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Transcriptional Activation and Increased mRNA Stability Contribute to Overexpression of *CDR1* in Azole-Resistant *Candida albicans*[▽]

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Many azole-resistant (AR) clinical isolates of *Candida albicans* display increased expression of the drug transporters *CDR1* and *CDR2*. In this study, we evaluate the molecular mechanisms that contribute to the maintenance of constitutively high *CDR1* transcript levels in two matched pairs of azole-susceptible (AS) and AR clinical isolates of *C. albicans*. To address this, we use reporter constructs of *GFP* and *lacZ* fused either to the *CDR1* promoter (P_{CDR1}-*GFP/lacZ*; transcriptional fusion) or to the *CDR1* open reading frame (P_{CDR1}-*CDR1-GFP/lacZ*; translational fusion) integrated at the native *CDR1* locus. It is observed that expression of the two reporter genes as a transcriptional fusion in the AR isolates is higher than that in matched AS isolates. However, the difference in the reporter activity between the AS and AR isolates is even greater for the translational fusions, indicating that the sequences within the *CDR1* coding region also contribute to its increased expression in AR isolates. Further analysis of these observations by transcription run-on assays demonstrated a ~5- to 7-fold difference in the transcription initiation rates for the AR isolates from those for their respective matched AS isolates. Measurement of mRNA stability showed that the half-life of *CDR1* mRNA in the AR isolates was threefold higher than that in the corresponding AS isolates. Our results demonstrate that both increased *CDR1* transcription and enhanced *CDR1* mRNA stability contribute to the overexpression of *CDR1* in AR *C. albicans* isolates.

Resistance of the human fungal pathogen *Candida albicans* to azole antifungals is often caused by increased expression of genes encoding multidrug efflux pumps, the ATP-binding cassette transporter genes *CDR1* and *CDR2* and/or the major facilitator gene Ca*MDR1* (1, 7, 23, 29, 34, 36, 37, 38, 44, 45, 46). However, the molecular mechanisms leading to the constitutive overexpression of efflux pump-encoding genes in drug-resistant, clinical *C. albicans* isolates are only beginning to be understood. In particular, the regulation of *CDR1* expression has been studied by many groups (2, 3, 4, 5, 10, 11, 17, 20, 32). Various unrelated stresses, including elevated temperature or the presence of drugs and steroids, induce a transient transcriptional activation of *CDR1* in drug-susceptible *C. albicans* strains (20). Several *cis* elements in the *CDR1* upstream region that affect basal as well as induced *CDR1* expression have been identified. Puri et al. (32) identified four upstream activating sequences and four upstream repressing sequences in the 5′ noncoding region of *CDR1*. A basal regulatory element and a negative regulatory element, in the proximal region of the promoter, have also been characterized and implicated in the basal expression of *CDR1* (10, 11). A specific steroid-responsive region in the distal part of the *CDR1* promoter, consisting of two progesterone-responsive sequences and one β-estradiol-responsive sequence, has been shown to contribute exclusively to steroid responsiveness of *CDR1* (17). Another basal expression element in the *CDR1* upstream region and a *d*rug *r*esponse *e*lement (DRE), which is present in the upstream region of *CDR1* and *CDR2*, have been identified by de Micheli et al. (5). The DRE was found to mediate both the transient upregulation of *CDR1* and *CDR2* by steroid hormones and drugs and their constitutive overexpression in a resistant strain (5).

Coste et al. identified a transcription factor, *TAC1* (*t*ranscriptional *a*ctivator of *CDR* genes), that binds to the DRE in the *CDR1* and *CDR2* promoters (3). Inactivation of *TAC1* resulted in the loss of fluphenazine-induced upregulation of *CDR1* and *CDR2*, with little impact on basal expression levels, and also abrogated the constitutive overexpression of these efflux pumps in a drug-resistant strain (3, 4, 5). CaNdt80p, a homolog of the meiosis-specific transcription factor Ndt80p of *Saccharomyces cerevisiae*, is another positive regulator of *CDR1*. Deletion of Ca*NDT80* abolished the induced expression of *CDR1* and increased the sensitivity of *C. albicans* to antifungals (2). Interestingly, the global repressor CaTup1p acts as a negative regulator of *CDR1* expression (26, 48).

Although transcriptional regulation is considered to be the key step accounting for complex basal and induced patterns of *CDR1* expression, the possibility of posttranscriptional control of *CDR1* expression, which could also affect drug resistance, still remains to be explored. The large amounts of Cdr1p, which correlate with high *CDR1* mRNA levels, in azole-resistant (AR) *C. albicans* strains not only may be due to increased

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CDR1 transcription but could also be caused by increased stability of its mRNA and protein. It is therefore of interest to compare the following: (i) *CDR1* transcription initiation rates, (ii) *CDR1* mRNA stability, and (iii) Cdr1 protein stability in drug-susceptible and *CDR1*-overexpressing, drug-resistant *C. albicans* strains. In this study, we have addressed these issues by exploiting two pairs of matched azole-susceptible (AS) and *CDR1*-overexpressing, AR isolates. By using transcriptional and translational reporter gene fusions, transcriptional run-on (TRO), thiolutin, and cycloheximide chase assays, we demonstrate that *CDR1* overexpression in *C. albicans* is caused by an increase in its transcriptional initiation rate and by increased mRNA stability.

TABLE 1. *C. albicans* strains used

Strain	Description	Reference
Gu4	Fluconazole-susceptible clinical isolate	Franz et al. (6)
Gu4G1	P_{CDR1}-*GFP* integrated at *CDR1* locus	This study
Gu4G2	P_{CDR1}-*CDR1-GFP* integrated at *CDR1* locus	This study
Gu4L2	P_{CDR1}-*lacZ* integrated at *CDR1* locus	This study
Gu4L3	P_{CDR1}-*CDR1-lac*Z integrated at *CDR1* locus	This study
Gu5	Fluconazole-resistant clinical isolate	Franz et al. (6)
Gu5G1	P_{CDR1}-*GFP* integrated at *CDR1* locus	This study
Gu5G2	P_{CDR1}-*CDR1-GFP* integrated at *CDR1* locus	This study
Gu5L2	P_{CDR1}-*lacZ* integrated at *CDR1* locus	This study
Gu5L3	P_{CDR1}-*CDR1-lacZ* integrated at *CDR1* locus	This study
DSY294	Fluconazole-susceptible clinical isolate	Sanglard et al. (38)
DSY294G1	P_{CDR1}-*GFP* integrated at *CDR1* locus	This study
DSY294G2	P_{CDR1}-*CDR1-GFP* integrated at *CDR1* locus	This study
DSY294L2	P_{CDR1}-*lacZ* integrated at *CDR1* locus	This study
DSY294L3	P_{CDR1}-*CDR1-lacZ* integrated at *CDR1* locus	This study
DSY296	Fluconazole-resistant clinical isolate	Sanglard et al. (38)
DSY296G1	P_{CDR1}-*GFP* integrated at *CDR1* locus	This study
DSY296G2	P_{CDR1}-*CDR1-GFP* integrated at *CDR1* locus	This study
DSY296L2	P_{CDR1}-*lacZ* integrated at *CDR1* locus	This study
DSY296L3	P_{CDR1}-*CDR1-lacZ* integrated at *CDR1* locus	This study

MATERIALS AND METHODS

Materials. Medium chemicals were obtained from HiMedia (Mumbai, India). Restriction endonuclease, DNA-modifying enzymes, ultra-pure deoxyribonucleotides (dATP, dGTP, dCTP, and dTTP) for PCR, and ribonucleotides (ATP, CTP, GTP, and UTP) for TRO were purchased from New England Biolabs (NEB, Inc.). Thiolutin (CP-4092) was a generous gift from Pfizer, Inc., Groton, CT. Radiolabeled [5,6-^{3}H]uridine, [α-^{32}P]dATP, and [α-^{32}P]UTP were obtained from Amersham Biotech and Bhabha Atomic Research Center, India. Polyclonal anti-Cdr1p antibody was custom synthesized from Covance Research Products, Inc., PA. Oligonucleotides used were commercially synthesized from Sigma-Aldrich. All molecular biology-grade chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO), and routinely used chemicals (Tris, sodium chloride, glycine, potassium acetate, sodium carbonate, magnesium chloride, sodium hydroxide pellets, methanol, glacial acetic acid, urea, sodium dodecyl sulfate [SDS], formamide, and ethanol) were obtained from Qualigens and Merck, Mumbai, India.

Bacterial and yeast strains and growth media. *Escherichia coli* DH5α was used as a host for plasmid constructions and propagation. *C. albicans* strains used in this study are listed in Table 1. *C. albicans* strains were maintained on yeast extract-peptone-dextrose (YEPD) medium. All strains were stored as frozen stocks with 15% glycerol at −80°C. Before each experiment, cells were freshly revived on YEPD plates from this stock.

Plasmid construction. All primers and plasmids used in this study are listed in Table 2. Plasmids pCPL51 and pCPG3, harboring the P_{CDR1}-*lacZ* and P_{CDR1}-*GFP* transcriptional fusions, were constructed as follows. A *CDR1* promoter fragment was first amplified from genomic DNA of isolate Gu5 with the primers CDR1F and CDR1R, digested at the introduced ApaI and XhoI sites, and substituted for the *CDR1* promoter fragment in the previously described plasmid pCPL1 (11) to generate pCPL5. A *CDR1* downstream fragment was then amplified from genomic DNA of strain SC5314 with the primers CDR29 and CDR30. The PCR product was digested at the introduced PstI and SacII sites and ligated with the PstI/SacII-digested plasmid vectors pCPL5 and pCPG1 (11) to generate pCPL51 and pCPG3, respectively. Notably, plasmid pCPG1 harbors the *CDR1* promoter isolated from SC5314 genomic DNA, which has been used in our previous studies (11). The SC5314-derived *CDR1* promoter showed sequence differences from the *CDR1* promoters in the matched AS or AR isolates (at nucleotide positions −21, −150, −171, −215, −238, −315, −368, −381, −418, and −455 relative to the transcription start point) which have been used in this study (unpublished observation). These differences, however, did not affect the β-galactosidase reporter activity of integrated cassettes derived either from pCPL1 (11), which harbors the SC5314 *CDR1* promoter, or from pCPL4 or pCPL5, which harbor the Gu4- or Gu5-derived *CDR1* promoter, respectively (data not shown). Plasmid pCPG2, which contains the *GFP* reporter gene fused in frame with the last codon of the *CDR1* open reading frame (ORF), was constructed as follows. The C-terminal region of *CDR1* was amplified from SC5314 genomic DNA with the primers CDR32 and CDR31, digested at the introduced KpnI and BamHI sites, and ligated together with a BamHI-PstI fragment from pADH1G3 (14) containing *GFP*, the *ACT1* transcription termination sequence, and the Ca*SAT1* marker into the KpnI/PstI-digested pCPL51. To generate pCPL52, in which the *lacZ* reporter gene instead of *GFP* is fused in frame to the *CDR1* ORF, the C-terminal part of *CDR1* was PCR amplified from SC5314 genomic DNA with the primers CDR32 and CT-CDR1-R-RML, digested at the introduced KpnI and XhoI sites, and ligated into the KpnI/XhoI-digested pCPL51. To ensure in-frame translational fusion of the *CDR1* ORF with *lacZ*, pCPL62 was generated, in which an additional "A" in pCPL52 before the *lacZ* ATG was removed by site-directed mutagenesis employing the A-del pCPL52-F and A-del pCPL52-R primers. All constructs were confirmed by appropriate restriction digestion analysis and by sequencing. The flanking *CDR1* sequences in all of these plasmids served for genomic integration of the reporter fusions at the native *CDR1* locus of clinical *C. albicans* isolates, and the dominant Ca*SAT1* marker (33) was used to select nourseothricin-resistant (Nou^r) transformants. Briefly, for the electroporation, 5 μl (approximately 1 μg) of the linearized DNA fragments were mixed with 40 μl of electrocompetent cells (33) and electroporated using a Bio-Rad Genepulser XL system (0.2-cm cuvette, 1.5 kV). After electroporation, *C. albicans* transformants were washed with 1 ml of 1 M sorbitol, resuspended in 1 ml YEPD medium, incubated for 3 to 4 h with shaking at 30°C prior to plating on YEPD plates containing 200 μg/ml of nourseothricin, and grown at 30°C (33). Nou^r transformants were picked up after 24 h of growth, and single-copy integration of each construct at the desired locus was confirmed by Southern hybridization with gene-specific probes (data not shown).

Fluorescence microscopy and flow cytometry of the cells. The strains were grown overnight in YEPD liquid medium, and aliquots were spotted on microscope slides. Fluorescence microscopy was performed with a Zeiss Axiolab microscope equipped for epifluorescence microscopy with a 50-W mercury high-pressure bulb and the Zeiss fluorescein-specific filter set 09. Imaging scans were acquired with an Argon laser of 488-nm wavelength and corresponding filter settings for green fluorescent protein (GFP) and parallel transmission images. For confocal microscopy, the cells were placed on glass slides and directly viewed with a Bio-Rad confocal microscope (Radiance 2100, AGR, 3Q/BLD; Bio-Rad, United Kingdom) under a 100× oil immersion objective (41). Fluorescence-

TABLE 2. Plasmids and oligonucleotides used

Name	Description	Source (reference) or sequence[a]
Plasmids		
pBS-KS(+)	Plasmid vector used for cloning purpose	Stratagene
pACT1	Plasmid harboring *ACT1* gene	Gift from Anand Bachhawat
pSAT1	Plasmid harboring *caSAT1* marker	Reuss et al. (33)
pLACZ6	Plasmid harboring *lacZ* reporter gene	Gaur et al. (11)
pADH1G3	Plasmid harboring *GFP* reporter gene and *caSAT1* marker	Hiller et al. (14)
pCPG1	Plasmid harboring P_{CDR1}-*GFP* fusion for *ACT1* locus integration	Gaur et al. (11)
pCPG3	Plasmid harboring P_{CDR1}-*GFP* fusion for *CDR1* locus integration	This study
pCPG2	Plasmid harboring *CDR1-GFP* fusion for *CDR1* locus integration	Prasad et al. (30)
pCPL1	Plasmid harboring P_{CDR1}-*lacZ* fusion for *ACT1* locus integration	Gaur et al. (11)
pCPL51	Plasmid harboring P_{CDR1}-*lacZ* fusion for *CDR1* locus integration	This study
pCPL52	Plasmid harboring *CDR1-lacZ* fusion for *CDR1* locus integration	This study
pCPL62	Plasmid harboring in frame *CDR1-lacZ* fusion for *CDR1* locus integration	This study
Oligonucleotides		
CDR1F	Forward primer for *CDR1* promoter amplification	5′-*GATC*GGGCCCTCGTTACTCAATAAGTAT-3′
CDR1R	Reverse primer for *CDR1* promoter amplification	5′-*AGCT*CTCGAGTTCTTTTTGACCTTTTAAAG-3′
CDR1-F-RML	Forward primer for *CDR1* promoter amplification	5′-GCTTCGCCTCAACTTCTTATAAAGTTTTGAAAG-3′
CDR1-R-RML	Reverse primer for *CDR1* promoter amplification	5′-CGTGGTATTCATTAATGGAATGGTTTTCTGAAG-3′
CDR29	Forward primer for amplification of *CDR1* downstream region	5′-*AATT*CTGCAGTTTGTTTTTTGACATGGTGGTATC-3′
CDR30	Reverse primer for amplification of *CDR1* downstream region	5′-*TCGTG*CCGCGGAACACTTTTTCTTTCTAATTATAA-3′
CDR32	Forward primer for amplification of *CDR1* C-terminal region	5′-*ATTT*GGTACCATACATTAAATTTGCTGGTGGG-3′
CDR31	Reverse primer for amplification of *CDR1* C-terminal region	5′-*GTTT*GGATCCTTTCTTATTTTTTTTCTCTCTGTTACCC-3′
CT-CDR1-R-RML	Reverse primer for amplification of *CDR1* C-terminal region	5′-*GTTT*CTCGAGTTTCTTATTTTTTTTCTCTCTGTTACCC-3′
A-del pCPL52-F	Forward primer for in-frame translational fusion of CDR1-ORF with *lacZ*	5′-GAGAGAAAAAAAATAAGAAACTCGAGATGAACATGACTGAAAAAATTCAAAC-3′
A-del pCPL52-R	Reverse primer for in-frame translational fusion of *CDR1*-ORF with *lacZ*	5′-GTTTGAATTTTTTCAGTCATGTTCATCTCGAGTTTCTTATTTTTTTTCTCTC-3′
KM1	Forward primer for amplification of *CDR1* N-terminal region	5′-CTTTTCCACTGGTAACTACT-3′
KM2	Reverse primer for amplification of *CDR1* N-terminal region	5′-CAATAAACCTGCTGACGAG-3′

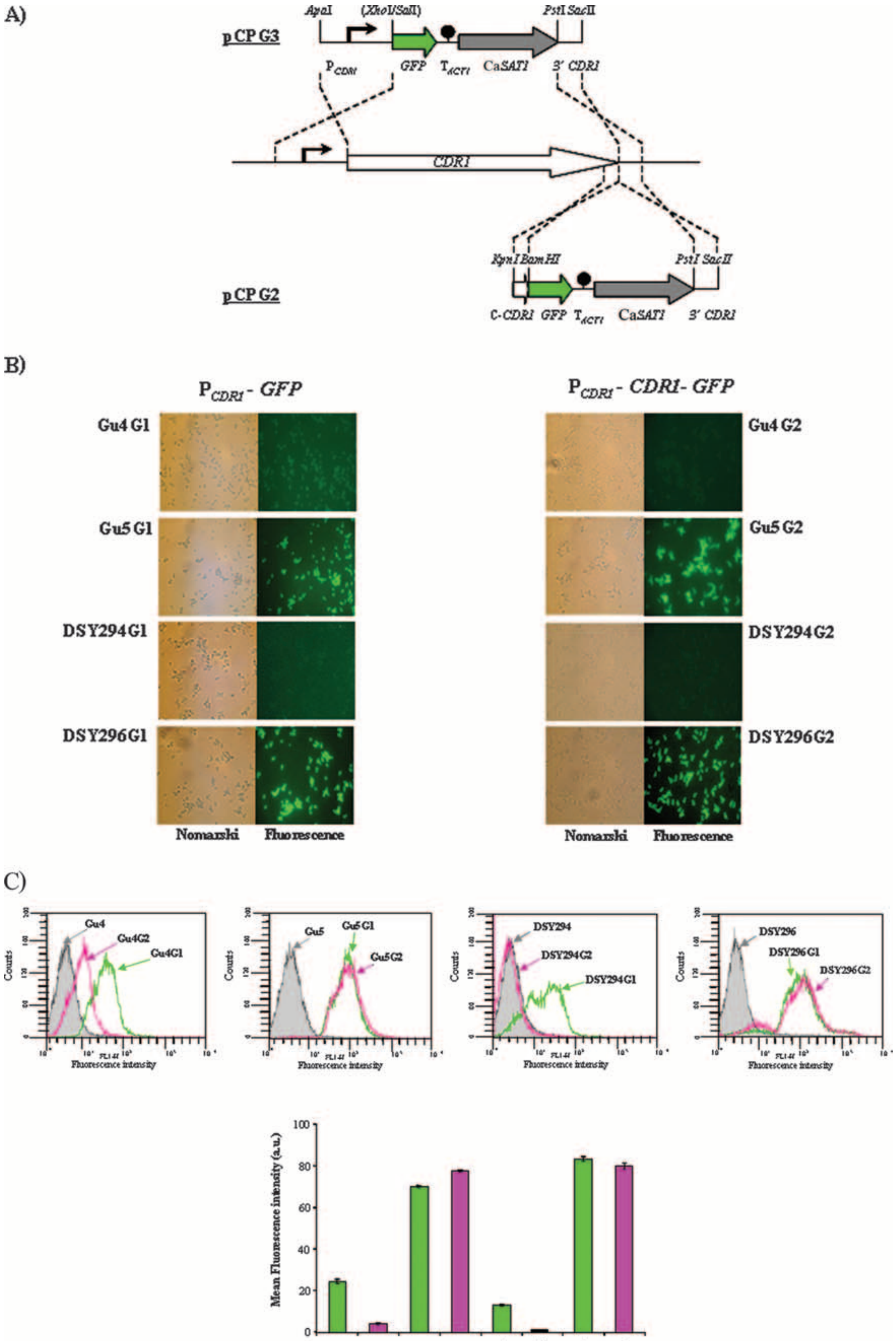
[a] Restriction sites introduced into the primers are underlined, while the flanking bases are written in italics.

activated cell sorter (FACS) analysis was performed using a FACSCalibur flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA) equipped with an argon laser emitting at 488 nm. Fluorescence was measured on the FL1 fluorescence channel equipped with a 530-nm bandpass filter. A total of 20,000 events were counted at the low flow rate. Fluorescence data were collected using logarithmic amplifiers. Data analysis was performed using CellQuest software (Becton Dickinson Immunocytometry Systems). The mean fluorescence intensity was calculated using the histogram statistics program.

Immunodetection of GFP in AS and AR reporter strains. Purified plasma membrane (PM) and crude-extract fractions of *GFP* reporter strains were prepared as described previously (13, 41). For Western blots, membranes were incubated with a 1:5,000 dilution of monoclonal anti-GFP antibody (JL-8) (BD Biosciences, Clontech) or a 1:1,000 dilution of polyclonal anti-Pma1p (plasma membrane ATPase) antibody. Immunoreactivity was detected using horseradish peroxidase-labeled secondary antibodies at a dilution of 1:5,000 (goat antimouse antibody for GFP and goat antirabbit antibody for Pma1p) using the enhanced chemiluminescence assay system (ECL kit; Amersham Biosciences, Arlington Heights, IL).

β-Galactosidase assays. β-Galactosidase assays were performed using duplicate samples of cells from three independent experiments as described previously (11, 21, 42). β-Galactosidase activity was determined by the standard equation (11, 21, 42) and is expressed in Miller units per mg of protein (Miller units are arbitrary units): β-galactosidase activity (Miller units) = $[OD_{420} \times 1{,}000]/[OD_{600} \times t \times v]$, where t is time of reaction expressed in min, v is volume of culture used in the assay, expressed in ml, and OD_{420} and OD_{600} are optical densities at 420 and 600 nm, respectively.

TRO analysis. TRO experiments were performed as described previously (8, 24) with the following modifications. Cells were grown at 30°C in YEPD with agitation until the culture reached an OD_{600} of 1.0. An aliquot of yeast cells (6×10^8 per ml) was used to perform TRO. The cells were centrifuged for 5 min at 4,000 × *g* and resuspended in 5 ml of cold TMN (10 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$; pH 7.4). The cells were again centrifuged for 5 min at 4,000 × *g*, and the cell pellet was resuspended in 900 μl of sterile cold diethyl pyrocarbonate (DEPC)-treated water. Next, the cell suspension was transferred to a fresh microcentrifuge tube containing 50 μl of 10% *N*-lauryl sarcosine sodium sulfate (sarkosyl) and was incubated for 20 min on ice. After the permeabilization step, cells were recovered by low-speed centrifugation at 6,000 rpm for 2 min at 4°C and the supernatant was removed. In vivo transcription was reinitiated by resuspending the permeabilized cell fraction in 120 μl of 2.5× transcription buffer (50 mM Tris-HCl [pH 7.7], 500 mM KCl, 80 mM $MgCl_2$), 16 μl of AGC mix (10 mM


A)
pCPG3
ApaI
(XhoI/SalI)
PstI SacII
P_{CDR1}
GFP
T_{ACT1}
CaSAT1
3′ CDR1
CDR1
pCPG2
KpnI BamHI
PstI SacII
C-CDR1 GFP T_{ACT1} CaSAT1 3′ CDR1
B)
P_{CDR1} - GFP
P_{CDR1} - CDR1- GFP
Gu4 G1
Gu5 G1
DSY294G1
DSY296G1
Gu4 G2
Gu5 G2
DSY294G2
DSY296G2
Nomarski
Fluorescence
Nomarski
Fluorescence
C)
Counts
Fluorescence intensity
Gu4
Gu4G2
Gu4G1
Gu5
Gu5G1
Gu5G2
DSY294
DSY294G2
DSY294G1
DSY296
DSY296G1
DSY296G2
Mean Fluorescence intensity (a.u.)
100
80
60
40
20
0
Gu4G1
Gu4G2
Gu5G1
Gu5G2
DSY294G1
DSY294G2
DSY296G1
DSY296G2
AS
AR
AS
AR

each of ATP, GTP, and CTP), 6 μl of dithiothreitol (0.1 M), 1 U of RNase inhibitor per μl, 10 mM phosphocreatine, 1.2 μg of creatine phosphokinase per μl, and 15 μl of [α-^{32}P]UTP (3,000 Ci/mmol, 10 μCi/μl). Cells were maintained on ice at all times. The final volume was adjusted to 300 μl with DEPC-treated water, and the mix was incubated for 15 min at 30°C to allow transcription elongation. The reaction was stopped by adding 1 ml of ice-cold DEPC-treated water to the mix. Cells were recovered by centrifugation to remove nonincorporated radioactive nucleotides. Total RNA was isolated using the Trizole reagent (Sigma) as per the manufacturer's specifications except that 200 μl of ice-cold acid-washed 0.4- to 0.6-mm-diameter glass beads (Sigma, St. Louis, MO) were also used for efficient and complete lysis of permeabilized cells. Isolated total labeled RNA was again precipitated by adding 2.5 M NH$_4$ acetate and an equal volume of isopropanol. The mixture was stored overnight at −20°C. To pellet the RNA, tubes were centrifuged at 14,000 rpm for 15 min in the microcentrifuge. The isopropanol was removed, and the labeled RNA pellet was washed twice with 70% ethanol, dried, and resuspended in 100 μl of DEPC-treated water. This double precipitation of RNA was used to minimize DNA contamination. Total extracted RNA was spectrophotometrically quantified. An aliquot was used for specific radioactivity determination in a Tri-CARB 2900 TR liquid scintillation analyzer (Packard instrument Co., Inc.). All of the in vivo-labeled RNA of each isolate ($\sim 2 \times 10^6$ to 2.5×10^6 cpm) was subsequently used for reverse Northern hybridization with a dot blotted Nylon membrane (Hybond-N$^+$; Amersham Pharmacia Biotech) containing PCR-amplified gene-specific N-terminal *CDR1* sequences (nucleotides −242 to +256 from the transcription start point), plasmid pACT1 (positive control), and pBlueScript-KS(+) (negative control) as probes, as per the manufacturer's recommendation. Northern blots were scanned with a phosphorimager scanner (FLA-5000 Fuji phosphorimager). Signal intensities of hybridized nuclear RNA were quantified and subsequently normalized to the actin intensities using densitometry scanning.

Thiolutin chase assay. In order to measure the *CDR1* mRNA half-life, a potent in vivo transcriptional inhibitor of *C. albicans*, thiolutin, was used (18, 40). AS and AR isolates of *C. albicans* were incubated with an optimized concentration (40 μg/ml) of thiolutin (data not shown). For this purpose, cultures were treated with 150 μM of the copper chelator bathocuprioinedisulphonic acid and incubated at 30°C for an additional 10 min at 200 rpm. Transcription was subsequently shut off by the addition of 150 nM of CuSO$_4$ and 40 μg/ml of thiolutin. Addition of bathocuprioinedisulphonic acid and CuSO$_4$ was found to enhance the response of the cells to thiolutin (40). Briefly, 100 ml of cells were grown to an OD$_{600}$ of 1.0 at 30°C. Aliquots of cells were taken at the indicated times after transcriptional shutoff. Total RNA was isolated using Ambion's RiboPure-Yeast RNA isolation kit (catalog no. 1926) as per the manufacturer's instructions. For Northern blots, approximately 20 μg of total RNA from the above samples was hybridized with probes derived from gene-specific sequences of the *CDR1* gene. Hybridization signal intensity was quantified with a phosphorimager and was normalized to the band intensity at time T_0 and plotted as a line graph.

Cycloheximide chase assay. Cycloheximide chase assays were performed as described earlier (9) with certain modifications that included the use of an optimized concentration of cycloheximide (16) (data not shown) and the alkaline extraction procedure used for the preparation of crude protein extract (13). Briefly, aliquots of mid-log-phase-grown cells were withdrawn for analysis after translational shutoff at the indicated times and lysed in solution containing 1.85 M NaOH and 7.5% β-mercaptoethanol. Crude proteins isolated were precipitated with 50% trichloroacetic acid and sedimented. The sediment was resuspended in loading buffer (40 mM Tris-HCl [pH 6.8], 8 M urea, 5% SDS, 0.1 M EDTA, 1% β-mercaptoethanol, and 0.1 mg/ml bromophenol blue) and incubated at 37°C for 10 min. Nonsolubilized material was cleared by a centrifugation step, and solubilized proteins (approximately 20 and 30 μg for AR and AS isolates, respectively) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane for Western blotting. Immunodetected Cdr1p signal intensity was quantified with a phosphorimager and was normalized to the band intensity at time T_0 and plotted as a line graph.

RESULTS

Expression of transcriptional and translational *GFP* reporter fusions in AS and AR isolates. To analyze the molecular basis of *CDR1* upregulation in azole-resistant, clinical *C. albicans* isolates in more detail, we employed two matched pairs of AS and AR isolates. The resistant isolates Gu5 and DSY296, which were obtained after fluconazole therapy for oropharyngeal candidiasis in two different human immunodeficiency virus-positive patients, were shown by DNA fingerprinting to be highly related to the susceptible isolates Gu4 and DSY294, respectively, which were recovered from earlier infection episodes in the same patients (6, 38). It was recently shown that a mutation in the transcription factor *TAC1* is responsible for *CDR1* and *CDR2* upregulation in DSY296 (4). However, it has not yet been explored if other mechanisms contribute to the overexpression of the efflux pumps in these isolates.

We compared the expression of two different *GFP* reporter fusions in these isolates, one in which *GFP* was expressed from the *CDR1* promoter (P$_{CDR1}$-*GFP*) and another where *GFP* was fused in frame to the last codon of the *CDR1* ORF and expressed from the *CDR1* promoter (P$_{CDR1}$-*CDR1-GFP*) (see Materials and Methods) (Fig. 1A). The reporter fusions were integrated at the native *CDR1* locus, and two transformants of each of the four parental strains were used for further analysis. The reporter strains were designated Gu4G1 (P$_{CDR1}$-*GFP*) and Gu4G2 (P$_{CDR1}$-*CDR1-GFP*); Gu5G1 (P$_{CDR1}$-*GFP*) and Gu5G2 (P$_{CDR1}$-*CDR1-GFP*); DSY294G1 (P$_{CDR1}$-*GFP*) and DSY294G2 (P$_{CDR1}$-*CDR1-GFP*); and DSY296G1 (P$_{CDR1}$-*GFP*) and DSY296G2 (P$_{CDR1}$-*CDR1-GFP*).

Expression of the P$_{CDR1}$-*GFP* transcriptional and P$_{CDR1}$-*CDR1-GFP* translational fusions in cells grown to mid-exponential phase (OD$_{600}$ of ~1.0) was detected by epifluorescence microscopy (Fig. 1B) and quantified by FACS analysis (Fig. 1C). As expected, the fluorescence intensities of the AR reporter fusion strains were higher than those of the corresponding AS strains (2.5-fold for Gu5G1 versus Gu4G1, 19-fold for Gu5G2 versus Gu4G2; 6-fold for DSY296G1 versus DSY294G1; and 80-fold for DSY296G2 versus DSY294G2), confirming the previously reported increased *CDR1* transcript and Cdr1p protein levels in the AR isolates (4, 6, 38). Inter-

FIG. 1. Schematic depiction of *GFP* reporter fusion integrants and their expression in AS and AR isolates. (A) Structure of the DNA cassettes which were used to integrate the transcriptional (P$_{CDR1}$-*GFP*, top) and translational (P$_{CDR1}$-*CDR1-GFP*, bottom) *GFP* reporter fusions into the *CDR1* locus of the clinical *C. albicans* isolates (middle). The *CDR1* and *GFP* coding regions are represented by white and green arrows, respectively, the *CaSAT1* marker by the gray arrow, and the transcription termination sequence of the *ACT1* gene (T$_{ACT1}$) by the filled circle. *CDR1* upstream and downstream regions are represented by solid lines, and the *CDR1* promoter (P$_{CDR1}$) is symbolized by the bent arrow. Only relevant restriction sites are shown. (B) Nomarski and corresponding fluorescence micrographs of transformants containing the chromosomally integrated P$_{CDR1}$-*GFP* (left) or P$_{CDR1}$-*CDR1-GFP* (right) reporter fusion. (C) Cells of the reporter strains grown to exponential phase in YEPD medium were diluted to a density of 2×10^7 cells per ml in phosphate-buffered saline (pH 7.0), and a total of 20,000 events were analyzed by flow cytometry. The parental strains of the transformants, which do not contain GFP, were used as a negative control. The mean fluorescence intensity is shown for each population of cells (bottom panel) after normalization with values for their corresponding negative controls. Since the normalized mean fluorescence intensity of DSY294G2 was a negative value, we designated it "1.0′" for calculating the degree of change for this particular strain. a.u., arbitrary units.

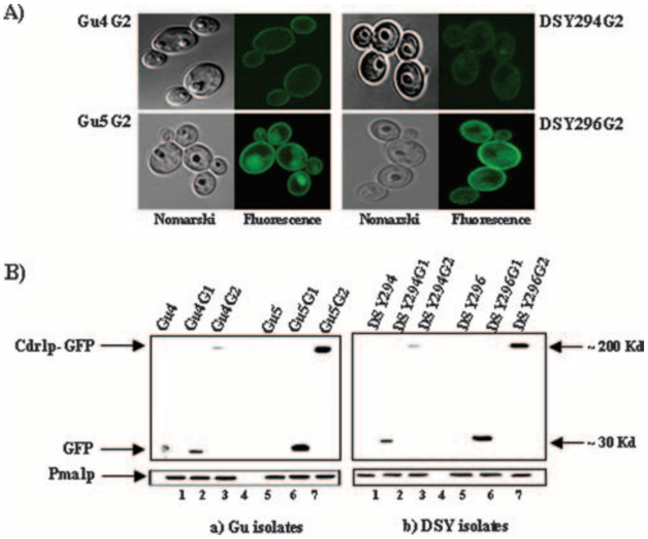


FIG. 2. Localization of Cdr1p and immunodetection of GFP in reporter fusion transformants. (A) Nomarski (left) and corresponding confocal (right) pictures of the transformants harboring the chromosomally integrated P_{CDR1}-*CDR1-GFP* (translational fusion) reporter construct are shown which indicate the proper plasma membrane localization of chimeric Cdr1p in clinical *C. albicans* isolates. The cells were viewed directly on a glass slide with a 100× oil immersion objective. (B) The Western blot analyses were done with an anti-GFP monoclonal antibody on both the transcriptional and translational fusion integrants. Equal loading of protein was assessed by using an anti-Pma1p polyclonal antibody.

estingly, however, expression of the P_{CDR1}-*CDR1-GFP* translational fusion resulted in much lower fluorescence than expression of the P_{CDR1}-*GFP* transcriptional fusion in AS isolates (6-fold for Gu4G2 versus Gu4G1 and 13-fold for DSY294G2 versus DSY294G1), whereas the two types of reporter fusions produced comparable fluorescence in AR isolates. Notably, confocal microscopy confirmed that the Cdr1p-GFP fusion protein was correctly localized to the cell membrane in all reporter strains expressing the translational fusion (Fig. 2A). Immunoreactive bands of the expected sizes were observed in whole-cell extracts and plasma membrane preparations of the P_{CDR1}-*GFP* and P_{CDR1}-*CDR1-GFP* reporter strains, respectively, after Western immunoblotting with an anti-GFP antibody (Fig. 2B). Additionally, the tagging of P_{CDR1} and P_{CDR1}-*CDR1* with *GFP* did not alter the drug resistance profiles of AS and AR isolates, which ruled out that the *GFP* fusions caused any selective impact on Cdr1p functionality for either AS or AR isolates (data not shown).

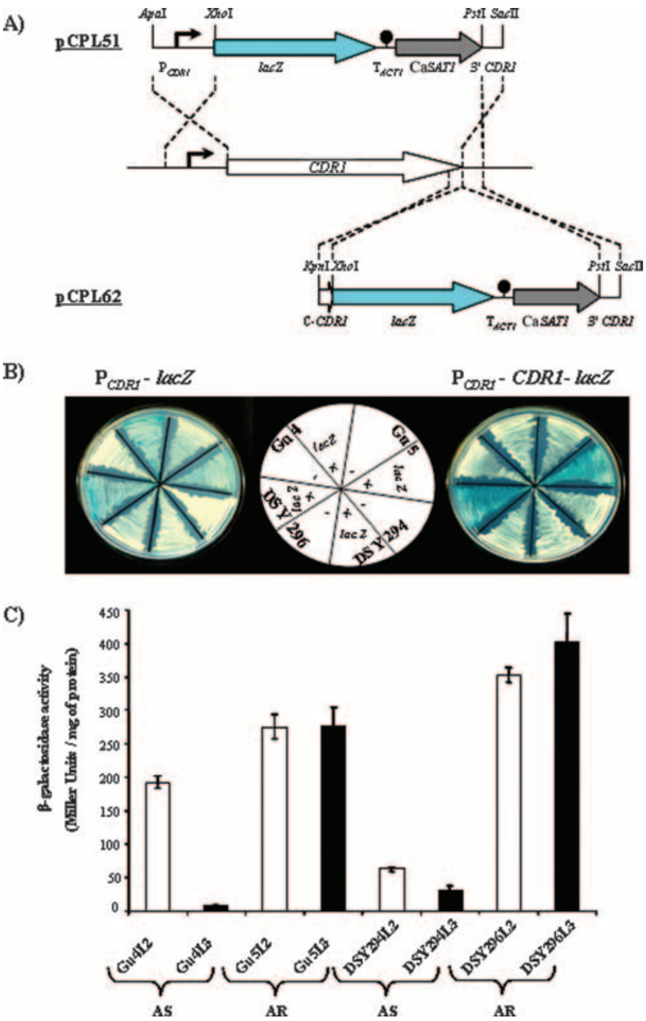


FIG. 3. Schematic depiction of *lacZ* reporter fusion integrants and qualitative and quantitative assay of β-galactosidase activity in AS and AR isolates. (A) Structure of the DNA cassettes which were used to integrate the transcriptional (P_{CDR1}-*lacZ*, top) and translational (P_{CDR1}-CDR1-*lacZ*, bottom) *lacZ* reporter fusions into the *CDR1* locus of the clinical *C. albicans* isolates (middle). The *CDR1* and *lacZ* coding regions are represented by white and blue arrows, respectively, the Ca*SAT1* marker by the gray arrow, and the transcription termination sequence of the *ACT1* gene (T_{ACT1}) by the filled circle. *CDR1* upstream and downstream regions are represented by solid lines, and the *CDR1* promoter (P_{CDR1}) is symbolized by the bent arrow. Only relevant restriction sites are shown. (B) Transformants harboring chromosomally integrated P_{CDR1}-*lacZ* (transcriptional fusion, left) and P_{CDR1}-*CDR1-lacZ* (translational fusion, right) and their corresponding parental strain (without *lacZ*) were streaked on minimal medium plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and photographed after 3 days' growth at 30°C. The positions of the individual strains on the plates are shown in the scheme (middle). (C) β-Galactosidase quantitative reporter activities of each transformant were determined as described previously (11, 21, 42). The values are means ± standard deviations (indicated by the bars) of three independent experiments with duplicate measurements of two independent clones. Empty and filled bars indicate transcriptional (P_{CDR1}-*lacZ*) and translational fusion (P_{CDR1}-*CDR1-lacZ*) transformants in both AS and AR backgrounds.

Expression of transcriptional and translational *lacZ* reporter fusions in AS and AR isolates. To rule out that the reduced expression of the P_{CDR1}-*CDR1-GFP* translational fusion in AS isolates was an artifact intrinsic to the Cdr1p-GFP fusion protein, we used codon-optimized *lacZ* (42) as an alternative reporter gene. As for *GFP*, transcriptional (P_{CDR1}-*lacZ*) and translational (P_{CDR1}-*CDR1-lacZ*) *lacZ* reporter fusions were generated and integrated at the native *CDR1* locus of the AS and AR isolates (see Materials and Methods) (Fig. 3A). The expression of Cdr1p in the P_{CDR1}-*CDR1-lacZ* construct was unaffected by its fusion to *lacZ* as tested by Western blotting with an anti-Cdr1p antibody (data not shown). Two transformants of each parental strain containing a single copy of the reporter fusion were used for further analysis. The reporter strains were designated Gu4L2 (P_{CDR1}-*lacZ*) and Gu4L3 (P_{CDR1}-*CDR1-lacZ*); Gu5L2 (P_{CDR1}-*lacZ*) and Gu5L3

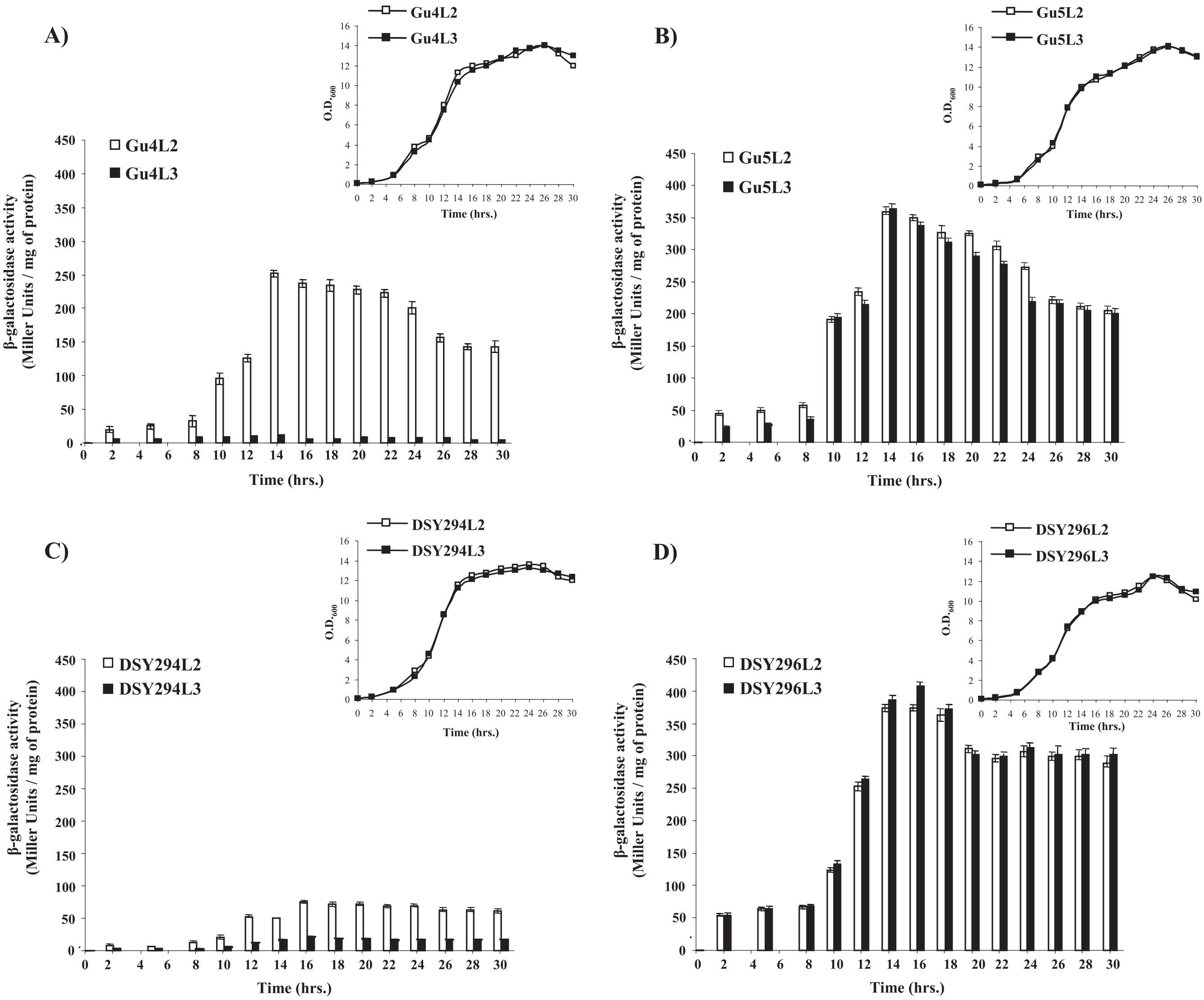


FIG. 4. β-Galactosidase reporter activity of *lac*Z reporter fusion integrants of AS and AR isolates during growth phase. Transcriptional fusion (P_{CDR1}-*lacZ*) and translational fusion (P_{CDR1}-*CDR1-lacZ*) reporter transformants of each isolates were grown from an initial OD$_{600}$ of 0.1 in YEPD broth and withdrawn at the indicated time points of growth for β-galactosidase reporter activity (Fig. 4A, B, C, and D). The inset depicts growth curves of the P_{CDR1}-*lacZ* (□) and P_{CDR1}-*CDR1-lacZ* (■) reporter transformants in AS and AR isolates. The negative-control parental strain (without *lacZ* fusion constructs) reporter activity value was always below 0.5 Miller units, and it was subtracted from the reporter activity of each corresponding transcriptional and translational fusion transformant. The values are means ± standard deviations (indicated by the bars) for three independent experiments with duplicate measurements of two independent clones. Gu4 transformants (A), Gu5 transformants (B), DSY294 transformants (C), and DSY296 transformants (D) were analyzed. Empty and filled bars indicate transcriptional (P_{CDR1}-*lacZ*) and translational fusion (P_{CDR1}-*CDR1-lacZ*) transformants in both AS and AR backgrounds.

(P_{CDR1}-*CDR1-lacZ*); DSY294L2 (P_{CDR1}-*lacZ*) and DSY294L3 (P_{CDR1}-*CDR1-lacZ*); and DSY296L2 (P_{CDR1}-*lacZ*) and DSY296L3 (P_{CDR1}-*CDR1-lacZ*).

Expression of the *lacZ* reporter gene in various strains was assessed by comparing the intensity of the blue color produced by cells grown on agar plates containing the indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Fig. 3B) and was quantified by determining β-galactosidase activities in liquid assays (Fig. 3C). The *lacZ* reporter study confirmed the results obtained with *GFP*. Higher *lacZ* expression levels were observed in transformants of the AR isolates than in transformants of the AS isolates (1.4-fold for Gu5L2 versus Gu4L2, 37-fold for Gu5L3 versus Gu4L3, 5.6-fold for DSY296L2 versus DSY294L2, and 13-fold for DSY296L3 versus DSY294L3). In addition, while the transcriptional and translational fusions yielded comparable activities in the AR isolates, expression of the translational fusion was much lower than that of the transcriptional fusion in the AS isolates (26-fold for Gu4L3 versus Gu4L2 and 2-fold for DSY294L3 versus DSY294L2). Of note, the integration of P_{CDR1}-*lacZ* reporter fusion constructs at the ectopic *ACT1* locus resulted in β-galactosidase activity comparable to that of native *CDR1* locus integrants (data not shown).

Growth phase versus β-galactosidase reporter activity. To investigate whether the observed differences in the expression

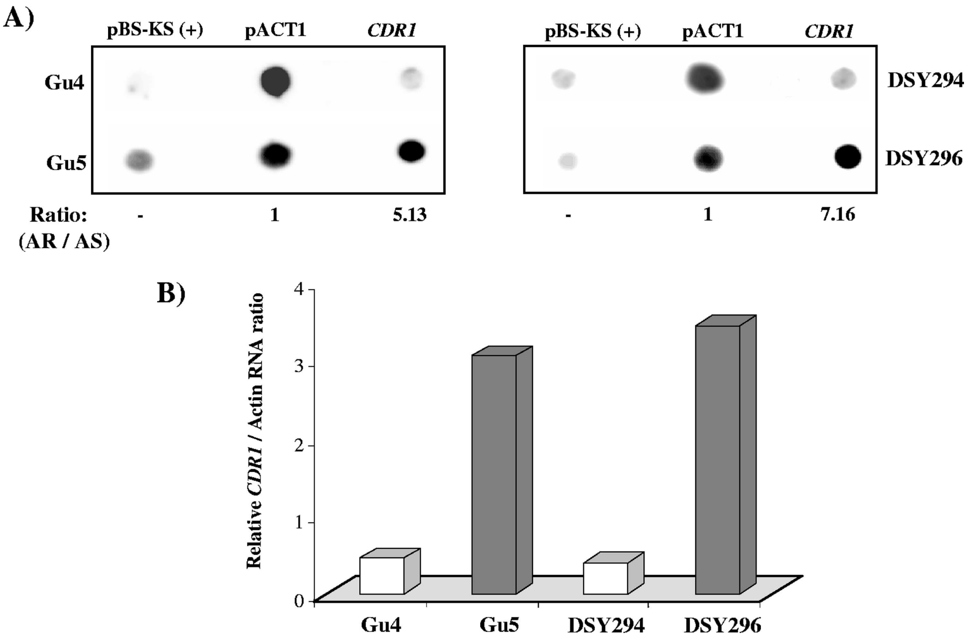


FIG. 5. TRO analysis of AS and AR isolates. (A) Approximately 2 μg (each) of *CDR1*, pACT1, and empty vector pBlueScript-KS(+) DNA was blotted and immobilized on charged nylon membranes (Hybond-N^+; Amersham Pharmacia Biotech) using a dot blot assembly apparatus. The blots were probed with total labeled nuclear run-on RNA as described in Materials and Methods. Hybridization signal intensities of nuclear RNA were quantified using densitometry scanning of phosphorimages. DNA from a pBlueScript-KS(+) plasmid was used as a negative control for nonspecific binding of nuclear RNA to a random DNA fragment. Signal intensities for each isolate were subtracted from the negative control values and subsequently normalized to the intensity corresponding to their AS isolate. The AR/AS ratio is the normalized nuclear RNA intensity between AR and AS isolates. (B) The relative intensity of *CDR1* with respect to actin RNA of each isolate is plotted.

of transcriptional and translational reporter gene fusions in AS and AR isolates depended on the growth phase, we quantitatively monitored β-galactosidase activities in the *lacZ* reporter strains at various times during growth in batch cultures. As can be seen in Fig. 4, the low reporter expression levels of the translational P_{CDR1}-*CDR1-lacZ* fusion compared with those of the transcriptional P_{CDR1}-*lacZ* fusion in the AS isolates were observed at all growth stages (Fig. 4A and C). In contrast, both types of reporter fusion produced comparable β-galactosidase activities in the AR isolates throughout growth (Fig. 4B and D).

Taken together, exploitation of reporter fusions and their expression analysis indicated that an increase in *CDR1* expression levels in the AR isolates compared to those in the corresponding AS isolates is contributed by affecting either *CDR1* promoter activity, mRNA stability, translational efficiency, or protein stability. Therefore, we performed further experiments on native *CDR1* (endogenous gene) to get a real insight into whether transcriptional/posttranscriptional control mechanisms are involved in the upregulation of *CDR1* expression in AR isolates.

Transcriptional rate for *CDR1* is increased in AR isolates. We first tested whether the transcription rate for *CDR1* was elevated in the AR isolates. For this purpose, TRO assays were performed. Both AS and AR isolates were grown to an OD_{600} of ~1.0, and the cells were permeabilized with the detergent *N*-lauryl sarcosine sodium sulfate (sarkosyl) for the isolation of intact nuclei (8, 24). The subsequent incubation of isolated nuclei with transcription buffer and radiolabeled $[\alpha\text{-}^{32}P]$UTP reinitiated the transcription (see Materials and Methods). The in vivo-labeled nascent RNAs were then used as probes in reverse Northern hybridizations with dot blotted *CDR1*-specific PCR-amplified DNA. As controls, pACT1 plasmid DNA, containing the constitutively expressed *ACT1* gene, and the empty vector pBluescript were also dotted on the membranes. As shown in Fig. 5A and B, the AR isolates exhibited an increased rate of transcription of *CDR1* compared with that for the AS isolates (fivefold for Gu5 versus Gu4 and sevenfold for DSY296 versus DSY294).

***CDR1* mRNA stability is increased in AR isolates.** To investigate if in addition to the increased transcription rates posttranscriptional events also contribute to the higher level of *CDR1* expression in drug-resistant strains, we determined *CDR1* mRNA stability in the AS and AR isolates. To this end, we exploited an effective sulfur-containing purine analogue, thiolutin, as a potent inhibitor of de novo transcription to determine mRNA stability in *C. albicans* (18, 40). Thiolutin affected $[^3H]$uridine incorporation into nascent RNAs in a concentration-dependent manner. About 40 μg/ml of thiolutin inhibited ~95% of the $[^3H]$uridine incorporation in total RNA (data not shown). Methylene blue staining revealed no decline in cell viability of AS and AR isolates treated with 40 μg/ml thiolutin, although growth was inhibited to a certain extent (data not shown). This optimized thiolutin concentration was subsequently used for the mRNA chase assays. Total RNA was isolated at different time points after transcriptional inhibition with thiolutin and analyzed by RNA gel blots (Fig. 6A). After probing the blots with a *CDR1*-specific probe, hybridization signals were quantified by densitometry scanning in a phosphorimager. Figure 6B depicts a typical *CDR1* mRNA decay profile

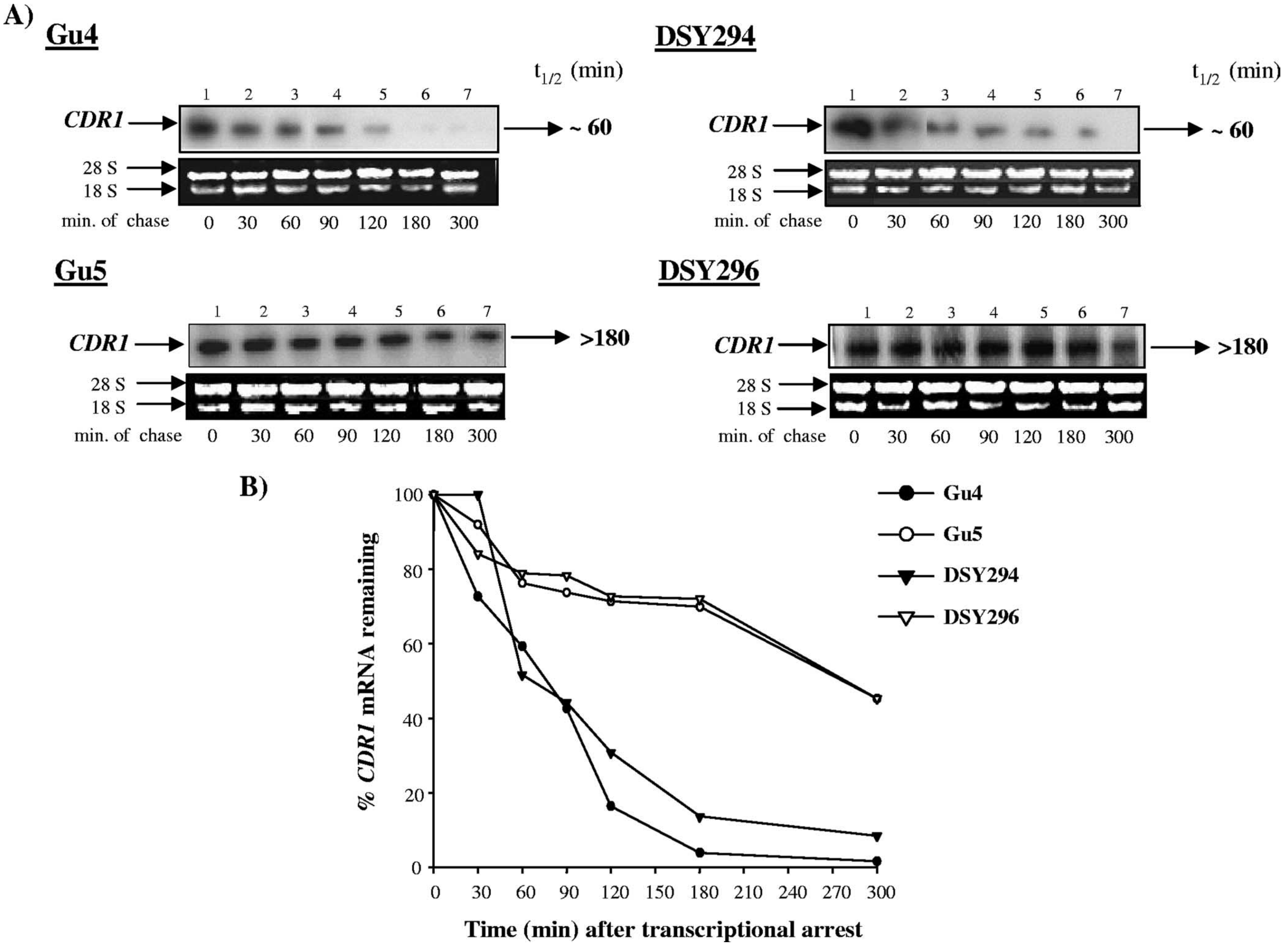


FIG. 6. *CDR1* mRNA decay assay. Exponentially growing cultures of *C. albicans* were incubated with the optimized thiolutin concentration (40 μg/ml) to inhibit ongoing in vivo transcription. Total RNA was isolated at the indicated times thereafter and fractionated on a 1% (wt/vol) agarose–2.2 M formaldehyde denaturing gel. (A) The gel was stained with ethidium bromide before blotting to monitor equal loading of the RNA and subsequently blotted onto a charged nylon membrane. The blot was hybridized with a *CDR1*-specific probe. Time points in minutes are indicated below each phosphorimage. (B) The hybridization signals were quantified using densitometry scanning in a phosphorimager. The signal intensity at each time point was normalized to that of time T_0 (expressed as a percentage) and plotted as described in Materials and Methods. $t_{1/2}$, half-life.

in the AS and AR isolates over a 300-min period from one of these experiments. *CDR1* mRNA could be detected in both AS isolates Gu4 and DSY294 at time T_0, and the signal intensity diminished progressively with time (mRNA half-life was approximately 60 min). The turnover of the *CDR1* transcript occurred much more slowly in the AR isolates Gu5 and DSY296, with a half-life of >180 min. These results demonstrated that *CDR1* mRNA stability was increased in the AR isolates over that in the AS isolates.

Cdr1 protein stability does not differ in AS and AR isolates. To test whether increased protein stability might also contribute to the high Cdr1p levels in AR isolates, cycloheximide chase assays were performed. Total crude protein extracts were isolated at different times after treatment of the cells with an optimized concentration (75 mM) of cycloheximide (16) and analyzed by Western immunoblotting with a rabbit polyclonal anti-Cdr1p antibody. Figure 7A shows the Western blot of the decay experiment, while Fig. 7B shows the quantitative decay profile. The half-life of Cdr1p was similar in AS and AR isolates and was calculated to be approximately 90 min.

DISCUSSION

In this study, we used two pairs of matched AS and AR *C. albicans* clinical isolates to study the mechanisms of *CDR1* overexpression in AR isolates. Our results demonstrate that both increased transcriptional activation and enhanced mRNA stability contribute to increased *CDR1* expression in these drug-resistant isolates. Interestingly, we found that in the AS isolates reporter fusions with the *CDR1* coding region were expressed at lower levels than fusions in which the reporter genes were directly fused to the *CDR1* promoter, whereas in the AR isolates the two types of reporter fusions were expressed at comparable levels. This would mean that sequences in the *CDR1* coding region can also contribute to the increased *CDR1* expression in AR isolates.

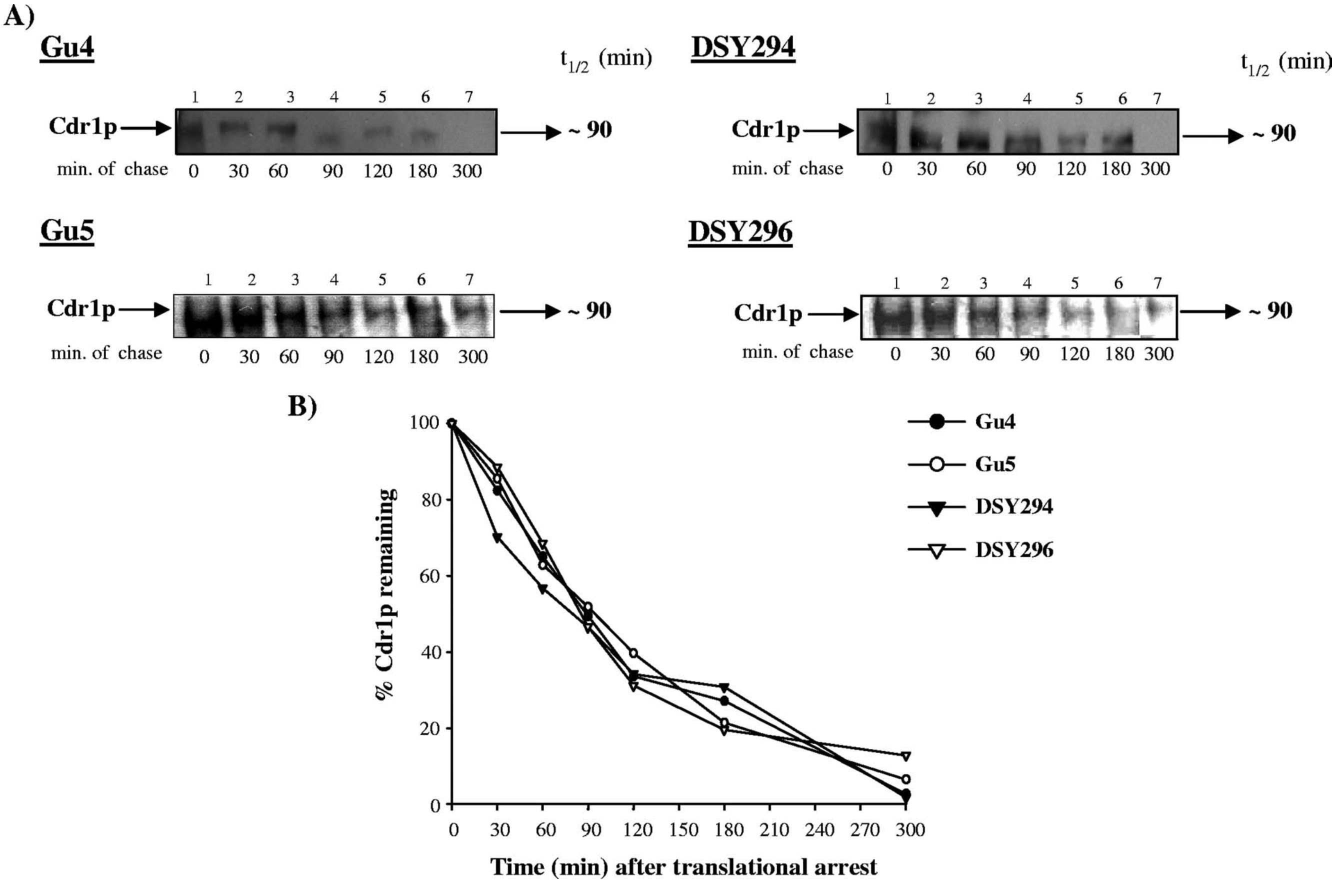


FIG. 7. Cdr1p decay assay. (A) Exponentially grown cultures of *C. albicans* were translationally halted at 30°C by addition of 75 mM of cycloheximide for 1 h. Whole-cell extracts were prepared at the indicated times after cycloheximide treatment. For AR isolates, ~20 μg, and for AS isolates, ~30 μg (because of relatively low expression of Cdr1p) of crude extract for each time was loaded and separated by SDS-polyacrylamide gel electrophoresis. Equal loading of protein was assessed using a Coomassie-stained gel (data not shown). Cdr1p was detected using a polyclonal anti-Cdr1p antibody. The Cdr1p-specific bands were subsequently quantified by densitometry scanning in a phosphorimager. (B) Band intensities (represented as percentages of the value at T_0) for each isolate were plotted against the chased time. $t_{1/2}$, half-life.

It has been shown previously that *CDR1* overexpression in *C. albicans* is caused by an increased *CDR1* transcription rate in AR isolates compared with that in AS isolates (24). Our TRO experiments confirmed that the transcriptional initiation rate from the *CDR1* promoter was five- to sevenfold higher in the AR isolates than in the AS isolates used in the present study (Fig. 5). The *CDR1* upstream region contains many sequence elements which are involved in the regulation of *CDR1* expression (5, 10, 11, 17, 32); however, no sequence differences were found in the *CDR1* upstream region of these matched pairs of AS and AR isolates (5, 11; also unpublished observations). In line with this, it has recently been shown that a gain-of-function mutation in the transcription factor *TAC1*, which controls *CDR1* expression, causes *CDR1* upregulation in the AR isolate DSY296 (4).

In order to evaluate if, in addition to transcriptional activation of *CDR1*, differential mRNA and protein stability also contribute to the enhanced Cdr1p levels in AR isolates, we performed thiolutin and cycloheximide chase assays and observed that the up-regulation of *CDR1* mRNA in AR isolates was due to an increase in the mRNA half-life (>180 min), which was approximately threefold greater than that in AS isolates (Fig. 6). In contrast, no difference in Cdr1p protein stability was observed between AS and AR isolates (Fig. 7). There are examples in other organisms where overexpression of efflux pumps can be caused by increased mRNA stability. An increase in the mRNA half-life of *MDR1* (a *CDR1* homologue in humans) has been shown to contribute to doxorubicin and colchicine resistance in the myelogenous leukemic cell line K562 (47). An enhanced mRNA stability of *bmr3*, encoding a multidrug transporter, also leads to a multidrug-resistant (MDR) phenotype in *Bacillus subtilis* (28). In addition, the reported MDR phenotype of *Entamoeba histolytica* trophozoites is also caused by transcriptional activation (27), as well as an increase in mRNA stability of the Eh*Pgp5* gene (22).

Notably, though, *cis* determinants located in the 3′ untranslated region (UTR) regulate the degradation of mRNA (35). Among these *cis* elements, adenylate-uridylate-rich-element motifs of the 3′ UTR involved in destabilization of their corresponding mRNAs are of prime importance (22, 31, 35). Several reports have also suggested a relationship between the relative affinity of a given RNA for RNA-binding protein(s) and the stability of an mRNA containing these sequences (31, 35). Our preliminary results reveal that the *CDR1* 3′ UTR is

~78% AU rich and also possesses several putative consensus binding sequences for a regulatory RNA-binding protein(s). Therefore, any contribution of *CDR1* 3′ UTR *cis* elements and of the mutation or alteration in *trans*-acting regulatory factor(s) corresponding to these conserved elements in determining mRNA stability between AS and AR isolates requires an in-depth analysis.

Our results with the reporter fusion transformants also suggest that sequences in the *CDR1* coding region could also be an important contributor for increased *CDR1* expression in AR isolates. In this context, it should be mentioned that synonymous and nonsynonymous nucleotide polymorphisms have been observed in the *CDR1* coding region, but so far none of these has been linked to *CDR1* overexpression (12, 15). Our present study did not consider the role of these allelic differences in sustained overexpression of *CDR1* in AR isolates. However, a recent study has reported that a silent polymorphism does not influence human *P-gp/MDR1* mRNA and protein expression but affects posttranslational events in terms of timing of cotranslational folding and membrane insertion (19, 43).

In conclusion, our results demonstrate for the first time that *CDR1* is regulated by both transcriptional and posttranscriptional events. Our finding that the acquisition of azole resistance involves transcriptional activation as well as decreased mRNA turnover opens up new possibilities for treatment regimes to circumvent MDR in *C. albicans*. In this context, it is worth mentioning that the intervention of overexpressing *MDR1* in MDR cell lines by verapamil (25) and ecteinascidin 743 (39) has been reported to be due to the transcriptional down-regulation of the gene.

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REFERENCES

1. **Akins, R. A.** 2005. An update on antifungal targets and mechanisms of resistance in *Candida albicans*. Med. Mycol. **43:**285–318.
2. **Chen, C. G., Y. L. Yang, H. I. Shih, C. L. Su, and H. J. Lo.** 2004. CaNdt80 is involved in drug resistance in *Candida albicans* by regulating *CDR1*. Antimicrob. Agents Chemother. **48:**4505–4512.
3. **Coste, A. T., M. Karababa, F. Ischer, J. Bille, and D. Sanglard.** 2004. *TAC1*, transcriptional activator of *CDR* genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters *CDR1* and *CDR2*. Eukaryot. Cell **3:**1639–1652.
4. **Coste, A., V. Turner, F. Ischer, J. Morschhäuser, A. Forche, A. Selmecki, J. Berman, J. Bille, and D. Sanglard.** 2006. A mutation in Tac1p, a transcription factor regulating *CDR1* and *CDR2*, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in *Candida albicans*. Genetics **172:**2139–2156.
5. **de Micheli, M., J. Bille, C. Schueller, and D. Sanglard.** 2002. A common drug-responsive element mediates the upregulation of the *Candida albicans* ABC transporters *CDR1* and *CDR2*, two genes involved in antifungal drug resistance. Mol. Microbiol. **43:**1197–1214.
6. **Franz, R., M. Ruhnke, and J. Morschhäuser.** 1999. Molecular aspects of fluconazole resistance development in *Candida albicans*. Mycoses **42:**453–458.
7. **Franz, R., S. L. Kelly, D. C. Lamb, D. E. Kelly, M. Ruhnke, and J. Morschhäuser.** 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. Antimicrob. Agents Chemother. **42:**3065–3072.
8. **Garcia-Martinez, J., A. Aranda, and J. E. Perez-Ortin.** 2004. Genomic run-on evaluates transcription rates for all yeast genes and identifies gene regulatory mechanisms. Mol. Cell **15:**303–313.
9. **Gardner, R. G., G. M. Swarbrick, N. W. Bays, S. R. Cronin, S. Wilhovsky, L. Seelig, C. Kim, and R. Y. Hampton.** 2000. Endoplasmic reticulum degradation requires lumen to cytosol signaling: transmembrane control of Hrd1p by Hrd3p. J. Cell Biol. **151:**69–82.
10. **Gaur, N. A., N. Puri, N. Karnani, G. Mukhopadhyay, S. K. Goswami, and R. Prasad.** 2004. Identification of a negative regulatory element which regulates basal transcription of a multidrug resistance gene *CDR1* of *Candida albicans*. FEMS Yeast Res. **4:**389–399.
11. **Gaur, N. A., R. Manoharlal, P. Saini, T. Prasad, G. Mukhopadhyay, M. Hoefer, J. Morschhäuser, and R. Prasad.** 2005. Expression of the *CDR1* efflux pump in clinical *Candida albicans* isolates is controlled by a negative regulatory element. Biochem. Biophys. Res. Commun. **332:**206–214.
12. **Haque, A., V. Rai, B. S. Bahal, S. Shukla, A. A. Lattif, G. Mukhopadhyay, and R. Prasad.** 2007. Allelic variants of ABC drug transporter Cdr1p in clinical isolates of Candida albicans. Biochem. Biophys. Res. Commun. **352:**491–497.
13. **Hiller, D., D. Sanglard, and J. Morschhäuser.** 2006. Overexpression of the *MDR1* gene is sufficient to confer increased resistance to toxic compounds in *Candida albicans*. Antimicrob. Agents Chemother. **50:**1365–1371.
14. **Hiller, D., S. Stahl, and J. Morschhäuser.** 2006. Multiple *cis*-acting sequences mediate upregulation of the *MDR1* efflux pump in a fluconazole-resistant clinical *Candida albicans* isolate. Antimicrob. Agents Chemother. **50:**2300–2308.
15. **Holmes, A. R., S. Tsao, S. W. Ong, E. Lamping, K. Niimi, B. C. Monk, M. Niimi, A. Kaneko, B. R. Holland, J. Schmid, and R. D. Cannon.** 2006. Heterozygosity and functional allelic variation in the *Candida albicans* efflux pump genes *CDR1* and *CDR2*. Mol. Microbiol. **62:**170–186.
16. **Imanishi, Y., K. Yokoyama, and K. Nishimura.** 2004. Inductions of germ tube and hyphal formations are controlled by mRNA synthesis inhibitor in *Candida albicans*. Nippon Ishinkin Gakkai Zasshi **45:**113–119.
17. **Karnani, N., N. A. Gaur, S. Jha, N. Puri, S. Krishnamurthy, S. K. Goswami, G. Mukhopadhyay, and R. Prasad.** 2004. SRE1 and SRE2 are two specific steroid-responsive modules of *Candida* drug resistance gene 1 (*CDR1*) promoter. Yeast **21:**219–239.
18. **Kebaara, B. W., L. E. Nielsen, K. W. Nickerson, and A. L. Atkin.** 2006. Determination of mRNA half-lives in *Candida albicans* using thiolutin as a transcription inhibitor. Genome **49:**894–899.
19. **Kimchi-Sarfaty, C., J. M. Oh, I. W. Kim, Z. E. Sauna, A. M. Calcagno, S. V. Ambudkar, and M. M. Gottesman.** 2007. A "silent" polymorphism in the *MDR1* gene changes substrate specificity. Science **315:**525–528.
20. **Krishnamurthy, S., V. Gupta, R. Prasad, S. L. Panwar, and R. Prasad.** 1998. Expression of *CDR1*, a multidrug resistance gene of *Candida albicans*: in vitro transcriptional activation by heat shock, drugs and human steroid hormones. FEMS Microbiol. Lett. **160:**191–197.
21. **Li, D., V. Gurkovska, M. Sheridan, R. Calderone, and N. Chauhan.** 2004. Studies on the regulation of the two-component histidine kinase gene *CHK1* in *Candida albicans* using the heterologous *lacZ* reporter gene. Microbiology **150:**3305–3313.
22. **Lopez-Camarillo, C., J. P. Luna-Arias, L. A. Marchat, and E. Orozco.** 2003. EhPgp5 mRNA stability is a regulatory event in the *Entamoeba histolytica* multidrug resistance phenotype. J. Biol. Chem. **278:**11273–11280.
23. **Lopez-Ribot, J. L., R. K. McAtee, L. N. Lee, W. R. Kirkpatrick, T. C. White, D. Sanglard, and T. F. Patterson.** 1998. Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. Antimicrob. Agents Chemother. **42:**2932–2937.
24. **Lyons, C. N., and T. C. White.** 2000. Transcriptional analyses of antifungal drug resistance in *Candida albicans*. Antimicrob Agents Chemother. **44:**2296–2303.
25. **Muller, C., F. Goubin, E. Ferrandis, I. Cornil-Scharwtz, J. D. Bailly, C. Bordier, J. Benard, B. I. Sikic, and G. Laurent.** 1995. Evidence for transcriptional control of human mdr1 gene expression by verapamil in multidrug-resistant leukemic cells. Mol. Pharmacol. **47:**51–56.
26. **Murad, A. M., C. d'Enfert, C. Gaillardin, H. Tournu, F. Tekaia, D. Talibi, D. Marechal, V. Marchais, J. Cottin, and A. J. Brown.** 2001. Transcript profiling in *Candida albicans* reveals new cellular functions for the transcriptional repressors *CaTup1*, *CaMig1* and *CaNrg1*. Mol. Microbiol. **42:**981–993.
27. **Nieto, A., D. Guillermo Perez, E. Orozco, F. Paz, and C. Gomez.** 2005. *Entamoeba histolytica EhPgp5* transcriptional activation depends on putative emetine response elements. Exp. Parasitol. **110:**233–237.

28. **Ohki, R., and K. Tateno.** 2004. Increased stability of *bmr3* mRNA results in a multidrug-resistant phenotype in *Bacillus subtilis*. J. Bacteriol. **186:**7450–7455.
29. **Prasad, R., P. D. Worgifosse, A. Goffeau, and E. Balzi.** 1995. Molecular cloning and characterization of a novel gene of *Candida albicans CDR1*, conferring multiple resistance to drugs and antifungals. Curr. Genet. **27:**320–329.
30. **Prasad, T., P. Saini, N. A. Gaur, R. A. Vishwakarma, L. A. Khan, Q. M. Haq, and R. Prasad.** 2005. Functional analysis of Ca*IPT1*, a sphingolipid biosynthetic gene involved in multidrug resistance and morphogenesis of *Candida albicans*. Antimicrob Agents Chemother. **49:**3442–3452.
31. **Prokipcak, R. D., A. Raouf, and C. Lee.** 1999. The AU-rich 3′ untranslated region of human *MDR1* mRNA is an inefficient mRNA destabilizer. Biochem. Biophys. Res. Commun. **261:**627–634.
32. **Puri, N., S. Krishnamurthy, S. Habib, S. E. Hasnain, S. K. Goswami, and R. Prasad.** 1999. *CDR1*, a multidrug resistance gene from *Candida albicans*, contains multiple regulatory domains in its promoter and the distal AP-1 element mediates its induction by miconazole. FEMS Microbiol. Lett. **180:**213–219.
33. **Reuss, O., A. Vik, R. Kolter, and J. Morschhäuser.** 2004. The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. Gene **341:**119–127.
34. **Riggle, P. J., and C. A. Kumamoto.** 2006. Transcriptional regulation of *MDR1*, encoding a drug efflux determinant, in fluconazole-resistant *Candida albicans* strains through an Mcm1p binding site. Eukaryot. Cell **5:**1957–1968.
35. **Ross, J.** 1995. mRNA stability in mammalian cells. Microbiol. Rev. **59:**423–450.
36. **Sanglard, D., and F. C. Odds.** 2002. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect. Dis. **2:**73–85.
37. **Sanglard, D., F. Ischer, M. Monod, and J. Bille.** 1997. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of *CDR2*, a new multidrug ABC transporter gene. Microbiology **143:**405–416.
38. **Sanglard, D., K. Kuchler, F. Ischer, J. L. Pagani, M. Monod, and J. Bille.** 1995. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob. Agents Chemother. **39:**2378–2386.
39. **Scotto, K. W., and R. A. Johnson.** 2001. Transcription of the multidrug resistance gene *MDR1*: a therapeutic target. Mol. Interv. **1:**117–125.
40. **Setiadi, E. R., T. Doedt, F. Cottier, C. Noffz, and J. F. Ernst.** 2006. Transcriptional response of *Candida albicans* to hypoxia: linkage of oxygen sensing and Efg1p-regulatory networks. J. Mol. Biol. **361:**399–411.
41. **Shukla, S., P. Saini, Smriti, S. Jha, S. V. Ambudkar, and R. Prasad.** 2003. Functional characterization of *Candida albicans* ABC transporter Cdr1p. Eukaryot. Cell **2:**1361–1375.
42. **Uhl, M. A., and A. D. Johnson.** 2001. Development of *Streptococcus thermophilus lacZ* as a reporter gene for *Candida albicans*. Microbiology **147:**1189–1195.
43. **Wang, D., A. D. Johnson, A. C. Papp, D. L. Kroetz, and W. Sadee.** 2005. Multidrug resistance polypeptide 1 (MDR1, ABCB1) variant 3435C>T affects mRNA stability. Pharmacogenet. Genomics **15:**693–704.
44. **White, T. C.** 1997. Increased mRNA levels of *ERG16*, *CDR*, and *MDR1* correlate with increased azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob. Agents Chemother. **41:**1482–1487.
45. **White, T. C., K. A. Marr, and R. A. Bowden.** 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin. Microbiol. Rev. **11:**382–402.
46. **Wirsching, S., S. Michel, G. Kohler, and J. Morschhäuser.** 2000. Activation of the multidrug resistance gene *MDR1* in fluconazole-resistant, clinical *Candida albicans* strains is caused by mutations in a *trans*-regulatory factor. J. Bacteriol. **182:**400–404.
47. **Yague, E., A. L. Armesilla, G. Harrison, J. Elliott, A. Sardini, C. F. Higgins, and S. Raguz.** 2003. P-glycoprotein (*MDR1*) expression in leukemic cells is regulated at two distinct steps, mRNA stabilization and translational initiation. J. Biol. Chem. **278:**10344–10352.
48. **Yang, Y. L., Y. H. Lin, M. Y. Tsao, C. G. Chen, H. I. Shih, J. C. Fan, J. S. Wang, and H. L. Lo.** 2006. Serum repressing efflux pump *CDR1* in *Candida albicans*. BMC Mol. Biol. **7:**22.