We have isolated a Candida albicans gene that confers resistance to the azole derivative fluconazole (FCZ) when overexpressed in Saccharomyces cerevisiae. This gene encodes a protein highly homologous to S. cerevisiae yap1, a bZip transcription factor known to mediate cellular resistance to toxicants such as cycloheximide (CYH), 4-nitroquinoline N-oxide (4-NQO), cadmium, and hydrogen peroxide. The gene was named CAP1, for C. albicans AP-1. Cap1 and yAP-1 are functional homologues, since CAP1 expression in a yap1 mutant strain partially restores the ability of the cells to grow on toxic concentrations of cadmium or hydrogen peroxide. We have found that the expression of YBR008c, an open reading frame identified in the yeast genome sequencing project and predicted to code for a multidrug transporter of the major facilitator superfamily, is dramatically induced in S. cerevisiae cells overexpressing CAP1. Overexpression of either CAP1 or YAP1 in a wild-type strain results in resistance to FCZ, CYH, and 4-NQO, whereas such resistance is completely abrogated (FCZ and CYH) or strongly reduced (4-NQO) in a ybr008c deletion mutant, demonstrating that YBR008c is involved in YAP1- and CAP1-mediated multidrug resistance. YBR008c has been renamed FLR1, for fluconazole resistance 1. The expression of an FLR1-lacZ reporter construct is strongly induced by the overexpression of either CAP1 or YAP1, indicating that the FLR1 gene is transcriptionally regulated by the Cap1 and yAP-1 proteins. Taken collectively, our results demonstrate that FLR1 represents a new YAP1-controlled multidrug resistance molecular determinant in S. cerevisiae. A similar detoxification pathway is also likely to operate in C. albicans.

Cells have evolved elaborate molecular mechanisms to protect themselves from injuries caused by environmental exposure to toxic compounds of different structures and functions.

One of these mechanisms, termed multidrug resistance (MDR)1 or pleiotropic drug resistance (PDR), operates in a wide variety of cell types, including bacteria, protozoans, fungi, and mammalian cells (1–4). It involves a network of membrane-associated transporters acting as multidrug efflux pumps and transcription factors regulating the expression of these pumps.

MDR has been extensively studied in the yeast Saccharomyces cerevisiae (3). Yeast transporters involved in MDR belong to two superfamilies: the ATP-binding cassette (ABC) and the major facilitator (MF) superfamilies (3, 5–7). These transporters utilize ATP hydrolysis (ABC) or proton electrochemical gradient (MF) as energy sources for active transport (8, 9). Yeast ABC transporters implicated in MDR include: Pdr5 (also known as Sts1/Ydr1/Lem1) and Snq2, two structurally similar proteins with overlapping but distinct drug specificities (10–14); Yef1, a vacuolar glutathione-cadmium conjugate pump closely related to the mammalian multidrug resistance-associated and to the cystic fibrosis transmembrane regulator proteins (15–17); and Yor1, a Ycf1 homologue conferring resistance to oligomycin and reveromycin (18, 19). MDR transporters of the MF superfamily in S. cerevisiae include Atr1/Snq1 and Sge1, conferring resistance to aminothiazole and 4-NQO and to crystal violet, respectively (20–22). Several additional open reading frames (ORFs) predicted to code for ABC and MF transporters have been identified with the yeast genome sequencing project, but their role in MDR has yet to be determined (5–7).

Transcription factors such as Pdr1, Pdr3, and yAP-1 are also implicated in MDR (23–25). Pdr1 and Pdr3, two homologous zinc finger proteins belonging to the Zn,Cb binuclear zinc cluster family, have been shown to control the expression of the ABC transporters PDR5, SNQ2, and YOR1 (23, 24, 26–29). yAP-1 (also known as Pdr4/Snq3/Par1) is also associated with resistance to a variety of toxicants, including CYH, 4-NQO, sulfofemuron methyl, and cadmium, and with tolerance to oxidative stress induced by compounds such as hydrogen peroxide and diamide (30–37). yAP-1 belongs to a family of basic domain-leucine zipper (bZip) transcription factors, which include S. cerevisiae Gcn4 and mammalian Fos and Jun (25). It binds to a conserved DNA sequence termed the yAP-1 response element (YRE) within the promoter of its targets to activate their transcription (34). yAP-1 targets include the cadmium resistance ABC transporter gene YCF1 (15, 16), as well as a number of genes involved in response to oxidative stress such as GSH1, encoding γ-glutamylcysteine synthase (38); TRX2,
one of the two genes coding for thioredoxin (36); and GLR1 encoding glutathione reductase (39). A YAP1 homologue, Cad1/yAP-2, has been isolated based on its ability to confer resistance to cadmium and to 1,10-phenanthroline, but the target genes mediating this resistance are still unknown (33, 40).

*Candida albicans* is an opportunistic yeast that causes severe infections in immunocompromised individuals (41). Among the different agents employed in antifungal therapy, the azole derivative FCZ is the most widely used because of its low toxicity and its high efficacy (42). However, the successful treatment of candidosis by FCZ has been impaired by the emergence of drug resistant strains in patients undergoing long term or prophylactic treatment, mostly AIDS patients (42–44). A number of studies investigating the mechanisms of FCZ resistance in* C. albicans* and other *Candida* species have shown that resistant strains fail to accumulate FCZ due to an increased drug efflux, suggesting the participation of transporter-mediated drug resistance mechanisms in these strains (45–49). Two *C. albicans* genes coding for transporters of the ABC superfamily, *CDR1* and *CDR2*, have been recently isolated by complementation of an *S. cerevisiae* pdr5 ABC superfamily, increased drug efflux, suggesting the participation of transport-erased by capillary to a Zeta-Probe nylon membrane (Bio-Rad, Hercules, CA). Hybridization was performed with the University of Wisconsin Genetics Computer Group programs (58).

**CAP1 and YAPI Expression Plasmids**—The FosBgl and pF1 plasmids were used throughout this study for expression of the full-length and truncated versions of the *CAP1* gene and were renamed YEp352/CAP1 and YEp352/CAP1-TR, respectively. A 2.5-kb DNA fragment overlapping the entire YAPI gene (positions 349 to +2129 with respect to the initiation codon) (25) was amplified by polymerase chain reaction (PCR) using *S. cerevisiae* genomic DNA as template, the high-fidelity *Pfu* DNA polymerase (Stratagene), and oligonucleotides 5’-CCAGCTGTGAT-3’ and 5’-CGATAGTAGTCTA. The resulting PCR fragment was gel-purified and cloned blunt into YEp352 cut with *Sma*I to generate plasmid YEp352/YAPI.

**Drug Resistance Assays**—For microtiter plate assays, cells grown for 48 h on selective SD medium were resuspended in a saline solution (0.85%) to an A600 of 0.1. These cells were then diluted 100-fold in SD medium. The diluted cell suspensions were added to round-bottom 96-well microtiter plates (50 μl/well; in duplicate) in wells containing equal volumes (50 μl) of medium with different concentrations of the drug tested or in drug-free medium. The plates were incubated at 30 °C for 48 h. Cell growth was evaluated by reading the optical density at 650 nm in a microplate reader (Vmax™, Molecular Devices). The relative growth was calculated as the percentage of growth in drug-containing medium relative to the control growth in drug-free medium. The chosen concentrations were 10 μM for FCZ, 0.1 μM for CV, and 1 μM for 4-NQO, which correspond to the minimal inhibitory concentration of these drugs for wild-type strain MRY13–1A transformed with control plasmid YEp352. For the cadmium resistance spot assay, transformants were grown overnight in SD medium and approximately 10^6 cells were spotted onto a YPD plate containing 0 or 10 μM cadmium. The plates were incubated for 3 days at 30 °C. Resistance to hydrogen peroxide was tested essentially as described (35). Briefly, transformants were grown to saturation in SD medium and approximately 10^6 cells were spread on YPD plates. A filter paper disc was placed in the center of each plate, 10 μl of hydrogen peroxide (30% w/v; Aldrich) was added to the disc and the plates were incubated for 2 days at 30 °C before measuring the diameter of the zones of growth inhibition.

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**DNA Preparation and Northern Blot Analyses**—W303 cells transformed with plasmids YEp352, YEp352/CAP1, or YEp352/CAP1-TR were grown in SD–ura medium to an A600 of 2.0. Total RNA was extracted using the glass bead extraction method (59). RNA samples (20 μg) were electrophoresed on a 7.5% formaldehyde, 1% agarose gel and transferred to nylon membranes (Bio-Rad, Hercules, CA). The hybridizations were performed with [α-32P]-labeled DNA probes (60). The CAP1 probe was a 614-bp XbaI-HindII DNA fragment (positions +47 to +661) isolated from clone pF1A. The PDR5 probe was generated by PCR with primers 5’-CATACA-GAGCTCGAATC and 5’-CCACAGTTGACTGATTAGG and overlaps region +111 to +447 of the PDR5 gene (positions are relative to the translation initiation codon) (10). The YBR008w probe, which overlaps a region from −2 to 579 of the YBR008w ORF (GenBank Z55877), was amplified by PCR with primers 5’-CATACA-GAGCTCGAATC and 5’-CCACAGTTGACTGATTAGG and overlaps region +111 to +447 of the PDR5 gene (positions are relative to the translation initiation codon) (10). The YBR008w probe, which overlaps a region from −2 to 579 of the YBR008w ORF (GenBank Z55877), was amplified by PCR with primers 5’-
FIG. 1. Restriction map of a C. albicans genomic DNA clone conferring resistance to FCZ (pF1, top). The thick black lines represent the vector YEp352 (57), and the thin lines represent the genomic DNA insert. The ability of five pF1 subclones (pF1a, -b, -c, -a1, and -a2) to confer FCZ resistance is indicated (+, confers resistance; -, does not confer resistance). The restriction sites used to generate the different subclones are shown in pF1. The arrow on the pF1 map indicates the position of the initiator methionine at the beginning of the incomplete ORF (identified in pF1a). A 3.2-kb BglII fosmid fragment overlapping the complete ORF was subcloned into YEp352 at the BamHI site (FosBgl, bottom). The initiator methionine (arrow) and stop codon (•) are shown in FosBgl.

AGAAAAACGTACACCACAATCA (forward) and 5'-CGAGCTCTGGTGTG-TAGCCCTTATAGTCT (reverse). The PCR fragment was constructed with SacI (a SacI site was introduced at the 3' end of the reverse primer) and cloned into pGEM7Z (Promega, Madison, WI) cut with Smal and SacI, to yield plasmid pGEM7YBR008. The ybr008c deletion plasmid (pGEMYbr008cΔ::HIS3) was constructed by replacing a 1.6-kb Spel-HindIII fragment of YBR008c (corresponding to positions -316 to +1339 relative to the translation initiation codon) by a 0.9-kb Smal-PstI HIS3 fragment isolated from plasmid pJF215 (61). A linear 2.3-kb ybr008cΔ::HIS3 deletion fragment was excised with EcoRI and SacI and used to transform W303 α/α. His + transformants were selected and sporulated. haploid strains MRY13-1A (YBR008cΔ::) and MRY13-1B (ybr008cΔ::HIS3) were obtained as meiotic segregants of the heterozygous strain MRY13 (YBR008cΔ::HIS3). These strains were analyzed by Southern blotting to confirm their genotype at the YBR008c locus.

β-Galactosidase Assays—An FLR1(YBR008c)-lacZ fusion plasmid was constructed using a PCR fragment overlapping the promoter region, the translation initiation codon, as well as a short portion of the coding region of the FLR1 gene (positions -828 to +25). This PCR fragment was generated using oligonucleotides 5'-CGGATCCGCCTGGA-TAGCCCTTATAGTCT (forward) and 5'-CCAGCACTTCTCTAAGTTGACGTTA (reverse). The PCR fragment was digested with SacI and HindIII and used to transform W303 α/α. His + transformants were selected and sporulated. haploid strains MRY13-1A (YBR008cΔ::) and MRY13-1B (ybr008cΔ::HIS3) were obtained as meiotic segregants of the heterozygous strain MRY13 (YBR008cΔ::HIS3). These strains were analyzed by Southern blotting to confirm their genotype at the YBR008c locus.

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RESULTS
Isolation and Characterization of the C. albicans CAP1 Gene—Molecular genetic techniques involving the isolation of C. albicans genes by functional overexpression in S. cerevisiae provide a powerful approach for the study of drug resistance mechanisms. We have used this approach to search for C. albicans genes that mediate resistance to the azole derivative FCZ when overexpressed in S. cerevisiae. Strain W303 was transformed with a YEp352-based C. albicans genomic DNA library (56). Ura + transformants were pooled and plated on agar plates containing FCZ (500 μg/ml), a concentration that corresponds to 10 × the minimal inhibitory concentration for W303 cells transformed with vector YEp352 alone. Plasmids from 12 randomly chosen resistant colonies were purified and analyzed by restriction mapping, revealing that all the isolated plasmids carried an identical 6-kb genomic insert. One of these plasmids (pF1) was selected for further characterization (Fig. 1). Functional testing of different pF1 subclones indicated that a 3-kb fragment (pF1a) was sufficient to confer resistance (Fig. 1). DNA sequence analysis of pF1a revealed the presence of an incomplete ORF coding for a C-terminal truncated protein. To obtain a full-length clone for this ORF, a C. albicans fosmid library was screened with a pF1a subfragment as a probe, yielding five positive fosmids. A 3.2-kb BglII fosmid fragment hybridizing with the probe was further subcloned into plasmid YEp352 (57) at the BamHI site (Fig. 1). The resulting plasmid (FosBgl) was also functional in conferring FCZ resistance, as determined by agar plate assay (not shown). DNA sequence analysis of the 3.2-kb BglII genomic DNA fragment revealed that it contains an ORF of 499 amino acids (Figs. 1 and 2). This protein is rather hydrophilic, with an isoelectric point of 5.15 and a predicted molecular mass of 55 kDa.

A computer search of sequence data bases revealed that the closest homology to the cloned ORF was observed with the S. cerevisiae transcription factor YAP1 (37% identity, 55% overall homology) (25). The gene was thus named CAP1, for C. albicans AP-1. Other yeast proteins of the AP-1 family found to share a high degree of sequence similarity with the Cap1 protein are S. cerevisiae yap-2/Cad1 (30% identity, 49% homology) (33, 40) and Schizosaccharomyces pombe pap1, which confers resistance to staurosporine (27% identity, 43% homology) (65). The general structure of these proteins includes an N-terminal basic domain involved in DNA binding to target sequences, followed by a leucine zipper motif implicated in dimerization, and a C-terminal acidic domain with regulatory function (25, 33, 37, 40, 65, 66).

A multiple sequence alignment of Cap1 with its homologues was generated using the Pileup program (58); the two regions displaying the highest degree of sequence conservation are presented in Fig. 3. These regions correspond to the N-terminal basic domain followed by the leucine zipper motif (Fig. 3A) and to the C-terminal acidic domain (Fig. 3B). Within the AP-1 family of transcription factors, Cap1 belongs to a specific subgroup that includes yap-1, yap-2, and pap1, according to three criteria. First, its basic domain is located at the N terminus, unlike Gcn4, Pap1, or Jun in which the basic domain is located at the C terminus (67). The basic domain contains a short segment highly conserved among the four proteins, which overlaps two clusters of basic residues separated by an alanine spacer motif (Fig. 3A). This specific cluster-spacer-cluster organization, found in all bZip transcription factors, is required for specific DNA binding activity (67). Second, the third position of the heptad repeat of the leucine zipper motif, which is a leucine
the four proteins (Fig. 3B). In yAP-1, these cysteines are part of three repeated cysteine-serine-glutamate (CSE) sequence motifs recently shown to be required for normal regulation of yAP-1 in mediating oxidative stress response (OSR) (37). Given that the truncated Cap1 protein that lacks the C-terminal region is functional in conferring FCZ resistance (Fig. 1), this domain appears to be dispensable, at least for this specific activity of Cap1.

Functional Complementation of yap1 by CAP1—yAP-1 has been shown to mediate cellular resistance to a large number of toxic compounds, including cadmium and hydrogen peroxide (33–35). To determine if the structural homology observed between Cap1 and yAP-1 translates into functional homology, we expressed YAP1 and CAP1 in DJY125 cells, which carry a yap1 disruption (35), and compared the ability of these cells to grow in the presence of toxic concentrations of cadmium or hydrogen peroxide. To this end, DJY125 cells were transformed with plasmid YEPL552/YAP1 (carrying the entire YAP1 gene under the control of its promoter), YEPL552/CAP1 (full-length), YEPL552/CAP1-TR (truncated), or YEPL552 alone. The S150-2B parental wild-type strain (35) transformed with YEPL552 was also included as a control. These transformants were then analyzed by a cadmium resistance spot assay (Fig. 4) and by a hydrogen peroxide filter disc assay (Table I). When compared with S150-2B (YEPL552), DJY125 (YEPL552) displayed hypersusceptibility to both cadmium and hydrogen peroxide, as reported previously for a yap1 mutant (33, 35). As anticipated, overexpression of YAP1 in DJY125 was able to restore the ability of the cells to grow in the presence of cadmium (Fig. 4) or hydrogen peroxide (Table I). Overexpression of CAP1 in DJY125 was also able to restore the ability of the cells to grow in the presence of both compounds, although with a lower efficiency than YAP1 (Fig. 4 and Table I). Finally, DJY125 cells transformed with YEPL552/CAP1-TR were as susceptible to the two compounds as DJY125 cells carrying the vector alone (Fig. 4 and Table I). These results demonstrate that Cap1 and yAP-1 are functionally related and that the C-terminal domain of Cap1 is required to mediate resistance to cadmium and hydrogen peroxide in S. cerevisiae DJY125 cells.

Identification of the YBR008c ORF as a Potential Transcriptional Target of CAP1—It has been shown that YAP1 confers resistance to toxic compounds and to oxidative stress by transcriptionally activating a number of downstream target genes encoding effector proteins mediating the resistance (34, 36, 38, 39). It is thus likely that Cap1 mediates FCZ resistance in S. cerevisiae through transcriptional activation of similar effector genes. What could these effectors be? In C. albicans, overexpression of CDR1, CDR2, and BEN+ has been shown to be associated with FCZ resistance (47). This raised the possibility that homologues of these genes could be the CAP1 target(s) mediating FCZ resistance in S. cerevisiae.

To test this hypothesis, we generated gene-specific probes for PDR5, the closest S. cerevisiae homologue of CDR1 and CDR2 (10), and YBR008c, the closest BEN+ homologue in S. cerevisiae identified through a Blast search. As for BEN+, the YBR008c gene product is predicted to be a member of the MF superfamily (5, 7, 68). Total RNA was extracted from S. cerevisiae W303 cells transformed with plasmids YEPL552, YEPL552/CAP1, or YEPL552/CAP1-TR. RNA samples were analyzed by Northern blotting using a CAP1 (Fig. 5A), a PDR5 (Fig. 5B), or a YBR008c (Fig. 5C) radiolabeled DNA fragment as probe. The membranes were hybridized concurrently with a LEU2 probe as a control (61). RNA analysis with the CAP1 probe showed that the full-length CAP1 gene was expressed at high levels in S. cerevisiae, giving rise to two RNA transcripts of 2.2 and 1.9 kb (Fig. 5A, lane 2), which were absent from the W303 control.
Fig. 3. Multiple sequence alignment of the N- and C-terminal domains of Cap1 with those of its closest homologues. *S. cerevisiae* yAP-1 (25) and yAP-2 (33, 40) and *S. pombe* pap1 (65) are shown. Identical (*) and conserved (•) residues are indicated. A, alignment of the basic regions followed by the leucine zipper motifs. The two clusters of basic residues (hatched boxes) and the alanine spacer motif (stippled box) are shown (67). Residues corresponding to the leucine zipper motif are underlined. B, Alignment of the C-terminal regions. The conserved cysteine residues are boxed.

![Image](86x291 to 270x434)

**A**

| lane 1 | lane 2 | lane 3 |
|--------|--------|--------|
| Cap1   | yAP-1  | yAP-2  |
| 26     | 45     | 24     |
| pap1   |        |        |
| 51     |        |        |

**B**

| lane 1 | lane 2 | lane 3 |
|--------|--------|--------|
| Cap1   | yAP-1  | yAP-2  |
| 425    | 576    | 354    |
| pap1   |        |        |
| 479    |        |        |

**YBR008c** is Essential for **CAP1** and **yAP1**-mediated **FCZ** Resistance—To determine whether **YBR008c** was this effector, we constructed a yeast strain carrying a chromosomal deletion of this gene. To this end, a chimeric gene was generated in which a 1.6-kb *Seq*-**HindIII** fragment overlapping the ATG initiator codon and most of the coding region of **YBR008c** was replaced with the **HIS3** gene, and was introduced into the isogenic diploid strain W303. A selected His⁺ transformant carrying one wild-type **YBR008c** and one disrupted **YBR008cΔ**:**HIS3** allele (as confirmed by Southern blotting analysis; data not shown) was sporulated. Tetrad dissection gave rise to viable **ybr008cΔ**:**HIS3** spores, demonstrating that **YBR008c** is not essential for growth. Two sister spores, **MYR13–1A** (**YBR008c**+) and **MYR13–1B** (**ybr008cΔ**), were selected for the following resistance studies.

Strains **MYR13–1A** and **MYR13–1B** were transformed with plasmids **YPE352**, **YPE352/YAP1**, **YPE352/CAP1**, and **YPE352/CAP1-TR**. These transformants were tested for their ability to grow in the presence or absence of 50 μg/ml **FCZ** (the minimal inhibitory concentration for wild-type strain **MYR13–1A** transformed with control plasmid **YPE352**), using a microtiter plate assay (Fig. 6A).

We found that the three genes (**YAP1**, **CAP1**, and **CAP1-TR**) were able to confer FCZ resistance when expressed in the wild-type **YBR008c** strain, demonstrating that the ability of **CAP1** to mediate FCZ resistance in *S. cerevisiae* is not restricted to the *C. albicans* gene but also extends to its *S. cerevisiae* homologue. This finding further expands the spectrum of drugs to which **YAP1** has been shown to confer resistance. Interestingly, the full-length **Cap1** protein was approximately 3 times less active than the truncated **Cap1** protein in this assay, indicating that the C-terminal domain of **Cap1** behaves as an inhibitor of **Cap1** activity with respect to the transcriptional regulation of **YBR008c** in *S. cerevisiae* (Fig. 6A).

Unlike **MYR13–1A** cells however, **MYR13–1B** cells transformed with plasmids **YPE352/YAP1**, **YPE352/CAP1**, or **YPE352/CAP1-TR** were completely unable to grow in FCZ, exhibiting a relative growth value identical to that of the **YPE352** control transformants (Fig. 6A). These results demon-

![Image](240x446 to 558x729)

**Fig. 4. Functional complementation of yap1 by cap1 for cadmium resistance.** DJY125 cells (35) transformed with plasmid **YPE352**, **YPE352/YAP1**, **YPE352/CAP1**, or **YPE352/CAP1-TR** and **S150-2B** cells (35) transformed with control plasmid **YPE352** were tested for resistance to cadmium by a spot test growth inhibition assay. Transformants were grown overnight in SD ura medium. Approximately 10⁴ cells were spotted onto a YPD plate containing 10 μM cadmium (+ **CdSO₄**) or no cadmium (– **CdSO₄**). The plates were incubated for 3 days at 30 °C.
Regulation of an MFS MDR Transporter by YAP1 and CAP1

Table I

Functional complementation of yap1 by CAP1 for resistance to hydrogen peroxide

DJY125 cells (35) transformed with plasmid YEp352, YEp352/YAP1, YEp352/CAP1, or YEp352/CAP1-TR and S150–2B cells (35) transformed with YEp352 were tested for their level of resistance to hydrogen peroxide. Strains were grown to saturation in SD–ura medium, and approximately 1 × 10⁶ cells were plated on YPD plates. A filter paper disc was placed in the center of each plate, 10 μl of H₂O₂ (30%) was added to the disc, and the plates were incubated for 2 days at 30 °C. The diameters of the clear zones were measured (cm). The data shown represent the means of three independent experiments performed in triplicate.

| Strains                                      | Diameter of zone of growth inhibition (cm) |
|---------------------------------------------|------------------------------------------|
| DJY125 (YEp352)                             | 5.2 ± 0.3                                |
| DJY125 (YEp352/YAP1)                        | 2.4 ± 0.1                                |
| DJY125 (YEp352/CAP1)                        | 3.9 ± 0.3                                |
| DJY125 (YEp352/CAP1-TR)                     | 5.1 ± 0.3                                |
| S150–2B (YEp352)                            | 2.2 ± 0.2                                |

Fig. 5. Northern analysis of potential CAP1 targets in S. cerevisiae. Total RNA was extracted from W303 a/a cells transformed with plasmid YEp352 (lanes 1), YEp352/CAP1 (lanes 2), or YEp352/CAP1-TR (lanes 3). RNA samples (20 μg) were separated in triplicate by electrophoresis on an agarose gel, transferred to a nylon membrane, and probed with a CAP1 (A), a PDR5 (B), or a YBR008c (C) radiolabeled fragment. A probe for the LEU2 gene (61) was used simultaneously with each probe as an internal control to monitor RNA loading and transfer. Autoradiography was for 24 h. The positions of the 26 S (3415 nt) and 18 S (1788 nt) ribosomal RNAs are indicated on the left. The lower band corresponds to the LEU2 transcript (1350 nt).

Fig. 6. YBR008c is essential for YAP1- and CAP1-mediated multidrug resistance. MRY13–1A (YBR008c; light grey) or MRY13–1B (ybr008c::HIS3; black) cells transformed with plasmid YEp352, YEp352/YAP1, YEp352/CAP1, or YEp352/CAP1-TR were grown in a 96-well microtiter plate at 30 °C in SD–ura medium containing (A) FCZ (50 μg/ml), (B) CYH (0.1 μg/ml), (C) 4-NQO (1 μM), or in drug-free medium. The cell growth, evaluated after 48 h by reading the optical density at 650 nm in a microplate reader, is presented as the percentage of growth in drug-containing medium relative to the control growth in drug-free medium. Values reported represent the average of three (A) or two (B and C) independent experiments performed in duplicate.

C. albicans results in hypersusceptibility to these two compounds (47, 53, 70). It was thus of interest to investigate if FLR1 was the YAP1 effector mediating CYH and 4-NQO resistance in S. cerevisiae.

To this end, strains MRY13–1A and MRY13–1B transformed with plasmids YEp352, YEp352/YAP1, YEp352/CAP1, and YEp352/CAP1-TR were examined for their ability to grow in the presence of CYH or 4-NQO (Fig. 6, B and C). We found that overexpression of the three genes (YAP1, CAP1, and CAP1-TR) in the wild-type FLR1 strain resulted in resistance to both CYH and 4-NQO (Fig. 6, B and C). As observed for FCZ (Fig. 6A), the full-length CAP1 gene was much less active than the truncated CAP1 in conferring resistance to these two compounds (Fig. 6, B and C). MRY13–1B cells transformed with plasmids YEp352/YAP1, YEp352/CAP1, or YEp352/CAP1-TR were as susceptible to CYH as the YEp352 control transformants (Fig. 6B), indicating that FLR1 is the only YAP1 target mediating CYH resistance in S. cerevisiae. In contrast to FCZ and CYH, however, MRY13–1B cells transformed with
lacZ fusion gene was constructed, consisting of 828 bp of 5\' noncoding sequence and 25 bp from the YAP1 and is one of the target genes for the shown YREs is presented below.

YEp352/YAP1 retained a significant level of residual resistance to 4-NQO (Fig. 6C), revealing the participation of other YAPI-controlled molecular determinant(s) of 4-NQO resistance in S. cerevisiae besides FLR1. Although almost as active as YAPI in conferring resistance to 4-NQO in FLR1 wild-type cells, the truncated CAP1 gene was unable to induce the expression of these other 4-NQO resistance determinants, as no residual resistance was observed in MRY13–1B (YEp352/CAP1-TR) as compared with MRY13–1B (YEp352/YAP1) transformants (Fig. 6C). Taken together, these results demonstrate that FLR1 is the only YAPI target mediating resistance to FCZ and CYH and is one of the YAPI targets conferring resistance to 4-NQO. Thus, we can conclude that FLR1 represents a new YAPI-controlled multidrug resistance molecular determinant in S. cerevisiae.

**FLR1 Is a Transcriptional Target for Proteins of the yAP-1 Family**

To determine whether FLR1 is under the transcriptional control of the yAP-1 and Cap1 proteins, we assessed their ability to activate transcription of a lacZ reporter gene under the control of the FLR1 promoter. To this end, an FLR1-lacZ fusion gene was constructed, consisting of 828 bp of 5\' noncoding sequence and 25 bp from the FLR1 gene fused to E. coli lacZ in plasmid YEp368 (62). This FLR1-lacZ fusion gene was then introduced into MRY13–1A cells carrying plasmids YE352/YAPI, YE352/CAP1, YE352/CAP1-TR, or YE352. The \( \beta \)-galactosidase activities were determined from the resulting cotransformants to measure the effect of these plasmids on the expression of the FLR1-lacZ fusion gene (Fig. 7). Presence of the FLR1-lacZ construct in cells carrying the control plasmid YE352 gave rise to 0.05 units of \( \beta \)-galactosidase activity, a small but significant value since use of a control YEp368 vector lacking the FLR1 promoter did not result in any measurable \( \beta \)-galactosidase activity in the same assay (data not shown). When the FLR1-lacZ plasmid was introduced in cells transformed with plasmid YE352/YAPI, a \( \beta \)-galactosidase activity of 5.2 units was obtained, representing an approximate 100-fold increase as compared with the value measured in the control YE352 transformants. This result is consistent with the proposition that FLR1 is under the transcriptional control of YAPI in S. cerevisiae. A similar level of \( \beta \)-galactosidase activity was observed with cells transformed with the YE352/CAP1 plasmid (5.7 units), confirming the functional relatedness between yAP-1 and Cap1. Finally, the \( \beta \)-galactosidase activity measured in cells transformed with the YE352/CAP1-TR plasmid was approximately 2-fold lower than that measured in cells transformed with YE352/CAP1 (3.0 units), indicating that the truncated Cap1 protein is less active than the full-length Cap1 protein in this transactivation assay. These results were unexpected, in light of our data showing that the truncated Cap1 protein was more active than full-length Cap1 in conferring FCZ, CYH, and 4-NQO resistance (Fig. 6). Nevertheless, these data show that both YAPI and Cap1 are capable of stimulating FLR1 transcription and provide evidence that this control is mediated through the FLR1 promoter.

These results prompted us to scan the promoter region of the FLR1 gene for the presence of a DNA sequence motif similar to previously identified YREs (34). Our analysis revealed that the FLR1 promoter indeed contains three potential YREs of sequence 5\'-TTAGTA/CA, one on the coding strand (position -149 with respect to the translation initiation codon at +1) and two on the anticoding strand (positions -161 and -358) (Fig. 8). Functional YREs of similar sequence have been shown to be present in the promoter of the YAPI target genes GSH1, YCF1, TRX2, and GLR1 (Fig. 8) as well as in the SV40 early enhancer element (34, 36, 38, 39, 71). A detailed functional analysis of the FLR1 promoter is currently under way to determine whether the control of FLR1 transcription by yAP-1 is direct or indirect and whether it is mediated through the YREs present in the promoter.

**DISCUSSION**

Investigation of C. albicans molecular determinants of FCZ resistance has allowed us to isolate a gene coding for a new member of the yeast AP-1 family that we have named \( \text{CAP1} \). A computer search of protein data bases identified S. cerevisiae \( \text{yAP-1} \) as being the closest homologue of the Cap1 protein (25). Other yeast proteins also found to share a high degree of sequence similarity with Cap1 are S. cerevisiae \( \text{yAP-2/Cad1} \) and S. pombe \( \text{pap1} \) (33, 40, 65). These proteins are apparently not only structurally but also functionally related, since their overexpression from a multicopy plasmid results in a similar phenotype, namely an increased cellular resistance to different toxicants (31, 33, 40, 65, 69). More specifically, we show that the sequence homology between the Cap1 and \( \text{yAP-1} \) proteins translates into functional homology, because (a) \( \text{CAP1} \) expression in a \( \text{yAP-1} \) mutant partially restores the ability of the cells to grow on otherwise toxic concentrations of cadmium or hydrogen peroxide (Fig. 4 and Table I) and (b) both \( \text{yAP-1} \) and Cap1 can transactivate FLR1 to confer MDR (Figs. 6 and 8). \( \text{yAP-1} \) is the best studied member of this family of fungal transactivators. In addition to its role in drug resistance, \( \text{yAP-1} \) has been shown to participate in OSR via the transcriptional activation of a spectrum of targets including GSH1, encoding FLR1, GSH1, YCF1, TRX2, and GLR1. Numbers refer to the position of the first nucleotide of the motif with respect to the adenine of the translation initiation codon (position +1). YREs are found either on the coding (+) or on the anticoding (−) strand. A consensus sequence for the shown YREs is presented below.
γ-glutamylcysteine synthase (38); TRX2, one of the two genes coding for thioredoxin (36); and GLR1, encoding glutathione reductase (39). Therefore, it is quite possible that Cap1 serves similar functions in C. albicans by transactivating a number of effector genes homologous to those controlled by yAP-1 in S. cerevisiae. The availability of the cloned CAP1 gene will now allow an analysis of its role in C. albicans with respect to both MDR and OSR.

An amino acid sequence comparison of the Cap1, yAP-1, yAP-2, and pap1 proteins indicates that the most conserved regions among these proteins overlap the N-terminal bZIP and the C-terminal acidic domains (Fig. 3). A bZIP domain is conserved in a large number of transcription factors of the AP-1 family, including S. cerevisiae Gen4 and mammalian Jun, Fos, C/EBP, and CREB, and its function in DNA binding and in protein dimerization has been extensively studied (67). However, the role of the C-terminal domain, which is present only in the yeast AP-1 subfamily of transcription factors, is much less understood. A short segment of approximately 60 amino acids located at the extreme C terminus contains a number of highly conserved residues, including three invariably conserved cysteines (33, 40, 65) (Fig. 3B). In yAP-1, these cysteines are part of three repeated cysteine-serine-glycylate (CSE) sequence motifs recently shown to be required for normal regulation by yAP-1 in mediating oxidative stress tolerance (37). It has been shown that truncated yAP-1, yAP-2, and pap1 proteins lacking the C-terminal domain are still functional in conferring resistance to cadmium, to 1,10-phenanthroline, and to staurosporine, respectively, indicating that this domain is dispensable at least for these specific phenotypes (40, 65, 66). During the course of our work, we have isolated a genomic DNA fragment carrying an incomplete version of the CAP1 gene coding for a truncated protein lacking the entire C-terminal domain (Fig. 1). On the one hand, we find that the truncated Cap1 protein is active in conferring FLR1-mediated resistance to FCZ, CYH, and 4-NQO upon overexpression in a wild-type strain (Fig. 6). On the other hand, when tested for its ability to suppress a yap1 mutation, the truncated Cap1 protein is completely inactive in restoring tolerance to cadmium and hydrogen peroxide (Fig. 4 and Table I). A potential explanation for these two different phenotypes is that the truncated Cap1 protein requires the presence of yAP-1 to bring about efficient transcriptional activation. Alternatively, it is possible that the C-terminal domain of Cap1 selectively modulates the protein activity, displaying either an inhibitory or an activating function depending on the transcriptional target. The fact that the truncated Cap1 protein (a) is active in inducing FLR1-dependent 4-NQO resistance but inactive in inducing FLR1-independent 4-NQO resistance (Fig. 6C) and (b) is able to confer FCZ resistance in yap1 DJY125 cells (data not shown) supports the second hypothesis. Finally, we find that the truncated Cap1 protein, which is more active than the full-length protein in conferring MDR, is less active than full-length Cap1 in transactivating an FLR1-lacZ fusion gene (compare Figs. 6 and 7).

One possible reason for this discrepancy may be that drugs, present in the drug resistance assay and absent from the transactivation assay, differentially modulate the activity of the two forms of Cap1. As C. albicans genes can behave as mutants when expressed in S. cerevisiae (72), it will be important to study the effect of the Cap1 C-terminal truncation in C. albicans and/or to test the effects of a similar deletion in yap1 on its ability to transactivate its different targets in S. cerevisiae to be able to draw definitive conclusions from these observations.

Several ORFs predicted to code for MDR transporters of the MF superfamily (MFS) based on structural considerations have been identified during the yeast genome sequencing project but their role in MDR remains to be determined (5, 7). Based on hydropathy analyses, these ORFs have been grouped into three clusters: members of cluster I (12 ORFs) are predicted to contain 12 TM domains, whereas members of cluster II (10 ORFs) and of cluster III (6 ORFs) are predicted to contain 14 TM domains (5, 7). So far, only two transporters of the MFS in S. cerevisiae have been demonstrated to function as MDR determinants, namely Atr1 (4-NQO and aminotriazole resistance) and Sge1 (crystal violet and ethidium bromide resistance), which both belong to cluster II (reviewed in Ref. 7). Our data, showing that YAP1-mediated FLR1 overexpression in S. cerevisiae results in cellular resistance to FCZ, CYH, and 4-NQO, demonstrates that FLR1 indeed functions as an MDR determinant (Fig. 6). Furthermore, overexpression of FLR1 from the strong constitutive glyceraldehyde-3-phosphate dehydrogenase promoter in S. cerevisiae results in high levels of resistance to these three compounds as well as to benomyl and 1,10-phenanthroline, confirming that FLR1 is an MDR determinant with broad substrate specificity.2 FLR1 (YBR008c) is predicted to code for an integral membrane protein with 12 potential transmembrane segments belonging to cluster I (5, 7). Interestingly, MFS proteins known to function as MDR efflux pumps in other yeasts also belong to cluster I, including C. albicans BEN, Candida maltosa CYHR, and S. pombe CAR1 (7). It will be of interest to determine whether other MFS MDR ORFs, identified based on structural considerations by the systematic analysis of the yeast genome, actually function as MDR determinants and whether some of them are under the transcriptional control of YAP1 as part of a yet unidentified MDR network in S. cerevisiae (see below).

A number of observations indicate that FLR1 expression is under the control of AP-1 proteins. First, the steady state level of FLR1 RNA is drastically increased in CAP1 and CAP1-TR transformants (Fig. 5C). Second, YAP1- and CAP1-mediated multidrug resistance in a wild-type strain is completely abrogated (FCZ and CYH) or strongly reduced (4-NQO) in an flr1 null mutant strain (Fig. 6). Third, the FLR1 promoter contains three DNA sequence elements matching a functional YRE (Fig. 8). Fourth, the FLR1 promoter fused to a reporter gene is strongly transactivated by the overexpression of YAP1 or CAP1 (Fig. 7). Overexpression of YAP1 in S. cerevisiae had been previously shown to confer resistance to different toxic compounds including CYH and 4-NQO, but the target(s) of yap1-1 mediating this resistance had so far remained unidentified (31, 69). The results presented here clearly identify FLR1 as being the YAP1 effector mediating CYH resistance (in addition to FCZ resistance) and one of the YAP1 effectors conferring resistance to 4-NQO. Taken collectively, these results allow us to conclude that FLR1 represents a new YAP1-controlled MDR molecular determinant in S. cerevisiae. This widens the spectrum of already known yap1 physiological targets to include an MFS MDR transporter and further substantiates that yap1-1 is a key regulator molecule involved in conferring MDR in addition to OSR. It also raises the possibility that yap1 regulates additional unidentified targets involved in MDR. Our results showing residual YAP1-mediated resistance to 4-NQO in an flr1 null mutant strain confirms this hypothesis (Fig. 6C).

Such YAP1 target(s) mediating 4-NQO resistance could well be involved in MDR. Our observations also indicate that FLR1 expression is under the control of AP-1 proteins. First, the steady state level of FLR1 RNA is drastically increased in CAP1 and CAP1-TR transformants (Fig. 5C). Second, YAP1- and CAP1-mediated multidrug resistance in a wild-type strain is completely abrogated (FCZ and CYH) or strongly reduced (4-NQO) in an flr1 null mutant strain (Fig. 6). Third, the FLR1 promoter contains three DNA sequence elements matching a functional YRE (Fig. 8). Fourth, the FLR1 promoter fused to a reporter gene is strongly transactivated by the overexpression of YAP1 or CAP1 (Fig. 7). Overexpression of YAP1 in S. cerevisiae had been previously shown to confer resistance to different toxic compounds including CYH and 4-NQO, but the target(s) of yap1-1 mediating this resistance had so far remained unidentified (31, 69). The results presented here clearly identify FLR1 as being the YAP1 effector mediating CYH resistance (in addition to FCZ resistance) and one of the YAP1 effectors conferring resistance to 4-NQO. Taken collectively, these results allow us to conclude that FLR1 represents a new YAP1-controlled MDR molecular determinant in S. cerevisiae. This widens the spectrum of already known yap1 physiological targets to include an MFS MDR transporter and further substantiates that yap1-1 is a key regulator molecule involved in conferring MDR in addition to OSR. It also raises the possibility that yap1 regulates additional unidentified targets involved in MDR. Our results showing residual YAP1-mediated resistance to 4-NQO in an flr1 null mutant strain confirms this hypothesis (Fig. 6C).

Such YAP1 target(s) mediating 4-NQO resistance could well include the ABC transporter SNQ2 and/or the major facilitator ATR1, as both genes have been shown to confer resistance to 4-NQO and to contain YRE motifs in their promoter regions (14, 20, 21, 73). Experiments are currently under way to test this proposition.

2 N. Mainville, I. Balan, and M. Raymond, unpublished data.
C. albicans strains can develop clinical resistance to FCZ in individuals undergoing long term therapy or prophylactic treatment, mostly AIDS patients (42–44). Two major mechanisms of FCZ resistance have been identified so far in these strains: (a) alterations in the drug target (14-a-sterol demethylase), the product of the ERG16 gene, resulting in an increased production of the enzyme or in its reduced binding affinity for FCZ (reviewed in Refs. 74 and 75); and (b) a reduced intracellular FCZ accumulation, which usually correlates with the overexpression of the ABC transporter genes CDR1 and CDR2 and of the MF transporter BEN*, (47, 51); this latter mechanism appears to be the most important. The molecular events up-regulating the expression of these transporters in C. albicans are not known. It has been shown by Northern blot analyses of C. albicans clinical isolates that CDR1 is constitutively expressed at low levels in FCZ-susceptible strains, while CDR2 and BEN* are not expressed in these strains, at least at levels detectable by this technique (47, 51). However, these genes become markedly overexpressed in FCZ-resistant isolates, which can exhibit either CDR1, concomitant CDR1 and CDR2, or BEN* overexpression (47, 48, 51), suggesting the participation of transporter-specific transactivating factors in the establishment of FCZ resistance in these isolates. In S. cerevisiae, the expression of PDR5, a close homologue of CDR1 and CDR2, is under the transcriptional control of the zinc finger proteins PDR1 and PDR3 (27); it is thus possible that PDR1 and PDR3 homologues in C. albicans also operate to up-regulate CDR1 and CDR2 expression. On the other hand, our isolation of a YAP1 homologue from C. albicans together with our demonstration that both Yap1 and Cap1 transcriptionally control the BEN* homologue FLR1 in S. cerevisiae suggest that Cap1 could be involved in controlling BEN* expression to cause FCZ resistance in C. albicans. We have found that overexpressing Cap1 (the truncated form) in C. albicans CAG4 cells results in BEN* overexpression and in FCZ resistance, confirming that indeed Cap1 participates in the transcriptional control of BEN* in C. albicans.3 What could be the molecular events leading to BEN* transcriptional activation? FCZ resistance in strains overexpressing BEN* is a stable phenotype, suggesting that FCZ resistance in these strains is due to genetic alterations rather than to transient cellular adaptations (47, 48). Such permanent alterations potentially include dominant activations rather than to transient cellular adaptations (47, 48).

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