of protein complexes binding to the DRE; however, their identity remains unknown (8). In the present study, we addressed the identification of *trans*-acting elements involved in the regulation of *CDR1* and *CDR2*. We explored the *C. albicans* genome for genes encoding putative regulators with Zn(2)-Cys(6) finger motifs, since these factors can potentially bind 5′-CGG-3′ triplets that are present in the DRE of both the *CDR1* and the *CDR2* promoters (24). Among three genes tandemly arranged near the mating type locus, the deletion of one of them, now named *TAC1*, resulted in a loss of transient *CDR1* and *CDR2* upregulation after drug exposure.

MATERIALS AND METHODS

Strains, plasmids, and media. The *C. albicans* and *S. cerevisiae* strains used in this study are listed in Table 1. The plasmids constructed in this study (see below for further details) are listed in Table 2. Yeast strains were grown either in complete medium (1% Bacto Peptone [Difco Laboratories, Basel, Switzerland], 0.5% yeast extract [Difco], 2% glucose [Fluka, Buchs, Switzerland]; YEPD) or in minimal medium (yeast nitrogen base [Difco], 2% glucose [Fluka]; YNB). For growth on solid media, 2% agar (Difco) was added to either of the media. *C. albicans* Gal$^-$ mutants were generated by the method described by Gorman et al. (14). *Escherichia coli* DH5α (16) was used as a host for plasmid constructions and propagation. DH5α was grown in Luria-Bertani broth or on Luria-Bertani agar plates supplemented with ampicillin (0.1 mg/ml) when required.

Yeast tranformation. *S. cerevisiae* was transformed by a standard lithium acetate protocol as reported previously (13). For *C. albicans* transformation, cells from 0.2 ml of a stationary-phase culture were resuspended in 0.1 ml of a solution containing 200 mM lithium acetate (pH 7.5), 40% (wt/vol) polyethylene glycol 8000, 15 mg of dithiothreitol (DTT)/ml, and 250 μg of denatured salmon sperm DNA/ml. Transforming DNA (1 to 5 μg) was added to the yeast suspension, which was then incubated for 60 min at 43.5°C. Transformation mixtures were plated directly on selective plates.

Construction of promoter-*lacZ* fusions and measurement of β-galactosidase activities. *CDR1*- and *CDR2-lacZ* fusions made for *C. albicans* were constructed by amplification of the *CDR1* and *CDR2* promoters with primers CDR1-KPN and CDR1-PST or primers CDR2-KPN and CDR2-PST (Table 3) and cloning into pAU36 (38) that had been digested with KpnI and PstI. The resulting plasmids, pDS958 and pDS959, were digested with SacI and SnaBI prior to transformation into *C. albicans*, thus resulting in their integration at the *CDR1* and *CDR2* loci, respectively. β-Galactosidase activities were measured as described by Uhl and Johnson (38).

The *CDR2-lacZ* fusion made for the expression of *CDR2* promoter activity in *S. cerevisiae* was prepared by inserting the product of PCR amplification of the *CDR2* promoter with primers CDR2-PST-900 and CDR2-HIND (Table 3) into the PstI and HindIII sites of YIp353, resulting in pDS1139. Deletion of the DRE (−221 to −220) within this promoter was carried out as described by De Micheli et al. (8) by sewing of PCR fragments obtained with primers CDR2-PST-900 and CDR2-DEL3, with primers CDR2-HIND and CDR2-DEL5 (Table 3), and with pDS246 (33) as a template. The final amplification product obtained with external primers CDR2-PST-900 and CDR2-HIND was inserted into YIp353, resulting in pDS1161. *TAC1* alleles were obtained from pDS1097 (see below) and cloned into the BamHI and XhoI sites of pDS1139 and pDS1161, resulting in pDS1157 and pDS1180. These plasmids, together with pDS1139, were linearized with StuI to facilitate integration at the *S. cerevisiae URA3* locus.

The DRE-containing *CDR2-lacZ* derivatives used for expression in *C. albicans* were prepared by sewing of PCR fragments amplified from the first 300 bp of the *CDR2* promoter with primers CDR2-SACI and CDR2-anti, with primers CDR2-LACZ and MAL2-BAM, and with pDS246 (33) as a template. The final amplification product obtained with external primers CDR2-SACI and MAL2-BAM was subcloned into the SacI and BamHI sites of pDS1097, which contains *TAC1*, to yield pDS1187. The DRE-negative construct was prepared by PCR amplification with the same primers as those used to generate pDS1187 but with DRE-negative plasmid pDS1180 as a template. The final DRE-negative *CDR2-lacZ* fusion was created by PCR with primers CDR2-SACI and MAL2-BAM and by subcloning into pDS1097 to yield pDS1190. pDS1187 and pDS1190 were

TABLE 2. Plasmids used in this study

Plasmid	Parent plasmid	Description[a]	Reference or source
pAU36	See reference 38	*lacZ* reporter system for *C. albicans*	38
pDS958	pAU36	*CDR1-lacZ* fusion cloned into pAU36	This study
pDS959	pAU36	*CDR2-lacZ* fusion cloned into pAU36	This study
pDS1093	pGEX4-2T	Insertion of the N-ternimal end of *TAC1* containing the DNA-binding domain into pGEX4-2T	This study
pDS178	See reference 8	pRC2312 with a pBluescript multiple-cloning site	8
pDS1097	pDS178	Insertion of *TAC1* from SC5314 into pDS178	This study
pDS1098	pDS178	Insertion of *TAC1-1* from strain DSY294 into pDS178	This study
pDS1099	pDS178	Insertion of *TAC1-2* from strain DSY296 into pDS178	This study
YIp353	See reference 27	*lacZ* reporter system in integrative *S. cerevisiae* plasmid	27
pDS1139	YIp353	*CDR2-lacZ* fusion cloned into YIp353	This study
pDS1157	pDS1139	*TAC1* cloned into pDS1139	This study
pDS1161	pDS1139	Deletion of the DRE within the *CDR2* promoter in pDS1139	This study
pDS1180	pDS1161	*TAC1* cloned into pDS1161	This study
pDS1187	pDS1097	*CDR2-lacZ* fusion cloned into pDS1097	This study
pDS1190	pDS1097	Deletion of the DRE within the *CDR2* promoter in pDS1097	This study
pDS1160	CIpACT-C-ZZ	*TAC1* fused to protein A in expression plasmid CIpACT-C-ZZ (5)	This study
pDS1202	pDS1160	*TAC1* fused to GFP in pDS1160	This study

[a] Detailed descriptions and constructions are given in Material and Methods.

linearized with SalI before transformation into *TAC1* mutant DSY2906, facilitating integration at the genomic *LEU2* locus.

Construction of gene disruption cassettes. For the disruption of *ZNC1*, *ZNC2* (*TAC1*), and *ZNC3*, a region containing the entire open reading frame (ORF) or a portion of the ORF was amplified from genomic DNA with the cloning primers shown in Table 4. PCR fragments were cloned into pBluescript KS(+) to yield the cloning constructs shown in Table 4. Deletions within cloned regions were created by PCR with the deletion primers shown in Table 5 and with cloning constructs as templates. The 3.7-kb PstI-BglII fragment comprising the *URA3*-blaster cassette from pMB7 (11) was cloned into PCR fragments that had been digested with PstI and BglII to obtain deletion constructs (Table 5). For transformation in *C. albicans*, linear fragments were obtained by digestion of deletion constructs with ApaI and SacI.

For deletion of a region of *TAC1*, the 3.7-kb PstI-BglII fragment comprising the *URA3*-blaster cassette from pMB7 was ligated to pDS1048 that had been digested with BglII and PstI, resulting in an internal deletion of 265 bp within the *TAC1* ORF and creating pDS1052. The linear fragment liberated after digestion of pDS1052 with ApaI and SacI was used to transform *C. albicans*.

Construction of revertant strains. Revertant strains of each homozygous mutant generated in this study were obtained by transformation of a *C. albicans ura3* derivative with pRC2312-derived plasmid pDS178, containing the *URA3* and *LEU2* markers described previously (8). To generate revertants of *TAC1* mutant

TABLE 3. Primers used in this study

Primer	Sequence[a]
Znc2-5-BamB	GCAAGGATCCAAGAAGAAGTGGATAATTTTGATTAAC
Znc2-3-Xho	GCAACTCGAGAGTATATTCTGTTGGGAAAGGGGTGAG
Znc2-GST1	AGCAAGGATCCATGGACACTTCACTGTCACTGGGA
Znc2-GST2	GCTTTCTCGAGACTTGATTTGTTGTCATTTATGCCG
CDR1-F	CATGGTCAAGCCATTTTGTG
CDR1-R	ATCCATTCTGCTGGATTTGC
CDR2-F	CATGGTCAAGCCATTTTGTG
CDR2-R	ATCCATTCTGCTGGATTTGC
IFU5-F	AAACCCACCACAAGTTCCTG
IFU5-R	CTTGGGGCATTAGACCTTGA
RTA3-F	TACAGAATGGACTCCTACCT
RTA3-R	GCCGTACGATTTAATCGA
CDR1-PST	GCGCAAACTGCAGAATTTTTTTCTTTTTGACCTTTTAAAGAAA
CDR1-KPN	GCGCAAAGGTACCGGATCCTCGTTACTCAATAAGTATTAATTG
CDR2-PST	GCGCAAACTGCAGATGTTTTTATTGTATGTGTTAATTAGTGAA
CDR2-KPN	GCGCAAAGGTACCGGTTCCTCTAAATAAAAACTAGAAGGTTAT
CDR2-HIND	GCGCAAAGCTTTGTTGTGACTTGCAGTAGCAT
CDR2-PST-900	GCAAACTGCAGGTTCCTCTAAATAAAAACTAGAAGG
CDR2-DEL5	GTATTAATTTTTACGTATTTTCTTTGTGTTATTCAATTCTTGTTTTCAAAGCCT
CDR2-DEL3	AGTATTCATAATAGAGGCTTTGAAAACAAA
CDR2-XHO	GCGCAAACTCGAGACATGAAAAATGAAATCAATTCAAACACAA
CDR2-ANTI	AATGTTTTTATTGTATGTGTTAATTAGTGAA
CDR2-LACZ	AACACATACAATAAAAACATATGAACATGACTGAAAAAATTCAAACTT
CDR2-SAC	GCGCAAAGAGCTCACATGAAAAATGAAATCAATTCAAACACAA
MAL2-BAM	GCGCAAAGGATCCCATACGCTTTGCAGGTGGTGTTGATCC
SALI-ZNC2C	GCGCAAAGTCGACATAATGGACACTTCACTGTCACTGGGA
SPHI-ZNC2C	GCGCAAAGCATGCAAATCCCCAAATTATTGTCAAAGAAAAA
GFP-5-SPHI	CGCGAAAGCATGCATTCTAAAGGTGAAGA
GFP-3-NHE	CGCGAAAGCTAGCTTATTTGTACAATTCATCCATACCATGGG

[a] Restriction sites are underlined.

TABLE 4. Cloning constructs from plasmid Bluescript KS(+)

Gene	Region amplified with respect to ATG	Cloning primer	Sequence[a]	Cloning construct (insert size, kb)
TAC1	+1 to +2947	CaZNC2-BamHI	GCGCAAAGGATCCTTAAATCCCCAAATTATTGTCAAAGAAAAA	pDS1048 (2.9)
		CaZNC2-Xho	GCGCAAACTCGAGATGGACACTTCACTGTCACTGGGAACTCAC	
ZNC1	+1 to +2770	CaZNC1-Xba2	GCGCAAATCTAGATGCTGGATGTTAATGATACACTTAATCCT	pDS1047 (2.7)
		CaZNC1-Xho	GCGCAAACTCGAGTTATATATTTTGCTCATTAAATCCTAAAGA	
ZNC3	+1 to +3034	CaZNC3-Xho2	GCGCAAACTCGAGTTAGTTATAAAATATATCAGGAAAGTTCAA	pDS1043 (3)
		CaZNC3-Xba	GCGCAAATCTAGATGGATCCTGCTTATGATATACAATCGCA	

[a] Restriction sites are underlined.

DSY2906, ORFs flanked by 500 bp were amplified from genomic DNAs of strains SC5314, DSY294, and DSY296 with primers Znc2-5-BamB and Znc2-3-Xho (Table 3) and inserted into pDS178 that had been digested with BamHI and XhoI to yield pDS1097, pDS1098, and pDS1099, respectively. These plasmids were linearized with SalI and transformed into *C. albicans*, allowing integration at the genomic *LEU2* locus.

Construction and visualization of a Tac1p-GFP fusion. Green fluorescent protein (GFP) was fused to Tac1p at the C-terminal end. The *TAC1* ORF was amplified with primers SALI-ZNC2C and SPHI-ZNC2C and with pDS1097 as a template and was cloned into compatible SalI-SphI sites of CIpACT-C-ZZ (5) to yield pDS1160. GFP was amplified from yEGFP3 (6) with primers GFP-5-SPH and GFP-3-NHE and was cloned into compatible NheI-SphI sites of pDS1160, replacing protein A and yielding pDS1202. The final Tac1p-GFP fusion was under the control of the *ACT1* promoter contained in CIpACT-C-ZZ. Plasmid pDS1202 was digested with StuI and transformed into DSY2906. Transformants were grown on selective medium to logarithmic phase, and GFP fluorescence was revealed by microscopy. Nuclear DNA was stained by the addition of Hoechst 33258 at a concentration of 2 μg/ml for 30 min at room temperature prior to microscopy. Fluorescence microscopy and phase-contrast microscopy were performed with a Zeiss Axioplan microscope equipped for epifluorescence microscopy with a 100-W mercury high-pressure bulb and Zeiss filter sets 9 (for GFP) and 15 (for Hoechst 33258). A DX30 digital camera with high resolution (Kappa Messtechnik GmbH, Gleichen, Germany) was used to record images.

Purification of Tac1p fused to GST. A Tac1p–glutathione *S*-transferase (GST) fusion plasmid was constructed by introducing the first 129 amino acids of Tac1p in frame with GST into pGEX4-2T (Amersham Biosciences, Otelfingen, Switzerland). The fragment encoding the first 129 amino acids was first amplified with primers Znc2-GST1 and Znc2-GST2 (Table 3) and with genomic DNA as a template and then introduced into the BamHI-XhoI sites of pGEX4-2T to obtain pDS1093. Purification of the Tac1p-GST fusion from *E. coli* BL21 (*E. coli* B F^- *ompT hsdS* [r_B^- m_B^-] *gal dcm*) transformed with pDS1093 was carried out by standard protocols provided by Amersham Biosciences.

Northern and Southern blotting. Northern blotting was carried out as described previously (35). DNA probes used in this study were generated by PCR with the pairs of primers shown in Table 3. ^{32}P-labeled DNA probes were generated by random priming (10). The *TEF3* probe, used as an internal standard, originated from a 0.7-kb EcoRI-PstI fragment from pDC1 (18). Southern blotting was performed as described previously (35). Radioactive signals were revealed by exposure to Kodak BioMax MR film (Amersham Biosciences).

Band shift assays. Total protein extraction from *C. albicans* was performed as described previously (8). The DNA-binding probe was obtained by annealing complementary oligonucleotides (7.5 μg) corresponding to probes 1 to 5 (see Fig. 4) in 10 mM Tris-HCl (pH 8.0)–100 mM NaCl–1 mM EDTA (100 μl) for 2 min at 85°C followed by stepwise 1-min incubations at 75, 65, 50, and 25°C. A 2-μl aliquot of the probe was labeled with 1.8 MBq of [γ-^{32}P]dATP by using polynucleotide kinase (Roche, Basel, Switzerland) in a 10-μl reaction for 1 h at 37°C. The probe was purified by using a QiaQuick nucleotide removal kit (Qiagen Inc., Chatsworth, Calif.). The binding solution was obtained by adding sequentially 7 μl of binding buffer (20 mM HEPES [pH 7.9], 50 mM KCl, 0.5 mM EDTA, 0.1% NP-40, 1 mg of bovine serum albumin/ml, 5% glycerol), 1 μl of 0.2 M phenylmethylsulfonyl fluoride, 1 μl of 0.1 M DTT, 1 μl of poly(dI-dC) at 1 mg/ml, proteins, competitor if needed, and 2 to 3 μl of probe (at least 10^6 cpm). The total volume was adjusted to 20 μl with H_2O. Binding was performed for 20 min at room temperature. Before samples were loaded onto the polyacrylamide gel, 3 μl of 40% glycerol was added to the samples and the gel was prerun for 30 min at 100 V. Samples were electrophoresed for 150 min at 200 V onto a 5% polyacrylamide gel (Protogel 37.5:1) in Tris-borate-EDTA buffer. The gel was dried on Whatman 3MM paper and exposed to Kodak BioMax MR film.

Immunoblotting. *C. albicans* cell extracts for immunoblotting were prepared from cells grown to mid-log phase by an alkaline extraction procedure. Briefly, cells (optical density at 540 nm, 5) were resuspended in an Eppendorf tube with 500 μl of water and 150 μl of a solution containing 1.85 M NaOH and 7.5% β-mercaptoethanol. This mixture was incubated on ice for 10 min. Proteins were precipitated with 150 μl of a 50% trichloroacidic acid solution, and the suspension was left on ice for 10 min. Precipitated proteins were sedimented by centrifugation at 13,000 × *g* in a microcentrifuge for 15 min. The sediment was resuspended in 50 μl of loading buffer (40 mM Tris-HCl [pH 6.8], 8 M urea, 5% sodium dodecyl sulfate [SDS], 0.1 M EDTA, 1% β-mercaptoethanol, 0.1 mg of bromphenol blue/ml) and incubated at 37°C for 10 min. Nonsolubilized material was cleared by centrifugation for 10 min. Ten-microliter samples of solubilized yeast proteins were separated by SDS–10% polyacrylamide gel electrophoresis (PAGE) and transferred by Western blotting to a nitrocellulose membrane. Immunodetection of Cdr1p and Cdr2p was performed as described previously (8) with rabbit polyclonal anti-Cdr1p and anti-Cdr2p antibodies by chemilumi-

TABLE 5. Deletion constructs from cloning vectors containing *URA3*-blaster from pMB7 (11)

Gene	Region deleted with respect to ATG	Deletion primer	Sequence[a]	Disruption construct (deletion size)
TAC1	+1154 to +1419	Deletion performed by removal of an internal PstI-BglII fragment		pDS1052 (265 bp)
ZNC1	+550 to +1993	CaZNC1-Pst	GCGCAAACTGCAGATCGTTATGGTTATATTCTATACTGCCATC	pDS1049 (1.4 kb)
		CaZNC1-Bgl	CAATCAATCATTAGCTGGAGCTAGATCTATGTTG	
ZNC3	+531 to +2543	CaZNC3-Bgl	GCGCAAAGATCTTGGAGAGGGCTAGATTTATCTGTCTTTGCT	pDS1065 (2 kb)
		CaZNC3-Pst2	GCGCAAACTGCAGCATCATCATCGACTTCTTTCCAACCACCTA	

[a] Restriction sites are underlined.

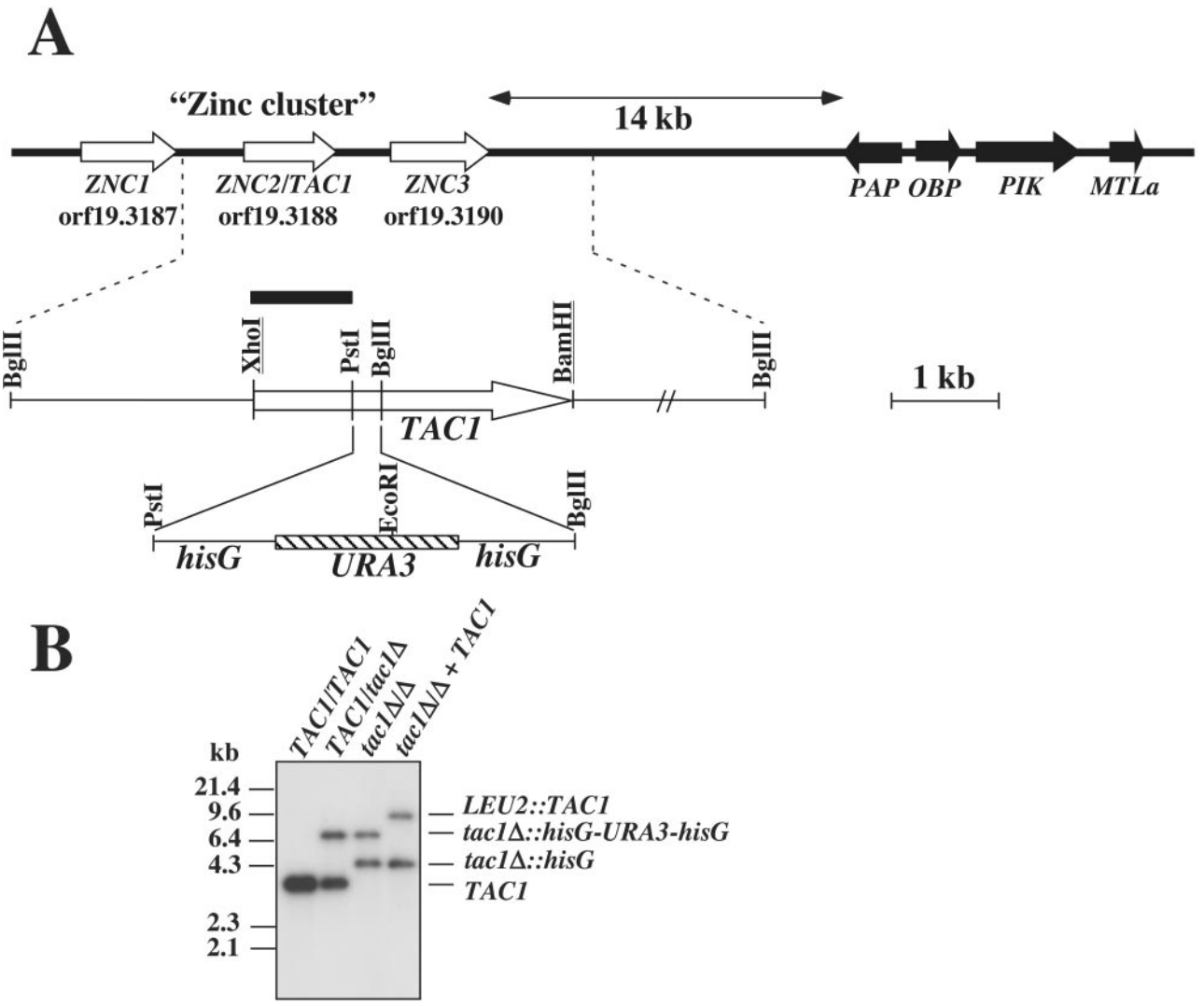

FIG. 1. (A) Restriction map of zinc cluster genes. The physical map shows the zinc cluster and the neighboring *MTL* locus (*MTL***a**). White arrows show the positions of *ZNC* ORFs, and black arrows show the positions of *MTL***a** locus genes. A second map with a higher scale for *ZNC2* (*TAC1*) focuses on the location of the *TAC1* deletion constructed with the *URA3*-blaster cassette. The entire *TAC1* ORF was used for cloning and construction of disruption cassettes (see Material and Methods for details). Underlined restriction sites were created by PCR cloning. (B) Southern analysis of the *TAC1* disruption. Genomic DNA was digested with BglII. The identity of each band of the expected size is shown at the right side of the Southern blot. The positions of molecular size standards are shown at the left side. The following strains correspond to the indicated genotypes: *TAC1*/*TAC1*, CAF2-1; *TAC1*/*tac1*Δ, DSY2875; *tac1*Δ/Δ, DSY2903; and *tac1*Δ/Δ + *TAC1*, DSY2937.

nescence with an ECL kit according to the recommendations of the manufacturer (Amersham Biosciences).

Drug susceptibility testing. Drug susceptibility testing was performed with microtiter plates and twofold serial dilutions of fluconazole (range, 128 to 0.06 μg/ml). Yeast cultures were grown overnight in YEPD and inoculated at a density of 10^4 cells/ml in a total volume of 200 μl containing a serial fluconazole dilution. Microtiter plates were incubated at 35°C for 24 h, and optical densities were read with a microtiter plate reader at a wavelength of 540 nm. The MIC was determined as the drug concentration required to decrease the optical density of the drug-free culture by at least 50%. Drug susceptibility testing was also performed by disk diffusion on YEPD agar plates onto which 10^5 cells had been dispersed by application of a filter disk containing 50 μg of fluconazole; the plates were incubated for 48 h at 30°C.

Microarray experiments. *C. albicans* cDNA microarray slides from Eurogentec, Seraing, Belgium, were used in this study. Briefly, 6,039 ORFs from PCR amplicons of 300 bp were spotted in duplicate on each aldehyde-coated glass slide in 32 blocks. The microarray covers nearly 98% of the total number of *C. albicans* genes and includes 27 control genes in each block, including, for example, negative controls (intergenic regions), cross-hybridization controls (*S. cerevisiae* genes), and dynamic-range controls (serial dilution of the *TEF3* gene from 1 to 1/32).

(i) RNA isolation and probe labeling. Each *C. albicans* strain was pregrown overnight in 5 ml of YEPD with constant agitation at 30°C. For RNA extraction, cultures were diluted to a density of 0.75×10^7 cells/ml in 15 ml of fresh YEPD and were grown at 30°C with agitation for 2 h to reach a density of 1.5×10^7 cells/ml. At this point, cultures of DSY294, DSY296, DSY2925, and DSY2926 were centrifuged for 5 min at 5,500 × *g* and 4°C for subsequent RNA isolation. Strains CAF2-1 and DSY2906 were exposed for 20 min to 10 μg of fluphenazine (Sigma-Aldrich, Buchs, Switzerland)/ml at 30°C with agitation. CAF2-1 also was cultivated in parallel under the same conditions but without drugs. After drug exposure, cultures were centrifuged at 4°C and 5,500 × *g* for RNA isolation. Total RNA was extracted by using glass beads as described previously (32). A least 200 μg of each RNA was purified as described by the RNeasy kit supplier (Qiagen). The concentration of purified RNA was measured spectrophotometrically at A_{260} and A_{280} and adjusted to 2.5 mg/ml. RNA was stored at −80°C until use.

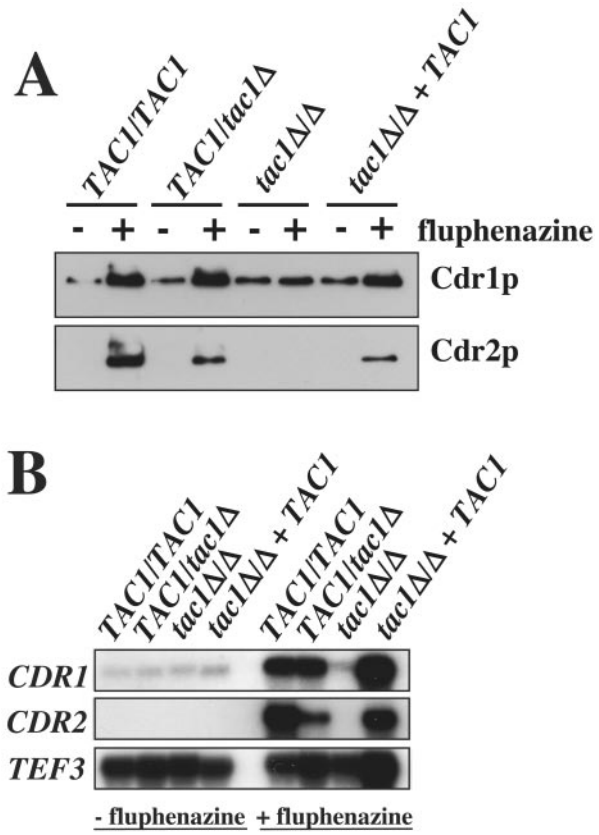

FIG. 2. *TAC1* functions as a transcriptional activator of *CDR1* and *CDR2*. (A) Immunodetection of Cdr1p and Cdr2p in *TAC1* mutant and revertant strains. Protein extracts of each strain were separated by SDS–10% PAGE and immunoblotted with rabbit polyclonal anti-Cdr1p and anti-Cdr2p antibodies as described previously (8). *C. albicans* strains were grown in YEPD to mid-log phase and exposed (+) or not exposed (−) to fluphenazine (10 μg/ml) for 20 min. See the legend to Fig. 1 for strain and genotype designations. (B) Northern analysis of *TAC1* mutant and revertant strains with *CDR1* and *CDR2* probes.

For RNA labeling, 25 μg of total RNA was mixed with 8 μl of 5× First-Strand buffer (Invitrogen, Basel, Switzerland), 1 μl of 0.1 μM *C. albicans*-specific Primer Mix Plus [including T20VN and oligo(dT)$_{18–21}$] (Eurogentec), 3 μl of 6.67 mM deoxynucleoside triphosphates (Roche), 1 μl of 1 mM dCTP (Roche), 1.5 μl of 1 mM cyanine 3 (Cy3)-dCTP or 1.5 μl of 1 mM cyanine 5(Cy5)-dCTP (Amersham Biosciences), 4 μl of 100 mM DTT (Invitrogen), 1 μl of RNasin (20 to 40 U/μl; Promega, Wallisellen, Switzerland), and diethyl pyrocarbonate-treated water to a volume of 40 μl. The mixture was heated at 65°C for 5 min and cooled at 42°C for 5 min. The reverse transcription reaction was performed for 1 h at 42°C with 1 μl of RNasin and 200 U of SuperScript II reverse transcriptase (Invitrogen). The reaction was stopped, and RNA was degraded by the addition of 5 μl of EDTA (50 mM, pH 8.0) and 2 μl of NaOH (10 N) and incubation at 65°C for 20 min. The reaction was neutralized with 4 μl of acetic acid (5 M). The labeled probes (cDNA) were purified according to the QiaQuick PCR purification kit protocol (Qiagen). The elution step was performed twice with prewarmed H_2O (42°C), and the samples were centrifuged at 13,000 × *g* for 1 min. The purified labeled probes were concentrated to a final volume of 5 μl by using a Microcon-30 filter (Amicon, Wallisellen, Switzerland).

(ii) Hybridization of microarrays. Cy5- and Cy3-labeled cDNAs synthesized from RNAs of cells for which transcription profiles were to be compared were pooled with 5 μl of heat-denatured salmon sperm DNA. Four different transcription profile comparisons were performed: (i) Cy3-labeled cDNA from CAF2-1 not exposed to fluphenazine with Cy5-labeled cDNA from the same strain exposed to fluphenazine (Flu experiment, as labeled in the data file array_TAC1_Sanglard.xls); (ii) Cy3-labeled cDNA from CAF2-1 with Cy5-labeled cDNA from DSY2906, both exposed to fluphenazine (TAC1 flu experiment); (iii) Cy3-labeled cDNA from DSY2925 with Cy5-labeled cDNA from DSY2926 (TAC1 allele experiment); and (iv) Cy3-labeled cDNA from DSY294 with Cy5-labeled cDNA from DSY296 (CDR experiment). Each DNA pool was boiled at 95°C for 2 min, chilled on ice, and mixed with 40 μl of hybridization buffer (DIG easy hyb; Roche). The mixture was applied to *C. albicans* microarray slides, covered with Lifterslips (25 by 44 mm; Erie Scientific Co., Portsmouth, N.H.) for all hybridization steps, and loaded into the hybridization chamber (Corning Inc., Corning, N.Y.), which was kept wet by loading 10 μl of H_2O in each existing well. After overnight hybridization at 42°C, coverslips were removed from slides by dipping in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS. Slides were washed for 5 min at room temperature with occasional agitation and rinsed with 0.2× SSC for 5 min at room temperature with occasional agitation. Slides were spin dried (500 × *g*, 5 min) and stored protected from light until scanning.

(iii) Quantitative analysis of microarrays. Hybridized microarray slides were scanned with a ScanArray 4000 scanner (Perkin-Elmer, Schwerzenbach, Switzerland) at a 10-μm resolution. The following wavelengths were used for photoexcitation: 532 nm for Cy3-labeled cDNA and 635 nm for Cy5-labeled cDNA. The intensity of each laser light was determined by equalizing the Cy3 and the Cy5 fluorescence signals of the dynamic-range controls (*TEF3*) in three different blocks for each slide. The resulting 16-bit TIFF files of each signal were quantified and converted to a text file by using ImaGene software, version 4.0 (BioDiscovery Inc., El Segundo, Calif.).

(iv) Normalization control. Data from each hybridization experiment were normalized by using a LOWESS (locally weighted scatter plot smooth) analysis for print-tip variability and for slide-to-slide variability with publicly available software (from Lund University; http://www.braju.com/R/com.braju.sma/) run in the R-project environment (http://cran.r-project.org/). In order to determine a significant threshold, LOWESS normalization was performed only on the *TEF3* spots, *TEF3* being a housekeeping gene. A significant threshold was designated as being above the mean of the *TEF3* ratios and twice the standard deviation. A twofold differential expression limit was therefore chosen for the comparison of pairwise experiments in this study. Normalized data can be downloaded from http://www.hospvd.ch/imul/ under the file name Microarray Data (array_TAC1_sanglard.xls).

RESULTS

Genome information as a tool for the discovery of *CDR1* and *CDR2* regulators. The cloning of transcriptional activators of *CDR1* and *CDR2* can be approached by several strategies. Since multidrug transporters of the ABC family share common functions in *C. albicans* and *S. cerevisiae*, it could be assumed that their regulation could be controlled by transcriptional factors with some degree of similarity. Attempts to clone *C. albicans* transcriptional activators of ABC transporters by use of *S. cerevisiae* resulted in the isolation of *FCR1* and *FCR3* (37, 40) and of three additional genes (*SHY1*, *SHY2*, and *SHY3* [suppressor of hypersusceptibility]) encoding proteins with Zn(2)-Cys(6) binuclear cluster domains. However, the deletion of these genes in *C. albicans* had no effect on *CDR1* and *CDR2* regulation (D. Sanglard, unpublished data).

Since the nucleotide sequence of the *C. albicans* genome is available, an alternative strategy consisting of a systematic search of ORFs encoding proteins with Zn(2)-Cys(6) binuclear cluster domains was undertaken. This strategy was based on the fact that transcription factors containing these motifs often recognized in *cis*-acting regulatory regions 5′-CGG-3′ triplets (22) that are present in the DRE of *CDR1* and *CDR2* (8). Using the data available from the most recent assembly of the *C.albicans* genome (http://www-sequence.stanford.edu/group/candida/search.html), we identified a total of 72 ORFs encoding proteins with Zn(2)-Cys(6) DNA-binding domains; of these gene products, 62 had these domains at their N-terminal ends. A cluster analysis of these ORFs revealed a group of related genes, i.e., orf19.3187 (*ZNC1* [zinc-Cys 1]), orf19.3188 (*ZNC2*), and orf19.3190 (*ZNC3*) (see the supplemental mate-

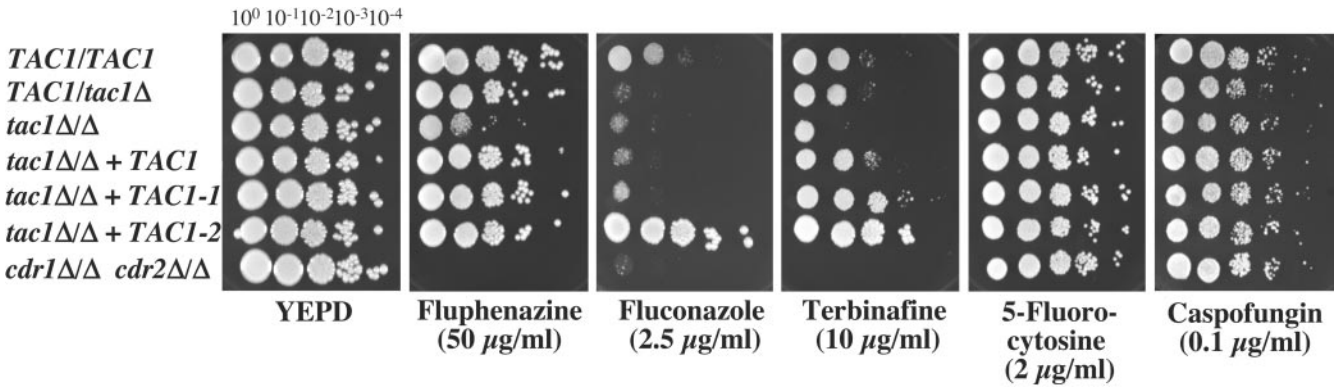

FIG. 3. Drug susceptibility testing of *C. albicans TAC1* mutant and revertant strains. Spotting assays were performed with serial dilutions of overnight cultures on YEPD containing the indicated drugs. Plates were incubated for 48 h at 35°C. The following strains correspond to the indicated genotypes: *tac1*Δ/Δ + *TAC1-1*, DSY2925; *tac1*Δ/Δ + *TAC1-2*, DSY2926; and *cdr1*Δ/Δ *cdr2*Δ/Δ, DSY654. See the legend to Fig. 1 for other strain and genotype designations.

rial). Interestingly, these genes are clustered physically within a 13-kb region present in the nucleotide sequences of contigs 19 to 10070 and contigs 19 to 20070. This region, the so-called zinc cluster region (Fig. 1A), is located on chromosome 5 of *C. albicans*, as deduced from data available at http://candida.bri.nrc.ca/candida/. Moreover, the zinc cluster region is situated approximately 14 kb upstream of the mating locus (either *MTL***a** or *MTL*α). Interestingly, homozygosity at the mating locus has been linked to the development of azole resistance in specific clinical strains (31). We therefore hypothesized that the genes contained in the zinc cluster region could be involved in this phenomenon. To test this hypothesis, individual mutants lacking each of the genes in this cluster were constructed and subjected to an analysis of *CDR1* and *CDR2* transcriptional regulation. The construction of homozygous mutants could be accomplished only with *ZNC2*. Only heterozygous mutants were obtained with *ZNC1* and *ZNC3*. Attempts to obtain homozygous mutants with these genes failed even after the screening of approximately 100 transformants from the disruption of the second allele of each gene.

TABLE 6. *CDR2-lacZ* reporter activities mediated by *TAC1* in *S. cerevisiae* and *C. albicans*

Type of promoter-*lacZ* fusion (plasmid)	Mean ± SD β-galactosidase activity (Miller units)[a]	
	Without fluphenazine	With fluphenazine
S. cerevisiae		
CDR2-lacZ	2.4 ± 0.4	ND
TAC1-CDR2-lacZ (pDS1157)[b]	96 ± 10	ND
TAC1-CDR2 (lacking DRE)-*lacZ* (pDS1180)[b]	0.7 ± 0.3	ND
YIp353 (parent plasmid)	<0.1	ND
C. albicans		
TAC1-CDR2-lacZ (pDS1187)[c]	0.3 ± 0.05	14 ± 4
TAC1-CDR2 (lacking DRE)-*lacZ* (pDS1190)[c]	<0.1	<0.1
pDS178 (parent plasmid)	<0.1	<0.1

[a] Values are from three measurements. ND, not determined. Fluphenazine treatment was performed with 20 μg/ml for 60 min.
[b] DSY669 was the recipient of pDS1157 and pDS1180.
[c] DSY2906 was the recipient of pDS1187 and pDS1190.

***ZNC2* (*TAC1*) disruption affects *CDR1* and *CDR2* upregulation.** Southern analysis of the *ZNC2* (now renamed *TAC1* [transcriptional activator of *CDR* genes]; see below) disruption revealed the expected restriction fragments in the analyzed mutants (Fig. 1B). These *C. albicans* strains, i.e., the heterozygous *TAC1*/*tac1*Δ mutant DSY2875, the homozygous *tac1*Δ/Δ mutant DSY2903, and the revertant (*tac1*Δ/Δ *TAC1*) DSY2937, were next subjected to an analysis of *CDR1* and *CDR2* expression under conditions of fluphenazine exposure or no fluphenazine exposure, which are known to affect the transporters. While the levels of Cdr1p and Cdr2p were elevated by fluphenazine exposure in wild-type strain CAF2-1, these levels remained unchanged in the *tac1*Δ/Δ strain (Fig. 2A). Intermediate increases in the levels of Cdr1p and Cdr2p were observed in the *TAC1*/*tac1*Δ strain and the revertant DSY2937. Northern analysis of the same strains with labeled *CDR1* and *CDR2* probes (Fig. 2B) demonstrated that *TAC1* controls the upregulation of *CDR1* and *CDR2* at the transcriptional level: while *CDR1* and *CDR2* were upregulated in CAF2-1 and the *TAC1*/*tac1*Δ strain, upregulation was absent in the *tac1*Δ/Δ strain after exposure to fluphenazine but was restored in the revertant DSY2937. Furthermore, relative β-galactosidase activities of *CDR1*- and *CDR2-lacZ* fusions introduced in the *ura3* derivative of the *tac1*Δ/Δ strain DSY2903 were not increased after fluphenazine exposure (data not shown). Taken together, these data strongly suggest that the gene previously named *ZNC2* is a positive regulator of both *CDR1* and *CDR2* and therefore can be renamed *TAC1* (transcriptional activator of *CDR* genes).

As expected from the role of *TAC1* in *CDR1* and *CDR2* upregulation, the disruption of *TAC1* alleles in *C. albicans* affected susceptibility to antifungal drugs and to other metabolic inhibitors: the *tac1*Δ/Δ strain was more susceptible to fluphenazine, fluconazole, or terbinafine than the wild-type strain, and this phenotype was reversed in strain DSY2937, into which the *TAC1* allele from SC5314 was reintroduced (Fig. 3). The reversal of the drug susceptibility phenotype was best observed in the revertant DSY2937 on plates containing

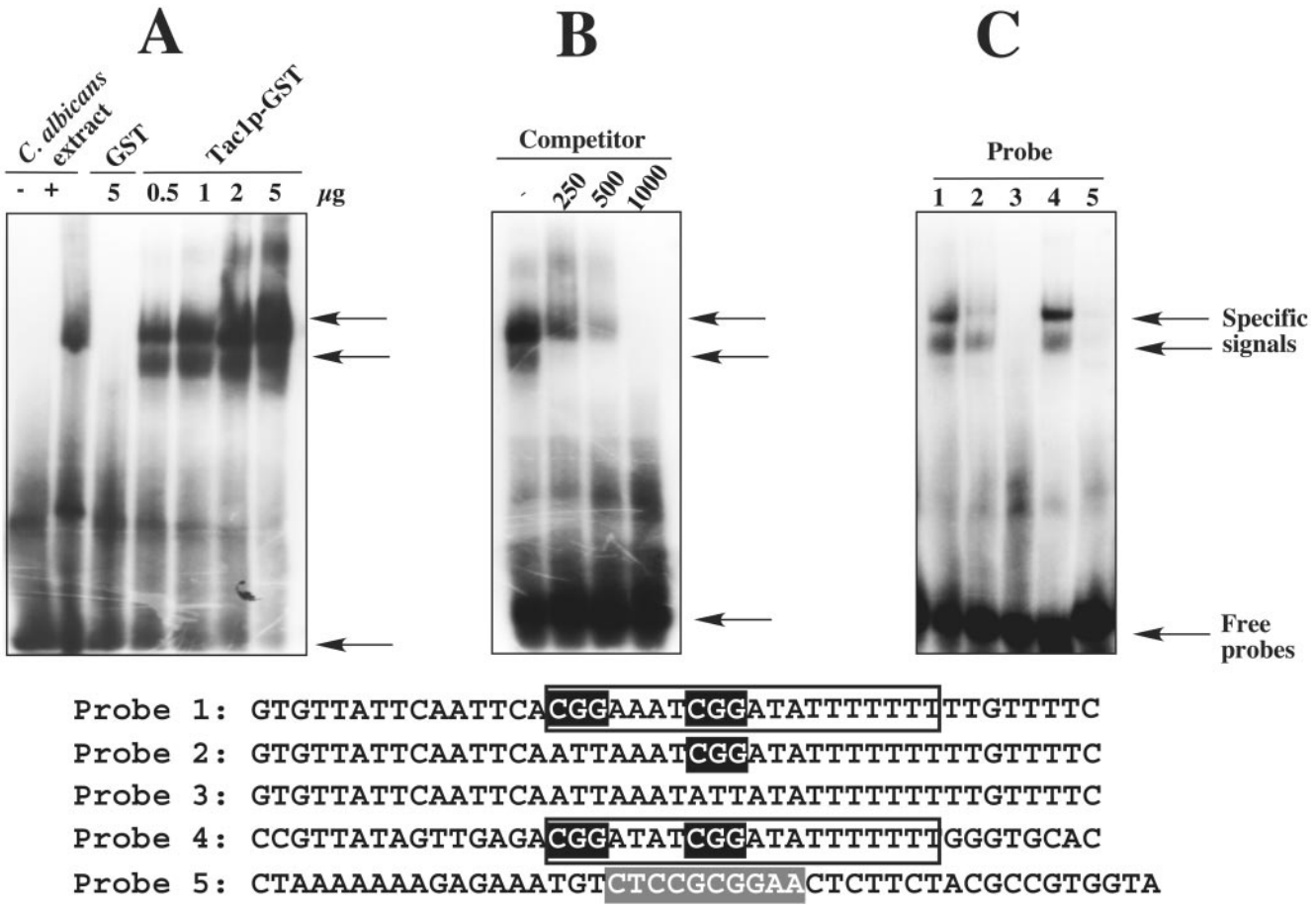

FIG. 4. Tac1p binds to the *CDR* DRE. Labeled probes were separated as described in Materials and Methods. Arrows indicate the positions of specific complexes and of free labeled probes. (A) Binding saturation of the DRE by Tac1p-GST. Increasing amounts of Tac1p-GST were added to the reaction mixtures before loading. (B) Competition experiments with unlabeled DRE. A total of 1 μg of Tac1p-GST was added to the labeled probe along with increasing amounts of unlabeled probe. Probe 1 corresponding to the DRE region (DRE consensus sequence boxed with two CGG triplets highlighted) of the *CDR2* promoter was used for the band shifts shown in panels A and B. (C) CGG triplet-dependent binding of Tac1p-GST. Probes 1, 2 (first CGG triplet changed), and 3 (first and second CGG triplets changed) correspond to the DRE region of the *CDR2* promoter. Probes 4 and 5 correspond to the DRE region (DRE consensus sequence boxed with two CGG triplets highlighted) of the *CDR1* promoter and to the PDRE (consensus sequence highlighted) of *PDR5*, respectively. Each probe was designed with two complementary oligonucleotides.

fluphenazine and terbinafine. However, the susceptibility of the *tac1*Δ/Δ mutant was not as pronounced as that of the *cdr1*Δ/Δ *cdr2*Δ/Δ mutant DSY654 in medium containing fluphenazine, fluconazole, or terbinafine: serially diluted cells of the *cdr1*Δ/Δ *cdr2*Δ/Δ mutant did not grow in the presence of these drugs, while residual growth was still observed for the *tac1*Δ/Δ mutant at low dilutions. These results can be explained by the contribution of the still measurable *CDR1* expression in the *tac1*Δ/Δ mutant to the observed differences in drug susceptibility. On the other hand, when compounds such as flucytosine or caspofungin were tested with the *tac1*Δ/Δ mutant, no difference in susceptibility between the mutant and the wild type could be observed (Fig. 3). This effect is consistent with studies reporting that these two antifungal agents are not considered to be substrates of Cdr1p and Cdr2p (2, 34).

***TAC1* binds to the DRE of *CDR1* and *CDR2* and is localized in the nucleus.** De Micheli et al. showed previously that *CDR1* and *CDR2* contain a *cis*-acting element, the so-called DRE, that is important for their upregulation after drug exposure or for their constitutive high level of expression in azole-resistant isolates (8). Since *TAC1* regulates *CDR1* and *CDR2*, it is possible that the protein encoded by this gene binds to the DRE. As a first approach, *TAC1* was expressed in both *S. cerevisiae* and *C. albicans*, and its activity was tested with the help of species-specific *CDR2-lacZ* chimeric constructs, one of which was devoid of the DRE. The *S. cerevisiae* integrative YIp353-derived plasmids contained the *CDR2* promoter with or without the DRE (pDS1157 or pDS1180, respectively) and *TAC1* from strain SC5314. The *C. albicans* integrative plasmids contained the *CDR2* promoter with or without the DRE (pDS1187 or pDS1190, respectively) fused to *Streptococcus thermophilus lacZ* (38). The *CDR2-lacZ* fusions were subcloned into pDS1097, which contained *TAC1* from strain SC5314. β-Galactosidase activities measured in *S. cerevisiae* revealed that the presence of *TAC1* in pDS1157 could elevate reporter activities by approximately 40-fold (Table 6). However, the absence of the DRE in the *CDR2* promoter subcloned in pDS1180 decreased reporter activities to almost background levels (Table

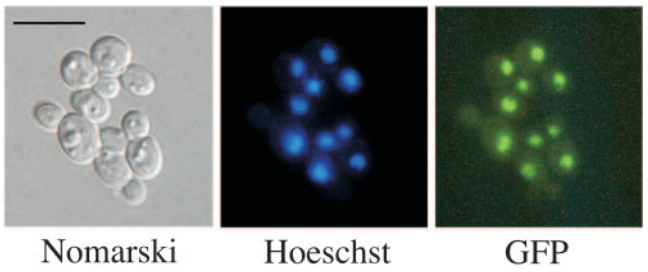

FIG. 5. Nuclear localization of Tac1p. DSY2906 transformed with pDS1202 was grown in liquid selective medium to a cell density of 10^7 cells/ml with constant agitation. Culture aliquots were sampled for nuclear staining as described in Materials and Methods and visualized by microscopy. Digital images were further processed with the computer program Adobe Photoshop 7.0 (Adobe Systems Incorporated, Mountain View, Calif.). Bar, 10 μm.

6). In *C. albicans*, β-galactosidase activities derived from the DRE-containing *CDR2-lacZ* fusion were near background levels under normal growth conditions; however, as expected, they were increased by fluphenazine treatment by approximately 50-fold. The absence of the DRE in plasmid pDS1190 abolished the increase in reporter activities (Table 6). Taken together, the data summarized in Table 6 demonstrate that *TAC1* needs the DRE to activate the transcription of *CDR2* not only in *S. cerevisiae* but also in *C. albicans*.

To show further that Tac1p is able to bind to the DRE, a Tac1p chimeric protein was constructed by fusion of the first 129 amino acids of Tac1p with GST. The first 129 amino acids of Tac1p contained the Zn(2)-Cys(6) binuclear cluster domain expected to interact with DNA. This fusion construct enabled the production of a soluble protein in *E. coli* and its recovery by affinity purification. After purification of the fusion protein, band shift assays were performed with Tac1p-GST and a labeled 43-bp DNA fragment based on the *CDR2* promoter sequence and containing the DRE. As shown in Fig. 4A and B, specific Tac1p-GST complexes with the labeled probe were visible. The formation of these complexes was dependent on the amount Tac1p-GST added to the reaction (Fig. 4A) and could be inhibited by increasing the addition of cold competitor from a 250- to a 1,000-fold excess (Fig. 4B). The binding of Tac1p-GST and a 43-bp fragment corresponding to the DRE-containing portion of the *CDR1* promoter was also observed (Fig. 4C, probe 4), indicating that the DRE consensus sequence (boxed in the sequences given at the bottom of Fig. 4) rather than nucleotide sequences upstream or downstream of the DRE consensus sequence is responsible for binding. The formation of two specific complexes in these band shifts could be due to the formation of protein multimers, either from the presence of GST or from the binding of several protein moieties to the DRE.

Since Zn(2)-Cys(6) fingers are thought to bind to *cis*-acting elements via CGG triplets, transversions were performed on those of the DRE and the resulting probes were incubated with Tac1p-GST. As shown in Fig. 4C, the removal of one CGG triplet (probe 2) decreased the binding of Tac1p-GST compared to the results obtained with probe 1, while the removal of both CGG triplets (probe 3) completely abolished the binding of the fusion protein. However, CGG triplets must be present in the context of the DRE, since Tac1p-GST could not bind to the PDRE of *PDR5* (probe 5), which also contains CGG triplets recognized by Zn(2)-Cys(6) binuclear cluster domains. That fact that Tac1p-GST can bind to the DRE strongly suggests that Tac1p is involved in the regulation of *CDR1* and *CDR2*. The binding of this factor is probably a necessary step for the activation process resulting in the upregulation of both genes. Due to its DNA-binding activity, Tac1p is expected to be localized in the nucleus. To address this notion, GFP was fused to the C-terminal end of Tac1p, and the resulting fusion protein was observed by fluorescence microscopy. The fusion protein was transcriptionally functional, since it was able to restore *CDR2* upregulation when present in the *tac1*Δ/Δ mutant (data not shown). Figure 5 shows that the fusion protein was localized in the nuclei of growing cells, since staining of nuclear DNA colocalized with GFP in the majority of the observed cells. The nuclear localization of Tac1p agrees with its function as a transcription factor.

A *TAC1* allele from an azole-resistant clinical isolate can confer azole resistance to a susceptible isolate. Azole resistance can be coupled to the upregulation of *CDR1* and *CDR2*. Several investigators demonstrated this feature in several azole-resistant isolates (28, 35, 39). One possible hypothesis to explain the constitutive upregulation of these transporter genes in *C. albicans* is that it exists in several *TAC1* alleles and some of them are in a hyperactive state. To test this hypothesis, *TAC1* alleles from an azole-susceptible strain (DSY294) and its azole-resistant parent (DSY296, a strain in which *CDR1* and *CDR2* are upregulated) were recovered and introduced into a *ura3* derivative of the *tac1*Δ/Δ mutant, as described for the revertant strain DSY2937. The transformants (DSY2925 [*tac1*Δ/Δ *TAC1-1*], from DSY294, and DSY2926 [*tac1*Δ/Δ *TAC1-2*], from DSY296) then were exposed to fluphenazine to induce the expression of *CDR1* and *CDR2*, as described above. Western blot analysis of the transformants with anti-Cdr1p and anti-Cdr2p antibodies revealed that, while the levels of these proteins were elevated by fluphenazine treatment in the revertant strain DSY2925 containing the *TAC1-1* allele from DSY294, these levels were already elevated in the transformant DSY2926 containing a *TAC1-2* allele from the azole-resistant strain DSY296 (Fig. 6). Our current data reveal that only *TAC1-2* alleles have been recovered so far from the azole-resistant strain DSY296.

Drug susceptibility testing of these strains was performed by using a microtiter plate format with YEPD, increasing concentrations of fluconazole, and incubation for 24 h. The fluconazole MICs for strain CAF2-1, the *tac1*Δ/Δ mutant, and strain DSY2925 were between 0.5 and 1.0 μg/ml, but the MIC for strain DSY2926 was 16- to 32-fold higher (16 μg/ml), indicating that *TAC1-2* mediates resistance to this specific agent. Furthermore, the presence of the *TAC1-2* allele decreased susceptibility to other compounds, as shown for strain DSY2926 (Fig. 3, serial dilutions of *tac1*Δ/Δ *TAC1-2*). Azole resistance in strain DSY2926 could be explained by the increased expression of *CDR1* and *CDR2* and corresponding proteins—a consequence of the presence of *TAC1-2*. Experiments are in progress to demonstrate this hypothesis through the expression of the *TAC1-2* allele in a *cdr1*Δ/Δ *cdr2*Δ/Δ mutant. Our present results demonstrate a direct relationship among the presence of *TAC1-2* from an azole-resistant isolate,

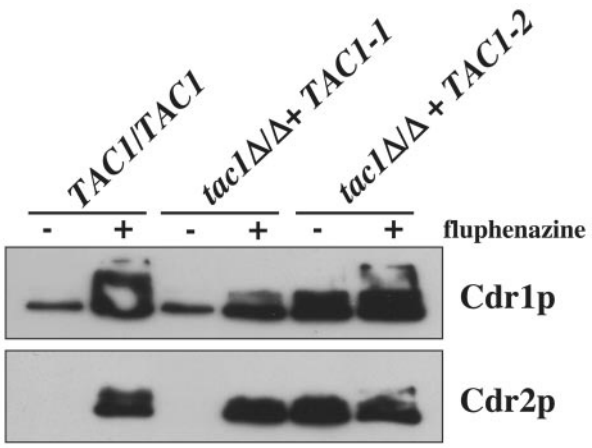

FIG. 6. The *TAC1-2* allele functions as a constitutive transcriptional activator of *CDR1* and *CDR2*. Protein extracts of each strain were separated by SDS–10% PAGE and immunoblotted with rabbit polyclonal anti-Cdr1p and anti-Cdr2p antibodies as described previously (8). *C. albicans* strains were grown in YEPD to mid-log phase and exposed (+) or not exposed (−) to fluphenazine (10 μg/ml) for 20 min. See the legends to Fig. 1 and 3 for strain and genotype designations.

high cellular levels of Cdr1p and Cdr2p (Fig. 6), and the development of azole resistance.

Codominance of azole resistance in *C. albicans*. It was previously reported that homozygosity at the mating locus was strongly linked to the development of resistance to azoles (31). We partially confirmed this observation, as we determined, using the method described by Rustad et al. (31), that from among our collection of clinical isolates and among 13 azole-resistant matched isolates upregulating *CDR1* and *CDR2*, 8 (2 *MTL***a**/*MTL***a** and 6 *MTL*α/*MTL*α) were homozygous at the mating locus (data not shown). In particular, the azole-resistant strain DSY296, from which only *TAC1-2* alleles were recovered, was homozygous for *MTL*α, while the matched azole-susceptible strain DSY294 was still *MTL***a**/*MTL*α. Since *TAC1* is located upstream of the mating locus and since *TAC1-2* is involved in *CDR1* and *CDR2* upregulation, it is possible that homozygosity at the mating locus can favor the development of azole resistance by rendering the *MTL*-associated *TAC1-2* locus homozygous. In other words, gain-of-function *TAC1-2* alleles could be codominant and need to be homozygous to confer sufficient *CDR1* and *CDR2* upregulation. To address this possibility, we crossed the Gal$^-$ derivative of DSY296 containing the *TAC1-2* allele (DSY296-3 [*MTL*α/*MTL*α Gal$^-$]) with the test strain CHY439 (*MTL***a**/*MTL***a** Gal$^+$ Ade$^-$ Ura$^-$). One of the resulting fusion products (DSY2781 [Gal$^+$ Ura$^+$ Ade$^+$]) (Fig. 7A) had an intermediate phenotype with respect to azole susceptibility, as shown by fluconazole disk diffusion assays and MIC determinations. The fluconazole MICs for strains DSY296-3 and CHY439 were ≥64 and 2 μg/ml, respectively, while that for strain DSY2781 was 16 μg/ml (Fig. 7B). Accordingly, Cdr1p and Cdr2p levels were intermediate between DSY296-3 and CHY439 (Fig. 7B). Since *TAC1-2* from DSY296-3 is responsible for *CDR1* and *CDR2* upregulation (Fig. 6), the reduced Cdr1p and Cdr2p levels in the fusion product DSY2781 are consistent with a codominance phenotype of the *TAC1-2* allele. Moreover, the codominance of the gain-of-function *TAC1-2* allele could explain the need for homozygosity at the MTL-associated *TAC1* locus in strain DSY296 for high-level expression of the *CDR1* and *CDR2* genes.

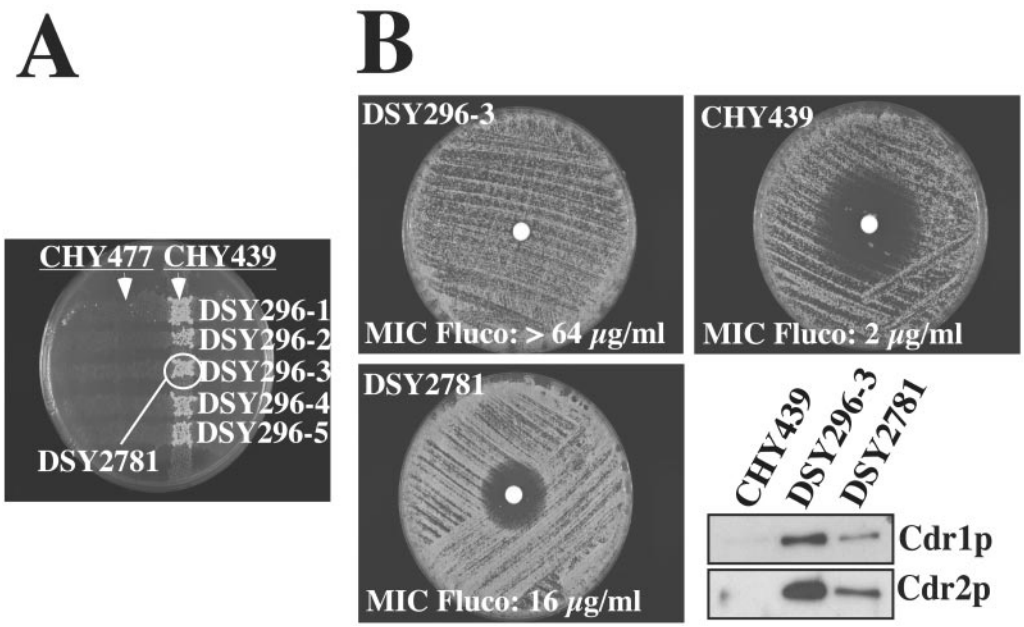

FIG. 7. Codominance of azole resistance in *C. albicans*. (A) Strain DSY296, which is homozygous at the *MTL*α locus, was mutagenized to obtain *gal1* strains (numbered 1 to 5). These strains were crossed with mating test strains CHY477 (*MTL*α) and CHY439 (*MTL***a**) (26) and replica plated on YNB containing galactose. (B) Fluconazole (Fluco) susceptibility of strain CHY439, strain DSY296-3, and fusion product DSY2781 tested by diffusion disk assays and by corresponding Western blotting with anti-Cdr1p and anti-Cdr2p antibodies.

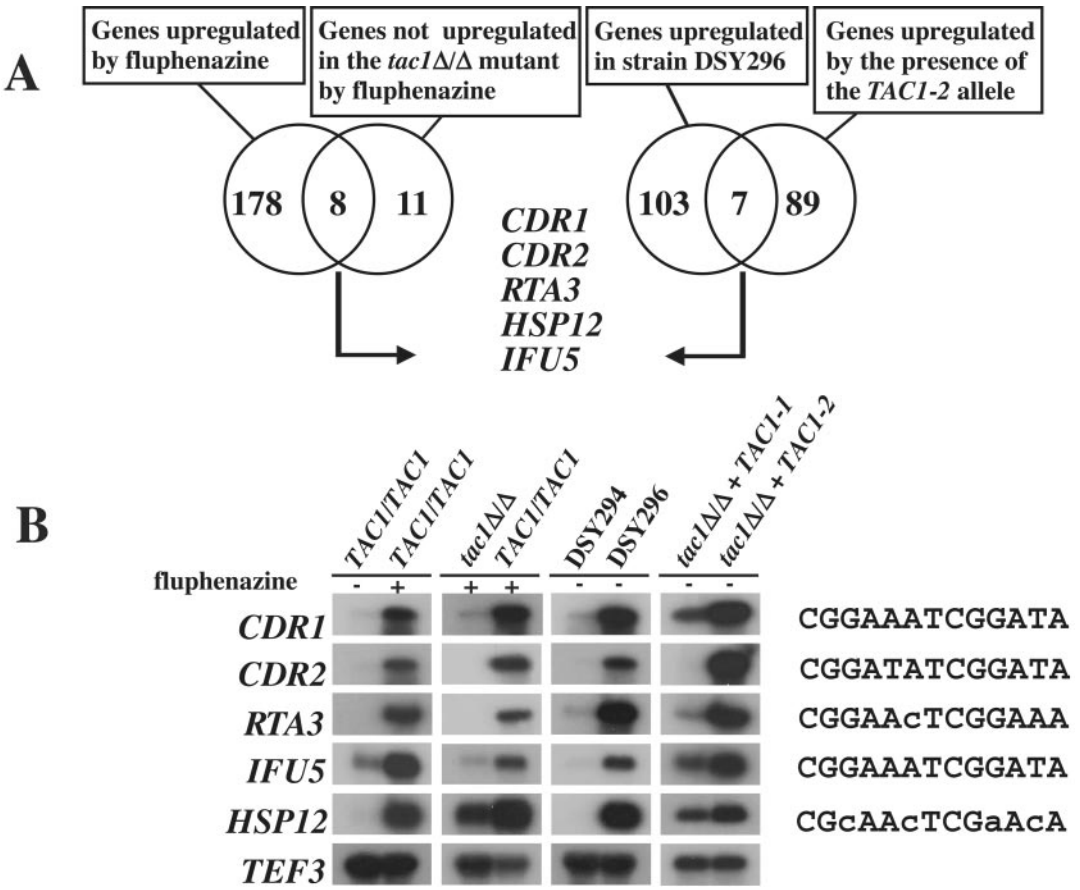

FIG. 8. Microarray analysis reveals a subset of *TAC1*-dependent genes. (A) Venn diagram analysis of genes upregulated by fluphenazine in strain CAF2-1 and of genes no more upregulated under the same conditions in a *TAC1* mutant strain (left side) and of genes commonly upregulated in strain DSY2926 expressing the *TAC1-2* allele and in azole-resistant strain DSY296 (right side). DSY296 upregulates *CDR1* and *CDR2* and is the strain from which *TAC1-2* originates. Genes common to these experiments are listed. A threshold of twofold was used to determine genes that were significantly upregulated. (B) Northern analysis of *TAC1*-regulated genes. DRE-like elements are shown for each gene investigated by Northern analysis. Lowercase letters show nucleotides different from those in the DRE of *CDR1* and *CDR2*. See the legends to Fig. 1 and 3 for strain and genotype designations. −, no fluphenazine; +, fluphenazine.

Gene expression profiling experiments reveal potential targets of *TAC1*. *CDR1* and *CDR2* are not the only DRE-containing genes in the *C. albicans* genome; thus, it is possible that *TAC1* could regulate these additional genes. *TAC1* might also regulate genes through other indirect processes. In order to determine additional *TAC1* gene targets, a gene expression profiling approach was used with microarrays of *C. albicans*. Several experiments were designed for this purpose. The numbers of genes upregulated in the presence of the hyperactive *TAC1* allele (in a comparison of *C. albicans* strains DSY2925 and DSY2926 containing *TAC1-1* and *TAC1-2* alleles in a *tac1*Δ/Δ mutant, respectively) and of genes upregulated in the azole-resistant strain DSY296, from which the *TAC1-2* allele originated (in a comparison of strains DSY294 and DSY296), were determined. We found 103 genes that were upregulated in the azole-resistant strain DSY296 compared to the azole-susceptible strain DSY294, 89 that were upregulated due to the presence of the *TAC1-2* allele compared to the presence of the *TAC1-1* allele in the *tac1*Δ/Δ mutant, and finally 7 genes that were upregulated in both experiments (Fig. 8A). We reasoned that genes upregulated in both experiments could be considered to be potential targets of *TAC1*. As expected, genes upregulated under both conditions included *CDR1* and *CDR2* but also *RTA3* (probable transmembrane protein similar to *S. cerevisiae* YOR049c), *HSP12* (heat shock protein), *IPF14285* (unknown function), *CDC23* (probable member of the anaphase-promoting complex), and *IPF8179* (protein with high similarity to *S. cerevisiae* YJL068p, a putative esterase).

We further extended our analysis by determining genes upregulated by fluphenazine treatment (in a comparison of strain CAF2-1 exposed to fluphenazine and strain CAF2-1 not exposed to fluphenazine) and next by identifying genes not upregulated by fluphenazine in a *tac1*Δ/Δ mutant strain compared to a wild-type strain (in a comparison of strain CAF2-1 exposed to fluphenazine and the *tac1*Δ/Δ mutant strain exposed to fluphenazine). We found 178 genes that were specifically upregulated by the addition of fluphenazine in the culture of the wild-type strain, 11 genes that were no more upregulated in the presence of fluphenazine in the *tac1*Δ/Δ mutant strain, and 8 genes that were upregulated by the addition of fluphenazine but that were also *TAC1* dependent (Fig. 8A). The combination of these conditions revealed *CDR1*, *CDR2*, *HSP12*, and

RTA3 but also *IPF885* [protein with weak similarity to Xog1p, an exo-β-(1,3)-glucanase involved in cell wall metabolism], *IPF1514* (unknown function), *IPF17283* (unknown function), *GRP2* (probable reductase similar to *S. cerevisiae* YOL151w), and *GPX1* (probable glutathione peroxidase similar to the *S. cerevisiae HYR1* gene product). Only four genes were common to these two microarray experiments, i.e., *CDR1*, *CDR2*, and *RTA3*, which all contain a consensus DRE in their promoters, and *HSP12* (Fig. 8A).

In order to confirm the microarray results, Northern blotting was performed with total RNAs extracted from strains grown under the conditions tested in the microarray analysis. As shown in Fig. 8B, mRNA signals obtained for *CDR1*, *CDR2*, *RTA3*, and *HSP12* confirmed the microaray results. These genes were upregulated in strain DSY2926 containing the hyperactive *TAC1-2* allele, in strain CAF2-1 exposed to fluphenazine, and in the azole-resistant strain DSY296. It was reported previously that *IFU5* (*LPG20*) was a DRE-containing gene (8). This gene failed to appear in the microarray analysis because it was flagged in two sets of experiments. However, *IFU5* was coordinately upregulated with *CDR1*, *CDR2*, *RTA3*, and *HSP12*, as shown by Northern analysis (Fig. 8B). Taken together, our results indicate that the *CDR1*, *CDR2*, *RTA3*, *IFU5*, and *HSP12* genes constitute a group of *TAC1*-dependent genes, consistent with the presence of a DRE-like region in these genes and with the ability of Tac1p to bind to the DRE.

DISCUSSION

Isolation of transcriptional activators of *CDR1* and *CDR2* by mining in the *C. albicans* genome. In this article, we report the isolation of a transcription factor involved in the regulation of *CDR1* and *CDR2* by inspection of genome data. We focused on Zn(2)-Cys(6) transcription factors because these factors are able to target 5′-CGG-3′ motifs that are present in the DRE of *CDR1* and *CDR2* promoters. These transcription factors have in common a Zn(2)-Cys(6) DNA-binding domain which targets sequences with direct, inverted, or everted palindromic repeats containing a 5′-CGG-3′ motif with various numbers of nucleotides in the intervening spacer region. Several examples can be cited from studies performed with *S. cerevisiae*: Hap1p recognizes the direct repeat 5′-CGGNNNTANCGG-3′; the heterodimer Pip2p/Oaf1p binds to inverted repeats of the consensus sequence 5′-CGGN$_{15–18}$-CCG-3′; Pdr8p and Yrr1p, which are factors involved in multidrug resistance, target DNA-binding motifs containing the 5′-CGG-3′ triplet in dyad symmetry; and Pdr1p and Pdr3p regulate their targets via the everted sequence motif 5′-TCCG/aC/tGG/cA/g-3′ (where lowercase letters indicate variable bases in a consensus motif) (9, 17, 23). More recently, it was shown that War1p binds the *cis*-acting element 5′-CGG-N$_{23}$-CCG-3′, which is essential for *PDR12* activation and resistance to weak acid stress (22).

Using the most recent assembly of the *C. albicans* genome, we were able to enumerate at least 72 ORFs with a Zn(2)-Cys(6) DNA-binding motif. Some ORFs might have been missed in this analysis; therefore, this number may be revised in future studies. Since no systematic collection of deletion mutants exists for *C. albicans*, potential candidates were selected based on their closest similarity to *C. albicans* genes that could interact with 5′-CGG-3′ triplets. Of particular interest was a cluster of three such genes (*ZNC1* to *ZNC3*) located in a tandem repeat (the zinc cluster) (Fig. 1A) but showing a low level of similarity to each other. The inactivation of *ZNC2* (*TAC1*) was sufficient to perturb the regulation of *CDR1* and *CDR2*. Several pieces of experimental evidence were provided here to establish *TAC1* as a positive activator of *CDR1* and *CDR2* through the *cis*-acting regulatory DRE: (i) a mutant lacking *TAC1* was not capable of upregulating *CDR1* and *CDR2* after drug exposure, and this phenotype was restored in a revertant containing a single *TAC1* copy (Fig. 2); (ii) a Tac1p-GST fusion containing the N-terminal DNA-binding domain could bind a 43-bp sequence containing a DRE previously identified as a *cis*-acting regulatory element of *CDR1* and *CDR2* (Fig. 4); (iii) a gain-of-function *TAC1* allele from an azole-resistant isolate (*TAC1-2*) resulted in constitutive high-level expression of *CDR1* and *CDR2* in the laboratory strain CAF4-2 (Fig. 6); and (iv) the reporter activities of a *CDR2-lacZ* chimeric promoter were dependent on the presence of *TAC1* and of the DRE either in *S. cerevisiae* or in *C. albicans* (Table 6).

Tac1p is a 981-amino-acid protein with typical features of zinc finger proteins of the Zn(2)-Cys(6) family. It contains, besides the DNA-binding domain (positions 33 to 80), an activation domain rich in acidic residues at the C terminus (from position 880) and a region (approximately from positions 400 to 510), called the middle homology region, that may be involved in the regulation of transcriptional activity (36). A BLAST search of the available GenBank data indicated the highest score (although the overall similarity was below 20%) for the *S. cerevisiae* protein Hal9p, which is a transcription factor that increases salt tolerance through the increased expression of *ENA1* (Na^+/Li^+ extrusion pump) (25). The closest partners of Tac1p in *C. albicans* were those of the zinc cluster, especially the *ZNC1* gene product (30% similarity); however, similarity to the other 71 members was generally below 20%. *TAC1* is composed of two ORFs in the most recent assembly of the *C. albicans* genome (orf19.3188 and orf19.3189), since a nucleotide change introduces a stop codon. Our own verification of the *TAC1* sequence resulted in a noninterrupted ORF. In conclusion, Tac1p is a protein with no known equivalent deposited in available protein databases, a result which could be expected from the motif of its DNA target, the DRE, which also has no equivalent among DNA-binding motifs in eukaryotes.

The removal of *TAC1* abolished the upregulation of *CDR2*; thus, this factor represents a major regulator of this gene, even though the possibility that other, not-yet-identified factors could contribute to *CDR2* upregulation cannot be excluded. The situation is slightly different for *CDR1*, which shows basal expression affected little by the absence of *TAC1*. Therefore, additional regulators for this gene can be expected. In a previous study, De Micheli et al. showed that the *CDR1* promoter contained a basal expression element (8). Moreover, Gaur et al. (12) reported that the *CDR1* promoter contained negative regulatory elements. Karnani et al. (19) described *cis*-acting elements that are different from the above-mentioned DRE and that are responsible for the upregulation of *CDR1* by steroids. The protein factors responsible for these effects are still unknown.

Functional aspects and relevance of *TAC1* for antifungal drug resistance. The pathway leading to the activation of *CDR1* and *CDR2* through Tac1p is not yet established. Here we described two different possibilities for the upregulation of *CDR1* and *CDR2*: either drug exposure or the expression of a mutant *TAC1-2* allele. Zinc finger regulators can be activated by mechanisms such as overexpression, nuclear-cytoplasmic shuttling, DNA binding, phosphorylation, and unmasking of the activation domain. Interestingly, it was shown earlier that the intensity of the band shift was increased in cells exposed to *CDR1* and *CDR2* inducers. Even though the amounts of Tac1p in *C. albicans* still cannot be precisely quantified, these results suggest that activation could be due to the enhanced expression of *TAC1* after drug induction (8). On the other hand, the fact that protein complexes probably containing Tac1p can bind to the DRE under normal growth conditions, as described by De Micheli et al. (8), suggests that Tac1p binds constitutively to the DRE. We showed here that Tac1p has a constitutive nuclear localization (Fig. 5); therefore, its activation is due not to migration into the nucleus but rather to a posttranslational modification(s). This feature was described recently for War1p. This zinc finger transcription factor has a nuclear localization and binds to its target in a constitutive manner. War1p can activate its target gene (the ABC transporter gene *PDR12*) through exposure to weak acid in a manner similar to that used here for Tac1p—exposure to fluphenazine. War1p is phosphorylated under these conditions, probably resulting in the activation of *PDR12* (22). Such an activation mechanism could be envisaged for Tac1p, and experiments are currently under way to address this hypothesis.

The *TAC1-2* allele isolated here from an azole-resistant strain does not require activation for the high-level expression of *CDR1* and *CDR2* (Fig. 6). Since the activation pathway for Tac1p is still not understood, it is difficult to provide explanations for this phenotype. However, since hyperactive alleles of transcription factors were described for *PDR1* and *PDR3* in *S. cerevisiae* and since these alleles contained gain-of function mutations, it is likely that *TAC1-2* contains one or several mutations enabling the constitutive upregulation of *CDR1* and *CDR2*. Preliminary data obtained from *TAC1-1* and *TAC1-2* sequencing revealed several substitutions for both alleles, but their individual contributions to *CDR1* and *CDR2* upregulation remain to be determined (Sanglard, unpublished).

In this study, we showed that the gain-of-function *TAC1-2* allele could be codominant and showed that homozygosity at the *MTL*-associated locus could be needed for the high-level expression of *CDR1* and *CDR2* (Fig. 7). The results of an analysis of *MTL* locus homozygosity in azole-resistant isolates from our collection are in good agreement with the results obtained by Rustad et al. (31), where a link between mating locus homozygosity and the development of azole resistance was observed in different sets of clinical isolates. However, Pujol et al. (30) reported contradictory results showing that mating locus homozygosity was not correlated with azole resistance in different sets of clinical isolates. The observations made by these authors led us to conclude that other gain-of-function *TAC1* alleles with a dominant phenotype can exist, thus making it possible that some azole-resistant isolates upregulating *CDR1* and *CDR2* are still heterozygous at the mating locus. To examine this possibility, current work in our laboratory is under way to characterize other *TAC1* alleles from several azole-resistant isolates as being homozygous or heterozygous at the *MTL* locus. These *TAC1* alleles will be also helpful for mapping of the mutations responsible for the constitutive high-level expression of *CDR1* and *CDR2*.

***TAC1* and gene targets.** We showed here that in addition to *CDR1* and *CDR2*, Tac1p has other putative target genes, as inferred from a microarray analysis. In a previous study, De Micheli identified by inspection of genome data two genes (*IFU5/LPG20*) and *RTA3/YFLO10C*) containing a DRE in their promoters (8). These genes were upregulated by exposure to estradiol, one of the agents that is able to upregulate *CDR1* and *CDR2*. Interestingly, through the use of a genome-wide approach with four microarray experiments (Fig. 8), these putative additional targets, i.e., *RTA3* and *IFU5*, were reconfirmed as *TAC1* targets. *HSP12* also belongs to this group of coregulated genes. This gene contains a DRE-like region with four mismatches compared to the consensus sequence for *CDR1*, *CDR2*, *IFU5*, and *RTA3*. It is difficult to predict whether this DRE-like region is a target of Tac1p, since the minimal requirements of a fully functional DRE are still not known. Therefore, it will be necessary to further verify the possible occupancy of Tac1p on the promoters of these genes by coimmunoprecipitation experiments. Moreover, it is still possible that additional *TAC1* targets will be identified in the future, since the microarrays used in this study do not include the entire collection of ORFs contained in the *C. albicans* genome.

In conclusion, we demonstrated here that *TAC1* is a major factor needed for the regulation of *CDR1* and *CDR2*, which are the main ABC transporter genes responsible for antifungal drug resistance in *C. albicans*. Other genes are under the control of *TAC1*, but their role in the development of antifungal drug resistance is still not known (but is under investigation). More important is that the identification of *TAC1* provides new perspectives for the characterization of additional elements linked to the regulatory circuit controlling antifungal drug resistance.

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ADDENDUM IN PROOF

Recent experiments have shown that the expression of the *TAC1-2* allele in the background of a mutant lacking *TAC1*, *CDR1*, and *CDR2* did not result in fluconazole resistance, thus demonstrating that *TAC1-2* mediates azole resistance through the presence of both *CDR1* and *CDR2*.

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