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CaNdt80 Is Involved in Drug Resistance in *Candida albicans* by Regulating *CDR1*

Chia-Geun Chen,[1,2] Yun-Liang Yang,[3] Hsin-I Shih,[3] Chia-Li Su,[2] and Hsiu-Jung Lo[2]*

Graduate Institute of Life Sciences, National Defense Medical Center,[1] and Division of Clinical Research, National Health Research Institutes,[2] Taipei, and Department of Biological Science and Technology, National Chiao Tung University, Hsinchu,[3] Taiwan, Republic of China.

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Overexpression of *CDR1*, an efflux pump, is one of the major mechanisms contributing to drug resistance in *Candida albicans*. *CDR1 p-lacZ* was constructed and transformed into a *Saccharomyces cerevisiae* strain so that the *lacZ* gene could be used as the reporter to monitor the activity of the *CDR1* promoter. Overexpression of *CaNDT80*, the *C. albicans* homolog of *S. cerevisiae NDT80*, increases the β-galactosidase activity of the *CDR1 p-lacZ* construct in *S. cerevisiae*. Furthermore, mutations in *CaNDT80* abolish the induction of *CDR1* expression by antifungal agents in *C. albicans*. Consistently, the *Candt80*/*Candt80* mutant is also more susceptible to antifungal drugs than the wild-type strain. Thus, the gene for CaNdt80 may be the first gene among the regulatory factors involved in drug resistance in *C. albicans* whose function has been identified.

The prevalence of fungal infections has increased significantly in the past few decades. Among the organisms causing these infections, *Candida albicans* is the most frequently isolated fungal pathogen in humans and has caused morbidity in seriously debilitated and immunocompromised hosts (6). Coincident with the increased use of antifungal drugs, the incidences of drug resistance have also increased (24, 33, 36). The limited variety of antifungal agents and emerging drug resistance highlight the need to identify potential targets and elucidate the molecular mechanisms involved in drug resistance for the development of new effective antifungal agents.

Overexpression of efflux pumps, either major facilitators or ATP binding cassette (ABC) transporters, has been shown to be one of the major mechanisms of drug resistance in clinical isolates (9, 18, 19). The *CDR1* gene, which encodes an ABC efflux pump, is identified by complementation of the *pdr5* mutant, which is hypersensitive to cycloheximide, chloramphenicol, and azole drugs, in *Saccharomyces cerevisiae* (25). Mutations in *CDR1* in *C. albicans* resulted in increased susceptibilities to azole drugs (29), which is consistent with the observation that overexpression of *CDR1* contributes to the drug resistance of clinical isolates of *C. albicans* (17, 36). The AP-1 site and the drug-responsive element of the *CDR1* promoter have been reported to be the *cis*-regulatory elements (5, 26). Furthermore, the existence of *trans*-regulatory factors of *CDR1* has also been suggested (26). However, the molecular mechanism and the gene network regulating the expression of *CDR1* and drug resistance are poorly understood.

In this study, as in previous studies (15, 16), we have successfully used *S. cerevisiae* as a model to study *C. albicans*, despite the differences between these two organisms. We have identified one predicted transcription factor, CaNdt80, the *C. albicans* homolog of *S. cerevisiae* Ndt80, which is a meiosis-specific transcription factor in *S. cerevisiae* (2, 3) and which is involved in drug resistance through the regulation of *CDR1* in *C. albicans*.

* Corresponding author. Mailing address: Division of Clinical Research, National Health Research Institutes, 128, Yen-Chiu-Yuan Rd., Section 2, Taipei 11529, Taiwan, Republic of China. Phone: 886-2-2652-4095. Fax: 886-2-2789-0254. E-mail: hjlo@nhri.org.tw.

MATERIALS AND METHODS

Strains and media. The *S. cerevisiae* strains, *C. albicans* strains, and plasmids used in this study are listed in Table 1, Table 2, and Table 3, respectively. Yeast peptone dextrose (YPD; 1% yeast extract, 2% peptone, and 2% dextrose) and synthetic dextrose (SD; 0.67% yeast nitrogen base without amino acids and with 2% dextrose) were prepared as described by Sherman (30).

Construction of *CDR1p-lacZ* strain. The *CDR1p-lacZ* fusion plasmid was constructed by using a DNA fragment generated by PCR from the promoter and the translation initiation codon ATG of *CDR1* from *C. albicans* SC5314. The PCR fragment was generated with primers HJL21 (5′-dTTTCCCGGGGGATCCTC GTTACTCAA) and HJL22 (5′-dCCCAAGCTTGCATAATTTTTTTCTTTTT GACCT), which introduced 5′ XmaI and 3′ HindIII sites (underlined), respectively, for directional cloning into the XmaI and HindIII sites of plasmid YIp363. Plasmid LOB42 contained the *CDR1p-lacZ* in-frame fusion, in which *lacZ* is the reporter gene for monitoring the activity of the *CDR1* promoter. Primers HJL44 (5′-TTTTCCCGGGCAGCAGTTTAGAAGCAAT) and HJL45 (5′-CCCCCCC GGGTGATTTGTCTTAACATT) were used to amplify the DNA fragment from the sequence 37 to 1648 bp downstream of the translation initiation site of the *ADE3* gene. The fragment of the *ADE3* gene was cloned in an antisense orientation into the XmaI site of the *CDR1p-lacZ* fusion of plasmid LOB42 to create plasmid LOB43. The LOB43 construct (Fig. 1) was digested with XhoI to linearize the DNA at position 337 bp downstream of the translation initiation site of the *ADE3* gene and transformed into *S. cerevisiae* (10560-2B). The *CDR1p-lacZ* fusion of plasmid LOB43 was integrated into the *ADE3* locus through homologous recombination to produce the $Leu2^+$ transformant, strain SLO1.

Screening for *trans*-regulators of *CDR1* in *S. cerevisiae*. A *C. albicans* genomic library constructed in high-copy-number *S. cerevisiae* plasmid 2μm-*URA3* has been prepared (15) and was transformed into SLO1 containing the *CDR1p-lacZ* fusion. The *lacZ* gene was used as the reporter to monitor the activity of the *CDR1* promoter. The β-galactosidase (β-Gal) activity was determined by both a filter assay and a liquid assay, as described previously (21). The library of transformants (approximately 500 colonies per 150-mm plate) was grown for 3 days before the transformants were replica plated onto filters laid on top of agar medium. When a Ura^+ candidate strain containing a library plasmid had elevated β-Gal activity, as shown by a darker blue color than the control strain with only plasmid 2μm (1) in the filter assay, it was considered to harbor a candidate plasmid for the positive *trans*-regulatory factor of *CDR1*. When this was the case, the β-Gal activity of the strain would be reduced to the basal level when the plasmid was removed from the cells. Thus, the candidate strains were grown on YPD liquid medium for 2 days to lose the library plasmid. The β-Gal activities of the candidate plasmids and those of their counterparts which had lost the library plasmids were determined by β-Gal liquid assay.

Cloning of *CaNDT80*. Primers HJL72 (5′-CGGGATCCTTGTGGCGATTT TCACTTTC) and HJL73 (5′-CCGGATCCTCAATGGGGGTGGATTGA)

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
10560-2B	*MAT***a** *his3*::*hisG ura3–52 leu2*::*hisG*	G. Fink laboratory collection
10560-5B	*MAT*α *trp1*::*hisG ura3–52 leu2*::*hisG*	G. Fink laboratory collection
SLO1	*MAT***a** *his3*::*hisG ura3–52 leu2*::*hisG ade3*::*CDR1p-lacZ-LEU2*	This study
SLO2	*MAT***a** *his3*::*hisG ura3–52, leu2*::*hisG ade3*::*CDR1p-lacZ-LEU2*(pRS426)	This study
SLO3	*MAT***a** *his3*::*hisG ura3–52 leu2*::*hisG ade3*::*CDR1p-lacZ-LEU2*(LOB44)	This study
SLO4	*MAT***a** *his3*::*hisG ura3–52 leu2*::*hisG ade3*::*CDR1p-lacZ-LEU2*	This study
SLO5	*MAT***a** *his3*::*hisG ura3–52 leu2*::*hisG ade3*::*CDR1p-lacZ-LEU2*(LOB45)	This study

were used to amplify the genomic DNA containing the *CaNDT80* gene of *C. albicans* from strain SC5314. The amplified DNA fragment starts from the position 578 bp upstream of the predicted start codon of the *CaNDT80* gene to the position 479 bp downstream of the predicted stop codon (TAA) of *CaNDT80*. After digestion with BamHI, the DNA fragment was introduced into the pRS426 vector to generate plasmid LOB45.

Deletion of *CaNDT80* in *C. albicans*. A homozygous *Candt80*/*Candt80* mutant was constructed on the basis of the gene disruption method described previously (7, 34, 35), as shown in Fig. 2. The region from the position 279 bp downstream of the translation initiation site to the position 359 bp downstream of the stop codon of the *CaNDT80* gene was replaced by *GFP-ARG4*. A DNA fragment containing the *GFP-ARG4* construct flanked by short homologous regions (70 bp) of *CaNDT80* at the two extremities was transformed into *C. albicans* strain BWP17. The region from the position 101 bp downstream of the translation initiation site to the position 148 bp upstream of the stop codon of the second copy of *CaNDT80* was replaced by the *URA3-dpl200*-based cassette. A PCR product containing the *URA3-dpl200* sequence with the *CaNDT80* short homologous regions (70 bp) at the two extremities was transformed into YLO131 (*CaNDT80*/*Candt80*::*GFP-ARG4*). AccI-digested pT7tetR-*HIS1* was integrated into the *ENO1* promoter of YLO132, the *Candt80*/*Candt80* homozygous mutant, to create strain YLO133. A BamHI DNA fragment containing wild-type *CaNDT80* from plasmid LOB44 was inserted into the pGEM-*HIS1* vector to generate pGEM-*HIS1-CaNDT80*, referred to as plasmid LOB49. Plasmid LOB49 was digested with SpeI, located in the promoter of *CaNDT80*, and was transformed into YLO132 to generate strain YLO137 (*Candt80*/*Candt80*::*CaNDT80*).

Antifungal susceptibility tests. The Etest (23, 32) was used to determine the susceptibilities of *S. cerevisiae* strains containing either 2μm-*CaNDT80* or plasmid 2μm to antifungal agents. Fluconazole (0.016 to 256 μg/ml) and ketoconazole (0.002 to 32 μg/ml) Etest strips (AB BIODISK, Solna, Sweden) were used. Homogenized colonies isolated from an overnight SD plate were transferred to 0.85% NaCl to achieve a density of 5×10^6 cells/ml. A sterile swab was dipped into the inoculum suspension and was then used to swab evenly the entire agar surface of an SD plate. The Etest strips were applied to the plate when the excess moisture had been absorbed completely. The agar dilution method was used to determine the susceptibilities of the *C. albicans* isolates to antifungal agents. Fluconazole and voriconazole were prepared to final concentrations of 25 and 1 μg/ml, respectively, in dimethyl sulfoxide (DMSO). Cells grown on medium containing an equal amount of DMSO in the absence of drug were used as controls. Strains were diluted to an optical density at 600 nm of 2 (approximately 2×10^7 cells/ml), and approximately 0.5 μl per spot was spotted onto plates containing different drugs with a replica plating device (Oxoid Inc., Nepean, Ontario, Canada). The strains were also serially diluted 10-fold.

Quantitative analysis of mRNA level by real-time PCR. The *C. albicans* cells were harvested after they were grown in 20 ml of SD liquid medium in the absence or presence of 100 μg of miconazole per ml at 30°C for 1 h (optical density at 600 nm, 0.7 to 1.0). A real-time hot-start PCR was performed with an LC FastStartDNA Master SYBR Green I kit in a LightCycler instrument (catalog no. 2239264; Roche, Mannheim, Germany) to determine the level of mRNA. The real-time PCR was performed according to the instructions of the manufacturer. The expression of *HGT4* in each strain was used as a control. The relative quantitation used two standard curves for the comparisons, and the results are given as a ratio (11). For the miconazole induction assay, the level of RNA isolated from different strains that had not been treated with miconazole was defined as 100%. The relative level of mRNA isolated from different strains that had been treated with miconazole was normalized to the level of mRNA isolated from the same strain that had not been treated with miconazole. To determine the effects of mutations in *CaNDT80* after miconazole induction, the level of RNA isolated from the wild-type cells was defined as 100%.

RESULTS

Screening the *trans*-regulators of *CDR1*. A total of approximately 24,000 independent library transformant colonies, which should cover about three times the *C. albicans* genome size, were screened. Of the 74 candidates picked initially, 16 were confirmed by the second filter assay to have β-Gal activity. Among these 16 candidates, 5 had higher β-Gal activities than the rest of the candidates and were chosen for further analysis. The higher β-Gal activities of two candidates resulted from mutations in a chromosome(s) in *S. cerevisiae*, since the strains still had the same level of β-Gal activity after they lost the plasmid. In contrast, the β-Gal activities of the remaining three candidates were reduced to the basal level when they lost the plasmid. Two of these three candidates harbored the same plasmid, named LOB44.

Overexpression of *CaNDT80* increases the expression of *CDR1p-lacZ*. *S. cerevisia*e strain SLO3 containing plasmid LOB44 had approximately sixfold higher β-Gal activity than strain SLO2 containing plasmid 2μm by the β-Gal liquid assay. Plasmid LOB44 contains a genomic insert of approximately 5 kb. This DNA fragment contains two full-length open reading

TABLE 2. *C. albicans* strains used in this study

Strain	Genotype	Reference or source
SC5314	Wild type	8
BWP17	*ura3*Δ::λ *imm434*/*ura3*Δ::λ *imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG*	35
DSY448	*ura3*Δ::λ *imm434*/*ura3*Δ::λ *imm434 cdr1*::*hisG*/*cdr1*::*hisG-URA3-hisG*	29
YLO131	*ura3*Δ::λ *imm434*/*ura3*Δ::λ *imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG CaNDT80*/*Candt80*::*GFP-Arg4*	This study
YLO132	*ura3*Δ::λ *imm434*/*ura3*Δ::λ *imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG Candt80*::*GFP-Arg4*/*Candt80*::*URA3-dpl200*	This study
YLO133	*ura3*Δ::λ *imm434*/*ura3*Δ::λ *imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG Candt80*::*GFP-Arg4*/*Candt80*::*URA3-dpl200 ENO1*/*eno1*::*ENO1-tetR-ScHAP4-3xHA-HIS1*	This study
YLO137	*ura3*Δ::λ *imm434*/*ura3*Δ::λ *imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG Candt80*::*GFP-Arg4*/*Candt80*::*URA3-dpl200*::*CaNDT80*::*HIS1*	This study

TABLE 3. Plasmids used in this study

Plasmid	Description	Source or reference
B2159	*ADE3 URA3 CEN* plasmid	G. Fink laboratory collection
pRS305	*LEU2* 2μm plasmid	31
pRS315	*LEU2 CEN* plasmid	31
pRS426	*URA3* 2μm plasmid	1
YIp363	β-Gal reporter *LEU2*-integrating plasmid	22
pGEM-*HIS1*	*CaHIS1*-integrating plasmid	35
pRS-*ARG4*Δ*SpeI*	*CaARG4 CEN* plasmid	35
pDDB57	*CaURA3-dpl200* cassette	34
pGFP-*HIS1*	*GFP-CaHIS1* cassette	7
LOB42	1.2-kb *CDR1* promoter-*lacZ* in-frame fusion in YIp363	This study
LOB43	1.6-kb *ADE3* in LOB42	This study
LOB44	*CaNDT80 URA3* 2μm plasmid	This study
LOB45	2.8-kb full length *CaNDT80 URA3* 2μm PRS426 plasmid	This study
LOB46	*NDT80 URA3* 2μm plasmid	This study
LOB47	*GFP-CaARG4* cassette	This study
LOB48	*tetR-ScHAP4-3xHA CaHIS1*-encoding plasmid	This study
LOB49	*CaNDT80 CaHIS1*-integrating plasmid	This study

frames. One is CaNdt80 and the other is orf6.1265, a short hypothetical protein of 106 amino acids (aa). The genomic insert was used for subcloning to produce LOB45, which harbored a 2.8-kb fragment with only the full-length gene of *CaNDT80* generated by PCR. The β-Gal activity of parental strain SLO1 containing plasmid LOB45 (strain SLO5) was as high as that of the strain containing plasmid LOB44. These results demonstrate that the increased β-Gal activity of *CDR1p-lacZ* in *S. cerevisiae* is dependent on the presence of the *CaNDT80* sequence.

***CaNdt80* in *C. albicans* and *Ndt80* in *S. cerevisiae* share the same DNA binding domain.** The *CaNDT80* gene encodes a putative transcription factor. The transcription factor is 592 aa in length and shares the DNA binding domain with Ndt80, a meiosis-specific protein in *S. cerevisiae*. A comparison of the CaNdt80 and Ndt80 sequences is shown in Fig. 3. Ndt80 in *S. cerevisiae* is 672 aa. The sequence from amino acids 1 to 330 has been shown to be important for DNA binding activity. Two different domains have been identified. The residues from amino acids 1 to 58 are needed for sequence-specific interactions, and the residues from amino acids 59 to 330 contain a DNA binding domain. The sequence from amino acids 223 to 572 of Candt80 and those from amino acids 3 to the 330 of Ndt80 share 37.6% identity and 57.9% similarity (Fig. 3A). There was no similarity between the N terminus of CaNdt80 and the C terminus of Ndt80 (Fig. 3A).

Overexpression of *CaNDT80* decreases susceptibility to fluconazole and ketoconazole in *S. cerevisiae*. Overexpression of *CaNDT80* increased the expression of *CDR1p-lacZ*, suggesting that CaNdt80 is an activator of *CDR1* involved in drug resistance. According to the results of Etest, the overexpression of *CaNDT80* decreased the susceptibility of *S. cerevisiae* to both fluconazole and ketoconazole (Fig. 4). The MIC of fluconazole increased from 24 to 64 μg/ml when cells overexpressed *CaNDT80* (Fig. 4b). Consistently, the MIC of ketoconazole increased from 6 μg/ml to greater than or equal to 32 μg/ml when cells overexpressed *CaNDT80* (Fig. 4d). The strain containing plasmid LOB45, which harbored *CaNDT80*, had similar susceptibilities to the two antifungal drugs. Thus, these data

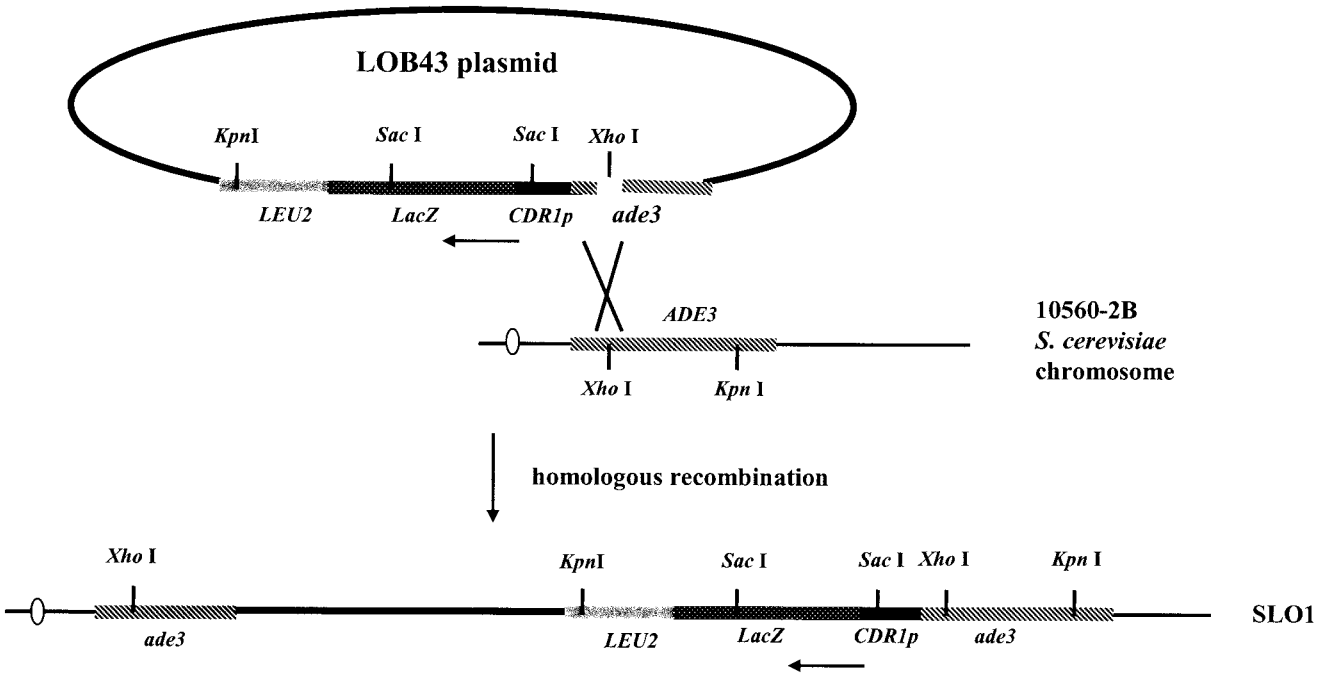

FIG. 1. Integration of the *CDR1p-lacZ* fusion in the *ADE3* locus in *S. cerevisiae*. Horizontal arrows indicate the direction of transcription from the *CDR1* promoter.

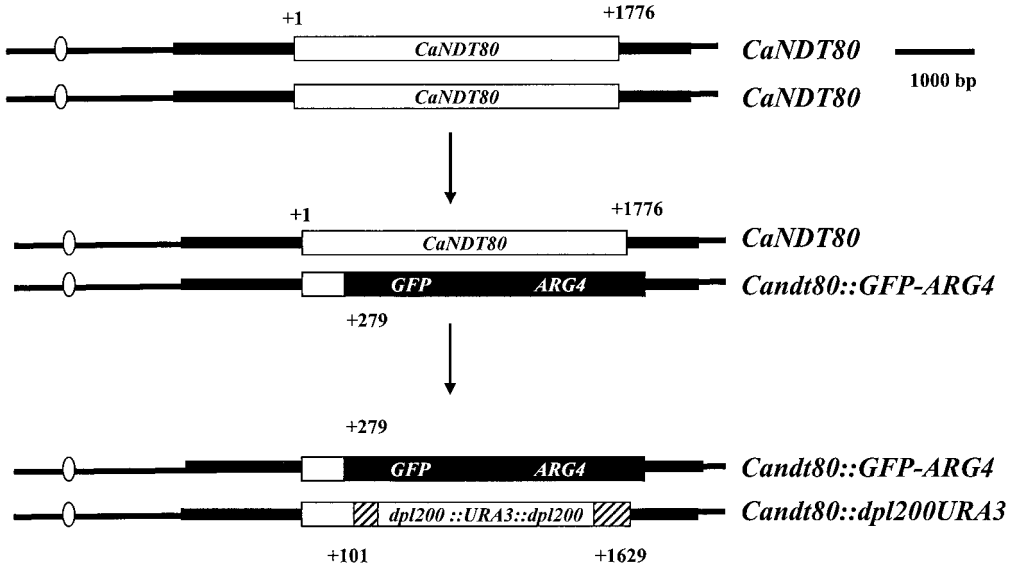

FIG. 2. Construction of a null mutation in *CaNDT80.* The two copies of the *CaNDT80* gene (open boxes) in the chromosome were sequentially replaced by the *GFP-ARG4* construct (solid boxes) and the *URA3-dpl200*-based cassette (hatched boxes).

A

B

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CaNdt80 :    1 MNQTLVRMMNMPNEDPNVSSEEAAAANVATIHNIKNEDSMHY  42
CaNdt80 :   43 QFGHHQPSIYDQQQQQQQQHHHQLPHHPIHYHGIAQQQQSQLPHFAMHPSAGVNNNQHLN 102
CaNdt80 :  103 HQPYQQMSHYNAQQMHQQQLHHQLMTPNPYQQHFQQQMHPQLHHEDHLNMHFNPMSYPQQ 162
CaNdt80 :  163 QQQQQQQQQQQQQQHLHHFGHQIPAPPAQQGPTPQQPHLHQQIPHPLSHHQTPQPTPQPLA 222
  Ndt80:    1 ------------------------------------------------------------MN   2
CaNdt80 :  223 QQQSPQPARQPRQTKKQKQQAQNQDQADAQSQAQQHHMAMMARANQNDMLESSTRKVAPR 282
               + ++  P  Q     K +++   + + D      Q    +      ++  +    K+APR
   Ndt80:    3 EMENTDPVLQDDLVSKYERELSTEQEEDTPVILTQ----LNEDGTTSNYFDKRKLKIAPR  58
CaNdt80 :  283 SSDLFRVGPPFSISKQHQPVYCVGTDMPVTPLLHARIDRGFEMGETGSWIGYKRNYFTLV 342
               S+  F+VGPPF + + + PV    T   +   + RIDRGF+  +   W+GYKRNYFTLV
   Ndt80:   59 STLQFKVGPPFELVRDYCPVVESHTGRTLDLRIIPRIDRGFDHIDE-EWVGYKRNYFTLV 117
CaNdt80 :  343 ASFTLQDFDFEKFIGNKFYTYDKVNNKVNGFPPHHPSHPQNQPQNHPGHPHHNQHAGESR 402
               ++F   + D + F+ + F    + ++ V                              ESR
   Ndt80:  118 STFETANCDLDTFLKSSFDLLVE-DSSV-----------------------------ESR 147
CaNdt80 :  403 VPISYFAIRLVAKCSDEDVAISLIQHTAKRDKGPQFPPPIYPAVPSELPDHETVKVSCNK 462
               + + YFAI++ AK  D+D  I+L+QHTAKRDKGPQF P + P VPS LP H+ ++ + N
   Ndt80:  148 LRVQYFAIKIKAKNDDDDTEINLVQHTAKRDKGPQFCPSVCPLVPSPLPKHQIIREASNV 207
CaNdt80 :  463 RNNNKIETMNKIFYFDRGNY-YQEYNLDSYKDQSILKSYPSQSISKVARFERIQFTSSIR 521
               RN  K++  +  FY  R +  Y+EY +D     S+L SYP  SI KVAR+ER+QF SSI
   Ndt80:  208 RNITKMKKYDSTFYLHRDHVNYEEYGVD-----SLLFSYPEDSIQKVARYERVQFASSIS 262
CaNdt80 :  522 VKSTNTTARYFTLHVELLGIIEDED-------------LQIQP------ILLSSIESPPL 562
               VK  +   ++F+LHV +LG + D D             L ++       + L  +++PPL
   Ndt80:  263 VKKPSQQNKHFSLHV-ILGAVVDPDTFHGENPGIPYDELALKNGSKGMFVYLQEMKTPPL 321
CaNdt80 :  563 IVRGRSPSSYHKDRTSGYRATNTPTPTPPQ*---------------------------- 592
               I+RGRSPS+Y
   Ndt80:  322 IIRGRSPSNYASSQRITVRTPSSVNSSQNSTKRKMPSMAQPLNESCLNARPSKRRSKVAL 381
   Ndt80:  382 GAPNSGASISPIKSRQSTPMEASKENEDPFFRPNKRVETLEHIQNKLGALKNQCPDSSLK 441
   Ndt80:  442 YPSSSSRGMEGCLEKEDLVYSSSFSVNMKQIELKPARSFEHENIFKVGSLALKKINELPH 501
   Ndt80:  502 ENYDITIEKKSMEQNYLRPEIGSRSECKTSYGNELSLSNISFSILPNSAENFHLETALFP 561
   Ndt80:  562 ATEEDVNRTFSRILETGSFQNYYQKMDAENADRVYSKGVKLIASGTLPSGIFNREELFEE 621
   Ndt80:  622 DSFYKY* 627
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FIG. 3. Comparison of CaNdt80 in *C. albicans* and Ndt80 in *S. cerevisiae*. (A) Overall comparison of CaNdt80 and Ndt80. The regions shared between these two proteins are shown as thick gray bars. The domains different between those two proteins are presented as thin black lines. (B) Protein sequences of CaNdt80 and Ndt80. The CaNdt80 sequence is shown in boldface.

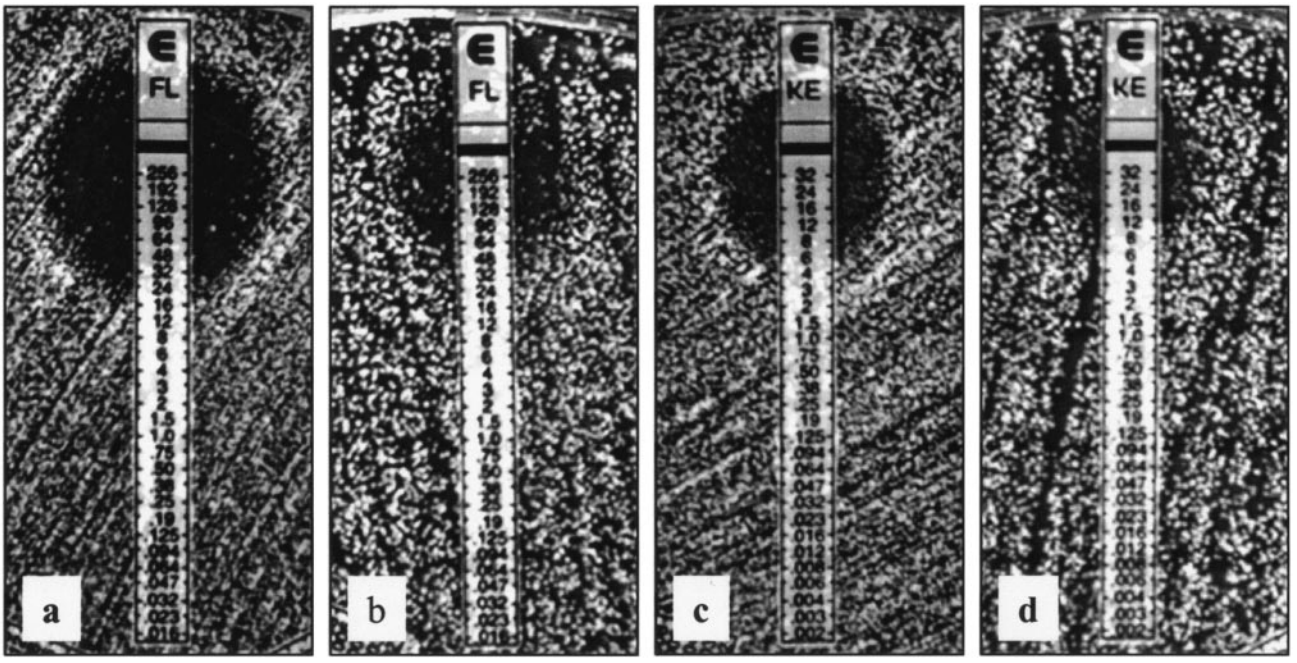

FIG. 4. Overexpression of *CaNDT80* reduces *S. cerevisiae* susceptibilities to fluconazole (FL) and ketoconazole (KE). Antifungal susceptibility was determined by Etest. (a and b) Fluconazole strips; (c and d) ketoconazole strips; (a and c) *S. cerevisiae* with the 2μm-*URA3* plasmid (1, 31) (SLO2); (b and d) *S. cerevisiae* with 2μm-*URA3*-*CaNDT80* plasmid LOB45 (SLO3). The results were photographed after 3 days of growth at 30°C.

suggest that CaNdt80 of *C. albicans* is capable of activating the gene involved in drug resistance in *S. cerevisiae*.

Mutations in *CaNDT80* reduce the expression of *CDR1* in *C. albicans*. The *CaNDT80* gene was expressed during vegetative growth in *C. albicans* (Fig. 5A, bar 7). The expression of *CaNDT80* was increased by treating the cells with miconazole (Fig. 5A; compare bars 7 and 8). To elucidate the functions of CaNdt80 in *C. albicans*, we have constructed a *Candt80*/*Candt80* homozygous mutant on the basis of the rapid gene disruption method (Fig. 2). No *CaNDT80* mRNA was detected in the *Candt80*/*Candt80* mutant, which therefore verified the presence of a null mutation in *CaNDT80*. As expected, *CDR1* expression was also induced by miconazole treatment (Fig. 5A; compare bars 5 and 6). Homozygous null mutations in *CaNDT80* abolished the induction of *CDR1* expression in the presence of the drug (Fig. 5A; compare bars 1 and 2). This result is consistent with the idea that CaNdt80 is an activator of *CDR1*. However, *CDR1* expression was not completely abolished by the null mutation in *CaNDT80*. In the presence of drugs, the level of *CDR1* mRNA in the *Candt80*/*Candt80* mutant strain was reduced to 15%, not 0%, of that in the wild-type strain (Fig. 5B; compare bars 1 and 3).

Mutations in *CaNDT80* increase susceptibilities to antifungal agents in *C. albicans*. According to the results of the agar dilution assay, strains with mutations in either *CDR1* or *CaNDT80* were more susceptible to fluconazole and voriconazole (Fig. 6). Cells grew in all spots in the absence of drugs (Fig. 6). The *Candt80*/*Candt80* mutant was more susceptible to drugs than the wild-type strain or the rescued strain containing a copy of wild-type *CaNDT80*. Few *cdr1*/*cdr1* cells grew on medium with drugs inoculated with 10^3 cells, while few *Candt80*/*Candt80* cells grew on medium with drugs inoculated with 10^2 cells (Fig. 6). Thus, the *cdr1*/*cdr1* mutant was more susceptible to drugs than the *Candt80*/*Candt80* mutant.

DISCUSSION

Potential targets of CaNdt80. In the present study, we have found that CaNdt80 is involved in drug resistance through regulation of the expression of *CDR1* in *C. albicans*. This observation is consistent with the findings from a previous report that the expression of *CaNDT80* in *C. albicans* is increased approximately threefold by itraconazole treatment (4). Overexpression of a fusion protein containing the potential *trans*-activation domain of CaNdt80 (the sequence from amino acids 1 to 216) and the DNA binding domain of Ndt80 (the sequence from amino acids 1 to 330) (13, 20) induced the expression of *CDR1p-lacZ* in *S. cerevisiae* (data not shown). These data suggest that the DNA binding domains of Ndt80 and CaNdt80 may recognize the same DNA sequence since the DNA binding domain of Ndt80 can replace that of CaNdt80. Ndt80 is autoregulated and activates its targets through the midsporulation element (MSE) consensus site (gNCRCAAA A/T, where g indicates a probable G, N indicates any nucleotide, and R indicates A or G) (3). One perfect MSE (gNCRC AAAA/T) is located 572 bp upstream of the translation-initiating codon (ATG) of *CaNDT80*; and three potential MSEs (CRCAAA) are located 70, 122, and 461 bp upstream of the translation initiation site. It will be interesting to determine whether CaNdt80, like Ndt80 in *S. cerevisiae*, also regulates its own expression.

Null mutations in *CaNDT80* abolish the induction of *CDR1* expression by drugs. Consistently, the promoter of *CDR1* contains three potential MSEs (CRCAAA) located 270, 438, and 835 bp upstream from the translation initiation site, respective-

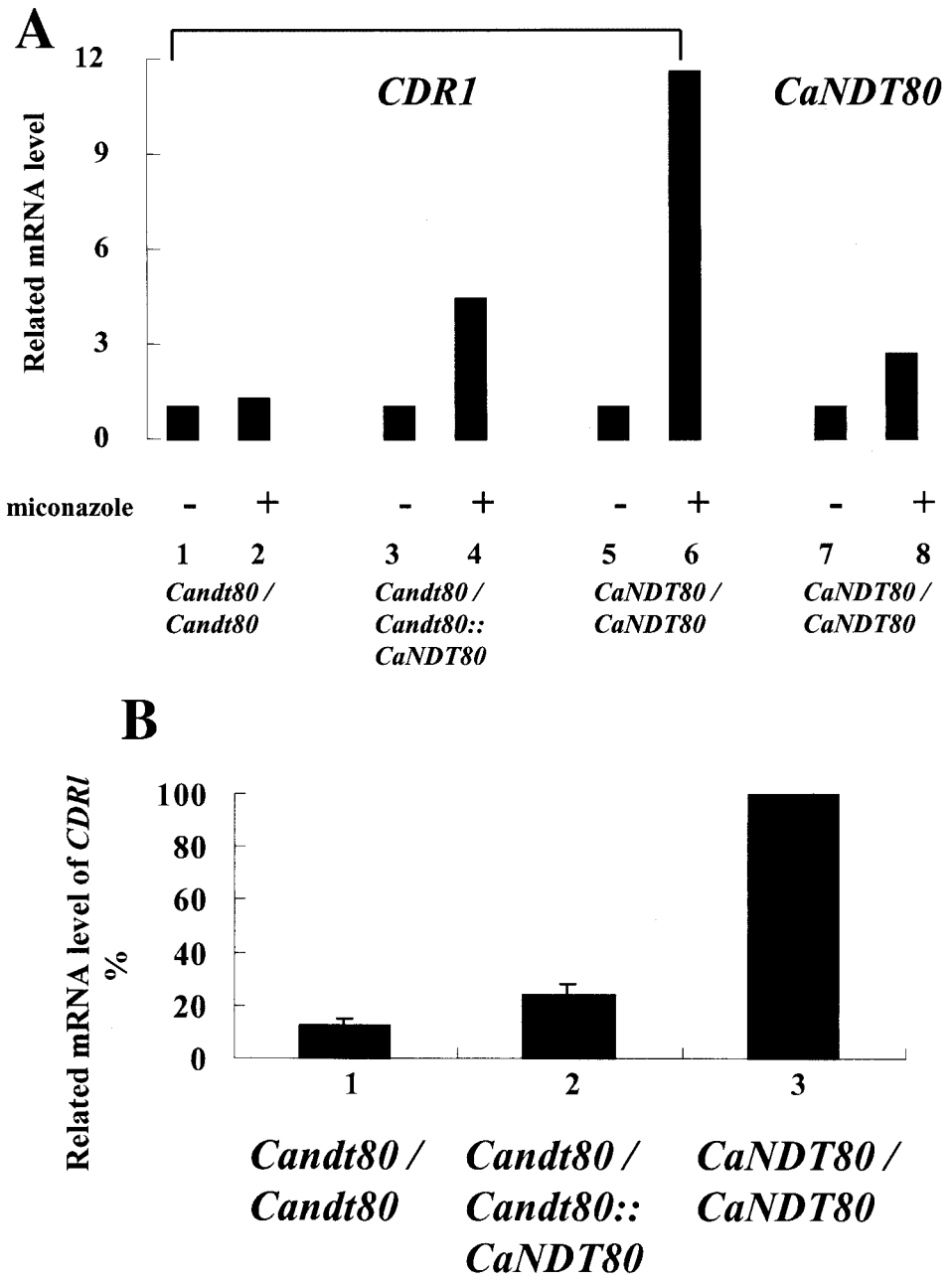

FIG. 5. Mutations in *CaNDT80* abolish the induction of *CDR1* by miconazole. (A) Total RNA was isolated from cells that were not treated (bars 1, 3, 5, and 7) or cells that were treated with 100 μg of miconazole per ml (bars 2, 4, 6, and 8) for 1 h at 30°C. The level of RNA isolated from different strains without miconazole treatment was defined as 100%. The relative level of mRNA isolated from different strains with miconazole treatment was normalized to the level of mRNA isolated from the same strain without miconazole treatment. (B) Bar 1, *Candt80/Candt80* (YLO133); bar 2, *Candt80*/*Candt80*::*CANDT80* (YLO137); bar 3, *CaNDT80*/*CaNDT80* (SC5314). The level of RNA isolated from wild-type cells was defined as 100%.

ly. The level of *CDR1* mRNA in the rescued strain is lower than that in the wild-type strain. Furthermore, the rescued strain (*Candt80*/*Candt80*::*CaNDT80*) is also more susceptible to fluconazole than the wild-type strain. These data suggest a dosage effect (+/+ > +/− > −/−) of *CaNDT80* in *C. albicans*, as has been documented in some other genes of *C. albicans* (12).

Overexpression of *CaNDT80* in *S. cerevisiae* decreased the susceptibility of the organism to antifungal agents, suggesting that in *S. cerevisiae* CaNdt80 may activate the genes involved in drug susceptibility, including *ERG11*, *PDR1*, and *TOP1*, whose promoters have either perfect or potential MSEs

Transcription factors with the DNA binding domain of CaNdt80. Ndt80 was identified as a meiosis-specific transcription factor required for *S. cerevisiae* to exit the pachytene stage (2, 3). Mutations in *NDT80* in *S. cerevisiae* did not alter the susceptibility to antifungal agents (unpublished data), which is consistent with previous findings that Ndt80 is active in *S. cerevisiae* diploid cells only during meiosis. The Orf6.4742 gene was

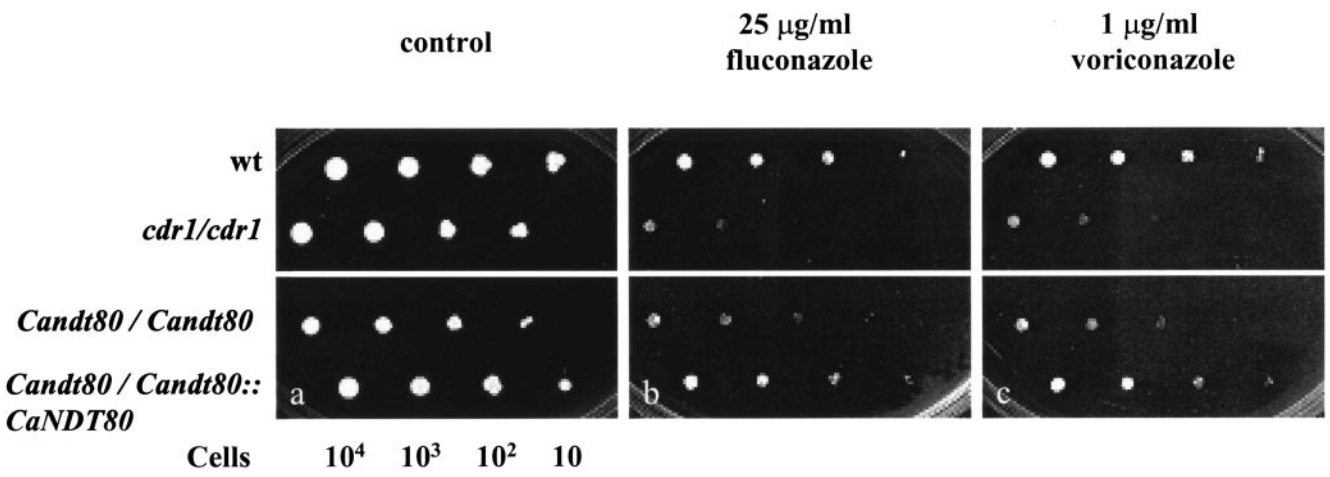

FIG. 6. Mutations in *CaNDT80* increase susceptibility to azole drugs in the agar dilution assay. Cells were grown on media in the absence of drug (control) and in the presence of fluconazole and voriconazole. The results for four strains, *CaNDT80*/*CaNDT80* (SC5314), *cdr1*/*cdr1* (DSY448), *Candt80*/*Candt80* (YLO133), and *Candt80*/*Candt80*::*CaNDT80* (YLO137) strains, were photographed after 2 days of growth at 30°C.

previously designated *CaNDT80* because of the highly conserved region of the novel DNA binding domain between CaNdt80 and Ndt80.

In addition to CaNdt80 and Ndt80, several proteins from higher eukaryotes, including *Neurospora crassa*, *Dictyostelium discoideum*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and humans, also contain sequences homologous to this novel DNA binding motif of CaNdt80 (20). Most metastatic tumors are more resistant to chemotherapeutic drugs than their primary counterparts, and many drug-resistant tumors are more invasive than nonresistant parental cells (14, 28). There seems to be a connection between the invasiveness (virulence) and the drug resistance of the tumor cells. Interestingly, C11orf9, a human transcription factor with a DNA binding domain similar to that of the novel DNA binding motif CaNdt80, is also highly expressed in invasive or metastatic tumor cells (10). Hence, this motif represents a new type of DNA binding domain and may consist of members with unique pathways.

The development of drug resistance and the limited variety of antifungal drugs available for clinical therapy are issues in treatments for infectious diseases. In the present study, we have shown that CaNdt80 regulates one of the drug resistance pathways of *C. albicans*. Hence, our findings may open a new doorway for the development and design of new effective agents for the treatment of microbial infections.

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