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Expression of the *CDR1* efflux pump in clinical *Candida albicans* isolates is controlled by a negative regulatory element

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Abstract

Resistance to azole antifungal drugs in clinical isolates of the human fungal pathogen *Candida albicans* is often caused by constitutive overexpression of the *CDR1* gene, which encodes a multidrug efflux pump of the ABC transporter superfamily. To understand the relevance of a recently identified negative regulatory element (NRE) in the *CDR1* promoter for the control of *CDR1* expression in the clinical scenario, we investigated the effect of mutation or deletion of the NRE on *CDR1* expression in two matched pairs of azole-sensitive and resistant clinical isolates of *C. albicans*. Expression of *GFP* or *lacZ* reporter genes from the wild type *CDR1* promoter was much higher in the azole-resistant *C. albicans* isolates than in the azole-susceptible isolates, reflecting the known differences in *CDR1* expression in these strains. Deletion or mutation of the NRE resulted in enhanced reporter gene expression in azole-sensitive strains, but did not further increase the already high *CDR1* promoter activity in the azole-resistant strains. In agreement with these findings, electrophoretic mobility shift assays showed a reduced binding to the NRE of nuclear extracts from the resistant *C. albicans* isolates as compared with extracts from the sensitive isolates. These results demonstrate that the NRE is involved in maintaining *CDR1* expression at basal levels and that this repression is overcome in azole-resistant clinical *C. albicans* isolates, resulting in constitutive *CDR1* overexpression and concomitant drug resistance.

Keywords: *Candida albicans*; *CDR1*; Multidrug resistance; Promoter; Regulation

Azole-resistant clinical *Candida albicans* isolates frequently exhibit reduced intracellular drug accumulation that correlates with enhanced expression of certain multiple drug resistance genes, the ATP-binding cassette (ABC) transporters, *CDR1* and *CDR2*, and the major facilitator, *CaMDR1* [1–5]. Fluconazole resistance is usually a stable phenotype that is maintained in the absence of selection pressure by the drug. This implies that certain genetic alterations have occurred in the resistant isolates that result in a constitutive overexpression of genes that encode the drug efflux pump proteins. Earlier studies suggested that *CDR1* is a highly regulatable gene, which is activated by several stresses like drugs, higher temperatures, and human steroids [6]. In silico analysis of the *CDR1* promoter region revealed the presence of several putative stress inducible *cis*-regulatory elements (HSE, MDR-NF1/YB-1, AP-1, etc.) [7]. Promoter deletion analyses and electrophoretic mobility shift assays (EMSAs) with *CDR1* promoter fragments confirmed the presence of a steroid responsive region (SRR) in the distal part of the promoter [8]. Deletion analysis within the SRR further delimited these steroid responsive sequences to two distinct elements, viz.,

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SRE1 and SRE2. Comparison of SRE1/2 with the promoter sequence of MDR (*CDR2* and *PDR5*) and non-MDR (*HSP90*) steroid responsive genes revealed a similarity with respect to conservation of three 5 bp stretches (AAGAA, CCGAA, and ATTGG) [8]. In addition, a common drug/steroid responsive element has also been identified in the *CDR1* and *CDR2* promoters of *C. albicans* [9].

The transcriptional activation of *CDR1* in the development of azole resistance is well known [4,6]. However, the mechanisms by which *CDR1* levels are altered in clinical resistant isolates are only partially understood [4]. *CDR1* expression in azole-resistant clinical isolates of *C. albicans* could be increased due to mutations in the promoter region of the gene, alterations in *trans*-regulatory factors controlling its expression, or molecular changes taking place during mRNA processing [10–13]. We had earlier identified a negative regulatory element (NRE) at positions −272 to −265 upstream of the transcriptional start point of *CDR1* [14]. Mutation or deletion of this sequence, 5′-CTGATTGA-3′, resulted in an activation of the *CDR1* promoter. A purified 55 kDa nuclear protein (NREBP) was shown to specifically bind to the NRE [14]. Considering the importance of the NRE in controlling expression of *CDR1*, in this study, we explored its clinical relevance. For this purpose, we investigated the role of the NRE in two matched pairs of azole-susceptible/-resistant clinical *C. albicans* isolates in which drug resistance correlated with the stable *CDR1* overexpression [4,15].

Materials and methods

Strains and media. All plasmids were maintained in *Escherichia coli* DH-5α. *E. coli* was cultured in LB (Luria–Bertani) broth or on LB plates, supplemented with ampicillin (0.1 mg/ml). *C. albicans* strains used in this study (Table 1) were maintained on YEPD (yeast extract 1%, bactopeptone 2%, and glucose 2%). When grown on solid media, 2.5% agar was added to the liquid media.

Plasmid construction and yeast transformation. Plasmids pCPL1, pCPLM, pCPLD, pCPG1, pCPGM, and pCPGD contain *lacZ* or *GFP* reporter genes under control of the wild type *CDR1* promoter or derivatives in which the NRE was mutated or deleted (see Figs. 1A and B). The flanking *ACT1* sequences in these plasmids serve for genomic integration of the reporter fusions into the *ACT1* locus of clinical *C. albicans* isolates and the dominant *caSAT1* marker was used to select nourseothricin-resistant transformants. For construction of pCPL1, an *Xho*I–*Pst*I fragment from plasmid pMEP2LACZ2 [16] containing the *Streptococcus thermophilus lacZ* gene [17], the transcription termination sequence of the *C. albicans ACT1* gene, and the *URA3* selection marker was first ligated between flanking *ACT1* sequences of plasmid pMPG2 (S. Stahl and J. Morschhäuser, unpublished) to obtain pLACZ6. An *Xho*I–*Pst*I fragment containing the *caSAT1* selection marker [18] was then substituted for the *Sal*I–*Pst*I fragment containing the *URA3* marker to generate pLACZ7. A *Kpn*I–*Apa*I fragment containing 0.5 kb of the *ACT1* coding region was PCR amplified from genomic DNA of *C. albicans* strain SC5314 with the primers ACT23

Table 1
C. albicans strains used in this study

Strain	Description	Source
Gu4	Fluconazole-susceptible clinical isolate	Franz et al. [15]
NGY4G	*CDR1*p-*GFP* (wild type) integrated in Gu4 at *ACT1* locus	This study
NGY4MG	*CDR1*p-*GFP* (NREmut) integrated in Gu4 at *ACT1* locus	This study
NGY4DG	*CDR1*p-*GFP* (NREdel) integrated in Gu4 at *ACT1* locus	This study
NGY4L	*CDR1*p-*lacZ* (wild type) integrated in Gu4 at *ACT1* locus	This study
NGY4ML	*CDR1*p-*lacZ* (NREmut) integrated in Gu4 at *ACT1* locus	This study
NGY4DL	*CDR1*p-*lacZ* (NREdel) integrated in Gu4 at *ACT1* locus	This study
Gu5	Fluconazole-resistant clinical isolate	Franz et al. [15]
NGY5G	*CDR1*p-*GFP* (wild type) integrated in Gu5 at *ACT1* locus	This study
NGY5MG	*CDR1*p-*GFP* (NREmut) integrated in Gu5 at *ACT1* locus	This study
NGY5DG	*CDR1*p-*GFP* (NREdel) integrated in Gu5 at *ACT1* locus	This study
NGY5L	*CDR1*p-*lacZ* (wild type) integrated in Gu5 at *ACT1* locus	This study
NGY5ML	*CDR1*p-*lacZ* (NREmut) integrated in Gu5 at *ACT1* locus	This study
NGY5DL	*CDR1*p-*lacZ* (NREdel) integrated in Gu5 at *ACT1* locus	This study
DSY294	Fluconazole-susceptible clinical isolate	Sanglard et al. [4]
NGY294G	*CDR1*p-*GFP* (wild type) integrated in DSY294 at *ACT1* locus	This study
NGY294MG	*CDR1*p-*GFP* (NREmut) integrated in DSY294 at *ACT1* locus	This study
NGY294DG	*CDR1*p-*GFP* (NREdele) integrated in DSY294 at *ACT1* locus	This study
NGY294L	*CDR1*p-*lacZ* (wild type) integrated in DSY294 at *ACT1* locus	This study
NGY294ML	*CDR1*p-*lacZ* (NREmut) integrated in DSY294 at *ACT1* locus	This study
NGY294DL	*CDR1*p-*lacZ* (NREdel) integrated in DSY294 at *ACT1* locus	This study
DSY296	Fluconazole-resistant clinical isolate	Sanglard et al. [4]
NGY296G	*CDR1*p-*GFP* (wild type) integrated in DSY296 at *ACT1* locus	This study
NGY296MG	*CDR1*p-*GFP* (NREmut) integrated in DSY296 at *ACT1* locus	This study
NGY296DG	*CDR1*p-*GFP* (NREdel) integrated in DSY296 at *ACT1* locus	This study
NGY296L	*CDR1*p-*lacZ* (wild type) integrated in DSY296 at *ACT1* locus	This study
NGY296ML	*CDR1*p-*lacZ* (NREmut) integrated in DSY296 at *ACT1* locus	This study
NGY296DL	*CDR1*p-*lacZ* (NREdel) integrated in DSY296 at *ACT1* locus	This study

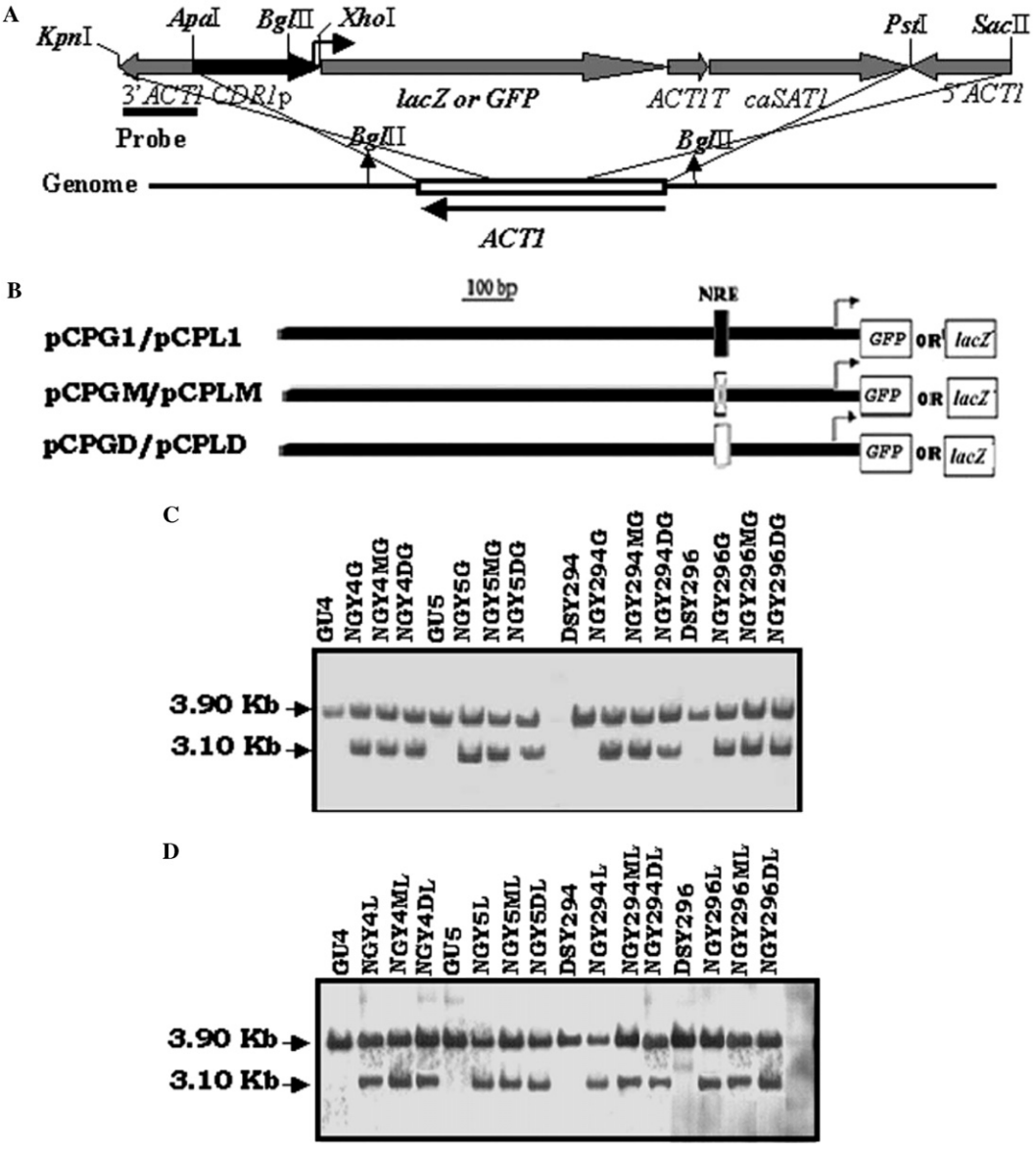

Fig. 1. Integration of *CDR1*p-*GFP* and *CDR1*p-*lacZ* fusions into the genome of clinical *C. albicans* isolates. (A) Structure of the DNA fragments used for integration of *CDR1*p-*GFP* or *CDR1*p-*lacZ* reporter fusions at the *ACT1* locus of the clinical *C. albicans* isolates. The genomic *ACT1* locus is represented by an open bar, the straight arrow indicating the direction of transcription. The dark arrow represents the *CDR1* promoter. The probe used to verify the correct integration is indicated by the thick line. Relevant restriction sites are shown. (B) Schematic representation of the *CDR1* promoter–reporter gene constructs. The crossed and open boxes indicate the mutation and deletion, respectively, of the NRE in the *CDR1* promoter. The names of the plasmids harboring the reporter fusions are shown on the left. (C,D) Southern hybridization of *Bgl*II-digested genomic DNA of the parental clinical *C. albicans* isolates and transformants containing the various *CDR1*p-*GFP* (C) and *CDR1*p-*lacZ* (D) reporter fusions with the *ACT1*-specific probe shown in (A). The sizes of the hybridizing fragments are given on the left side of the blots. The appearance of a 3.1 kb *Bgl*II fragment in the transformants in addition to the 3.9 kb wild type *ACT1* fragment confirms correct integration of the reporter gene fusions in one of the *ACT1* alleles.

(5′-GACATAACAATGGTACCGTATAATTC-3′) and ACT34 (5′-CGGACGATATGGGCCCAATCTGGCATCAC-3′) (the introduced *Kpn*I and *Apa*I restriction sites are underlined). In addition, an *Apa*I–*Xho*I fragment containing 1196 bp of *CDR1* upstream sequences was amplified from SC5314 genomic DNA with the primers CDR1F (5′-GATCGGGCCCTCGTTACTCAATAAGTAT-3′) and CDR1R (5′-AGCTCTCGAGTTCTTTTTGACCTTTTAAAG-3′). The *ACT1* fragment and the *CDR1* promoter fragment were ligated together into the *Kpn*I/*Xho*I-digested vector pLACZ7 to generate pCPL1, which contains the *lacZ* reporter gene under control of the wild type *CDR1* promoter. Substitution of a *C. albicans*-adapted *GFP* gene [19] for the *lacZ* gene generated pCPG1. A mutation (CTGATTGA to CCCCGGGG) or deletion of the NRE was introduced in pCPL1 and pCPG1 by site directed mutagenesis using the primer pairs FMF/FMR and FDLF/FDLR for NRE mutation and deletion, respectively [14]. The resulting plasmids were named pCPLM (NRE mutated), pCPLD (NRE deleted), pCPGM (NRE mutated), and pCPGD (NRE deleted). All constructs were confirmed by sequencing. The gel-purified *Kpn*I–*Sac*II fragments from pCPL1, pCPG1, pCPLM, pCPLD, pCPGM, and pCPGD, containing the *CDR1*p-*lacZ* and *CDR1*p-*GFP* fusions

(see Fig. 1A), were used to transform the fluconazole-susceptible clinical *C. albicans* isolates Gu4 and DSY294 as well as the corresponding fluconazole-resistant matched isolates Gu5 and DSY296. Transformations were performed as described earlier [14,18] and transformants were selected on YPD agar plates containing 200 μg/ml of nourseothricin after 24 h of incubation at 30 °C.

Microscopy. The strains were grown overnight in YPD liquid medium and aliquots were spotted on microscopic slides. Fluorescence was detected with a Zeiss Axiolab microscope equipped for epifluorescence microscopy with a 5-W mercury high-pressure bulb and the Zeiss fluorescein-specific filter set 09 [8,11].

β-Galactosidase assays. β-Galactosidase assays were performed as described by Uhl and Johnson [17]. Visual screening was carried out by streaking the *C. albicans* strains onto minimal media plates containing X-Gal. For plates, 1.70 g yeast nitrogen base without amino acids or ammonium sulfate, 20 g glucose, 5 g ammonium sulfate, and 20 g agar were dissolved in 930 ml H_2O. After autoclaving, 70 ml of 1 M potassium phosphate, pH 7.0, and 2.0 ml of a 20 mg/ml X-Gal solution were added [17].

For liquid assays, 1 ml of cell culture was pelleted and the cells were resuspended in an equal volume of *lacZ* buffer and placed on ice. The OD_{600} was determined for each sample [17]. Then 100 μl of cells was added to the *lacZ* buffer to a final volume of 1.0 ml and the cells were permeabilized with 15 μl of 0.1% SDS and 30 μl chloroform. Cells were equilibrated at 37 °C for 15 min and then 0.2 ml of ONPG (4 mg/ml) was added, and the cells were mixed and incubated at 37 °C. Reactions were stopped by addition of 0.5 ml of 1 M Na_2CO_3 and centrifuged for 5 min at 10,000*g* and the OD of the samples was measured at 420 and 550 nm. Units of activity were determined by the standard equation given by Ausubel [17].

Electrophoretic mobility shift assay. The EMSA was performed essentially as reported earlier [14]. The double stranded oligonucleotide harboring the NRE, generated by annealing of complementary strands (NRE1: 5′-GATCTGCCAACTGATTGAGGTTGA-3′, NRE2: 5′-GATCTCAACCTCAATCAGTTGGCA-3′), was end labeled with [γ-^{32}P]ATP and incubated with 30 μg of nuclear extracts isolated from each susceptible and resistant isolate for 20 min at room temperature [14]. The protein content of nuclear extracts for each isolate was estimated by Bradsford method and also checked on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [14].

Results

Expression of CDR1p-GFP reporter fusions in matched azole-susceptible and resistant clinical C. albicans isolates

In order to explore the significance of the NRE in the regulation of *CDR1* expression in drug-resistant clinical isolates of *C. albicans*, we monitored the activity of the wild type *CDR1* promoter and derivatives in which the NRE was either mutated or deleted in two matched azole-susceptible and resistant clinical *C. albicans* isolate pairs using *GFP* as a reporter gene. The wild type *CDR1* promoter was amplified from the commonly used azole-susceptible *C. albicans* model strain SC5314 (*CDR1*p-*GFP*, wild type) and an NRE mutation (*CDR1*p-*GFP*, NREmut) or deletion (*CDR1*p-*GFP*, NREdel) was introduced by site directed mutagenesis (see Materials and methods). To avoid recombination between the cloned *CDR1* promoters and the resident *CDR1* promoter of the host strains, the reporter fusions were integrated at the *ACT1* locus (see Fig. 1A). Nourseothricin-resistant transformants of the azole-susceptible *C. albicans* isolates Gu4 and DSY294, and of the corresponding matched, azole-resistant isolates Gu5 and DSY296, respectively [4,15], were selected using the recently developed dominant selection marker *caSAT1* [18]. Single copy integration was confirmed by Southern hybridization (Fig. 1C) and the resulting reporter strains were designated as NGY4G (*CDR1*p-*GFP*, wild type), NGY4MG (*CDR1*p-*GFP*, NREmut), NGY4DG (*CDR1*p-*GFP*, NREdel), NGY5G (*CDR1*p-*GFP*, wild type), NGY5MG (*CDR1*p-*GFP*, NREmut), NGY5DG (*CDR1*p-*GFP* NREdel), NGY294G (*CDR1*p-*GFP*, wild type), NGY294MG (*CDR1*p-*GFP*, NREmut), NGY294DG (*CDR1*p-*GFP*, NREdel), NGY296G (*CDR1*p-*GFP*, wild type), NGY296MG (*CDR1*p-*GFP*, NREmut), and NGY296DG (*CDR1*p-*GFP*, NREdel) (see Table 1 for details). No growth differences were observed between transformants and their respective parental strains in the absence of nourseothricin (data not shown).

The expression of the *CDR1*p-*GFP* fusions was analyzed by epifluorescence microscopy of cells grown to mid-log phase ($OD_{600} = 1.0$) in YPD liquid medium. Two transformants of each strain were analyzed. As shown in Fig. 2, all transformants of susceptible (Gu4 and DSY294) and resistant (Gu5 and DSY296) isolates (NGY4G, NGY294G, NGY5G, and NGY296G) containing the wild type *CDR1*p-*GFP* fusion showed a green fluorescent phenotype, but the fluorescence intensity of the cells was much higher in azole-resistant strains (NGY5G and NGY296G) as compared with their matched sensitive counterparts (NGY4G and NGY294G). This observation is in agreement with the previously described increased *CDR1* transcript levels in the resistant isolates [4,15]. The fact that the *CDR1* promoter from the azole-susceptible *C. albicans* strain SC5314 displayed stronger activity in resistant isolates than in the matched sensitive isolates demonstrated that in both resistant isolates *CDR1* overexpression was caused by alterations in *trans*-regulatory factors [20,21]. Interestingly, in the azole-susceptible strains expressing *GFP* from *CDR1* promoters in which the NRE was mutated or deleted (NGY4MG, NGY4DG and NGY294MG, NGY294DG), the intensity of fluorescence was found to be much stronger than in the corresponding strains that expressed *GFP* from the wild type *CDR1* promoter (NGY4G and NGY294G). These results matched well with our previous *CDR1* promoter deletion analysis in which NRE deletion/mutation led to derepression of *Renilla* luciferase reporter activity in a *C. albicans* laboratory strain [14]. In contrast, mutation or deletion of the NRE did not result in a further increase in the fluorescence intensity of azole-resistant strains expressing the *CDR1*p-*GFP* fusions (Fig. 2).

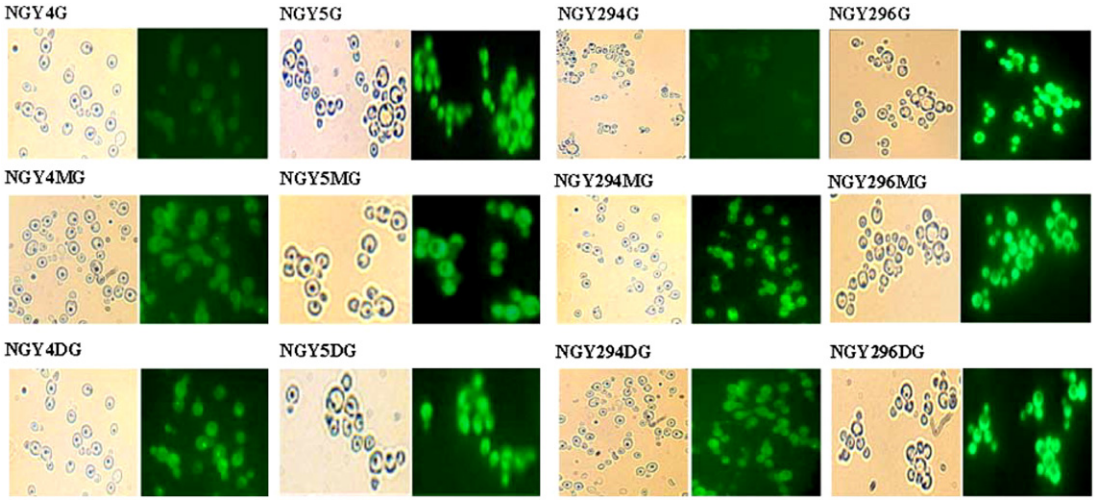

Fig. 2. Expression of the *GFP* reporter gene from wild type as well as NRE mutated and deleted *CDR1* promoters in azole-susceptible and resistant *C. albicans* isolates. Phase contrast (left) and corresponding fluorescence (right) micrographs of representative transformants harboring the chromosomally integrated *CDR1*p-*GFP* fusions.

Azole-resistant C. albicans isolates overcome the negative effect of the NRE on CDR1 expression

The activity of the various *CDR1* promoter derivatives in the clinical *C. albicans* isolates was confirmed using *lacZ* instead of *GFP* as a reporter gene in plate and liquid assays [17]. For this purpose, the *lacZ* gene from *S. thermophilus* [17] was fused to the wild type *CDR1* promoter (*CDR1*p-*lacZ*, wild type) and the derivatives in which the NRE was mutated (*CDR1*p-*lacZ*, NREmut) or deleted (*CDR1*p-*lacZ*, NREdel) (see Materials and methods). The *CDR1*p-*lacZ* reporter constructs were integrated at the *ACT1* locus of the two pairs of matched azole-susceptible and resistant isolates in the same way as the *CDR1*p-*GFP* fusions (see Figs. 1A and B). Single copy integration was confirmed by Southern hybridization (Fig. 1D) and the resulting reporter strains were designated as NGY4L (*CDR1*p-*lacZ*, wild type), NGY4ML (*CDR1*p-*lacZ*, NREmut), NGY4DL (*CDR1*p-*lacZ*, NREdel), NGY5L (*CDR1*p-*lacZ*, wild type), NGY5ML (*CDR1*p-*lacZ*, NREmut), NGY5DL (*CDR1*p-*lacZ*, NREdel), NGY294L (*CDR1*p-*lacZ*, wild type), NGY294ML (*CDR1*p-*lacZ*, NREmut), NGY294DL (*CDR1*p-*lacZ*, NREdel), NGY296L (*CDR1*p-*lacZ*, wild type), NGY296ML (*CDR1*p-*lacZ*, NREmut), and NGY296DL (*CDR1*p-*lacZ*, NREdel), respectively (see Table 1 for details).

Expression of the *CDR1*p-*lacZ* reporter fusions was first qualitatively observed by streaking two transformants of each strain and the parental control (without *lacZ*) on X-Gal plates. All transformants of resistant strains exhibited a dark colored phenotype after 48 h incubation at 30 °C, demonstrating high *lacZ* expression, whereas transformants of the sensitive isolates showed a less intense staining, indicative of a comparably lower *lacZ* expression (Fig. 3A, left panel). However, the sensitive strains exhibited an increased staining when the NRE was mutated or deleted in the *CDR1*p-*lacZ* reporter fusion (Fig. 3A, middle and right panels).

For better comparison of *CDR1* promoter activities, expression of the *CDR1*p-*lacZ* fusions was quantified in liquid β-galactosidase assays. As depicted in Fig. 3B, *lacZ* expression from the wild type *CDR1* promoter was about fivefold higher in transformants of the resistant isolate Gu5 (NGY5L) than in transformants of the matched susceptible isolate Gu4 (NGY4L) and approximately 21-fold higher in transformants of the resistant isolate DSY296 (NGY296L) than in transformants of the corresponding susceptible isolate DSY294 (NGY294L), which exhibited the lowest basal *CDR1* expression levels. In agreement with the results obtained with the *GFP* reporter gene, mutation as well as deletion of the NRE from the *CDR1* promoter resulted in higher *CDR1*p-*lacZ* expression levels in transformants of the susceptible isolates, but not in transformants of the resistant isolates. The β-galactosidase activity in the Gu4 transformants NGY4ML (NREmut) and NGY4DL (NREdel) was about threefold higher than in NGY4L (wild type), and the DSY294 transformants NGY294ML (NREmut) and NGY294DL (NREdel) exhibited approximately ninefold higher *lacZ* expression levels than NGY294L (wild type). Altogether, these results demonstrate that the NRE is involved in maintaining *CDR1* expression at basal levels in azole-susceptible *C. albicans* isolates and that this inhibitory function is overcome in some way in azole-resistant isolates, resulting in constitutive *CDR1* overexpression.

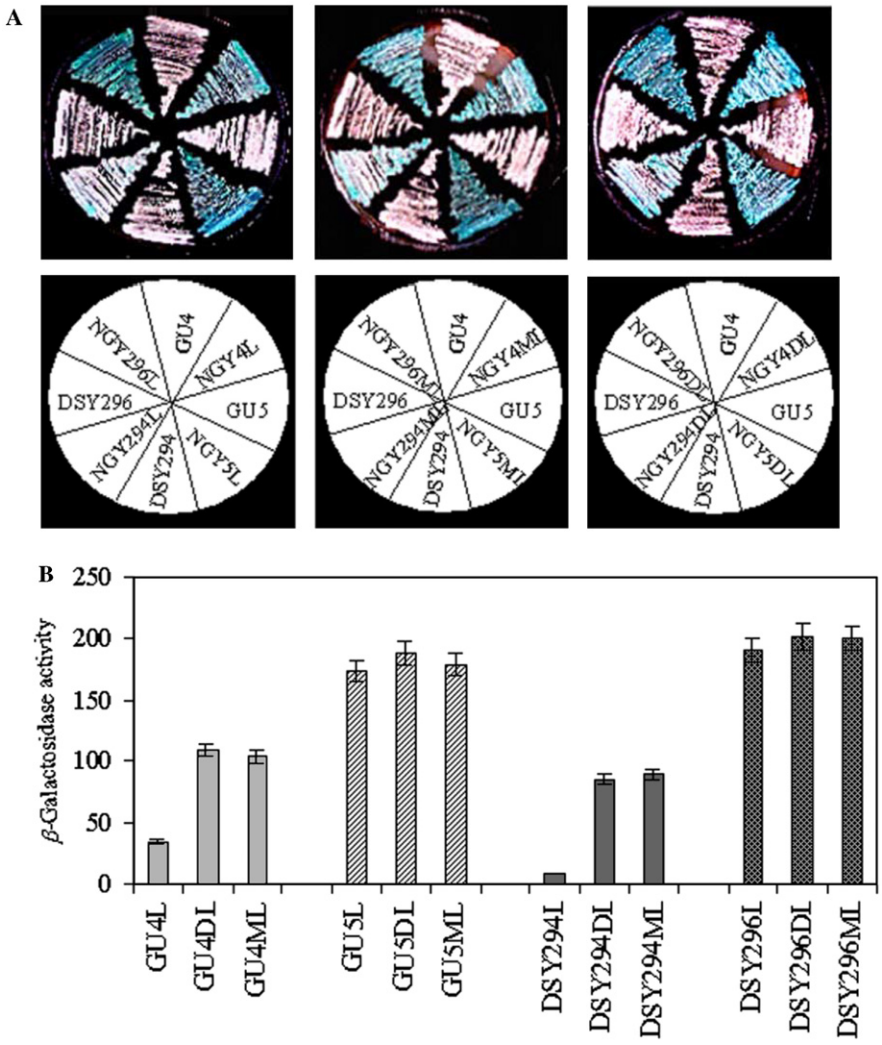

Fig. 3. Effect of mutation or deletion of the NRE in the *CDR1* promoter on β-galactosidase activity in clinical isolates of *C. albicans*. (A) Transformants harboring the chromosomally integrated *CDR1*p-*lacZ* fusions and their parental strains (without *lacZ*) were streaked on minimal media plates containing X-gal and photographed after 48 h of growth at 30 °C (upper panels). The positions of the individual strains on the plates are shown in the scheme below. (B) β-Galactosidase activities of strains harboring the various *CDR1*p-*lacZ* reporter fusions were determined as described in Materials and methods. The values are means ± SD (indicated by the bars) of three independent experiments with duplicate measurements with two independent clones. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Azole-resistant C. albicans isolates display reduced binding of NRE-binding protein (NREBP) to the CDR1 promoter

Since mutation as well as deletion of the NRE resulted in enhanced *CDR1* promoter activity only in azole-susceptible isolates, we hypothesized that the NRE-binding protein keeps *CDR1* expression down-regulated in susceptible isolates but may fail to do so in azole-resistant isolates, resulting in derepression of the *CDR1* promoter. Therefore, we performed electrophoretic mobility shift assays (EMSAs) with a radioactively labeled synthetic double stranded oligonucleotide harboring the NRE and equal amounts of nuclear extract proteins isolated from the fluconazole-susceptible (Gu4 and DSY294) and resistant (Gu5 and DSY296) *C. albicans* isolates. As shown in Fig. 4, a single, specific DNA–protein complex was formed with nuclear extracts from sensitive and resistant isolates. However, the signal intensity of the shifted DNA fragment was strongly reduced when the nuclear extracts from the azole-resistant isolates Gu5 and DSY296 were used in the binding reaction as compared with the signal intensity of the corresponding complex formed with nuclear extracts from the azole-susceptible isolates Gu4 and DSY294.

Discussion

In this study, we have explored the clinical relevance of a negative regulatory element (NRE) which we had

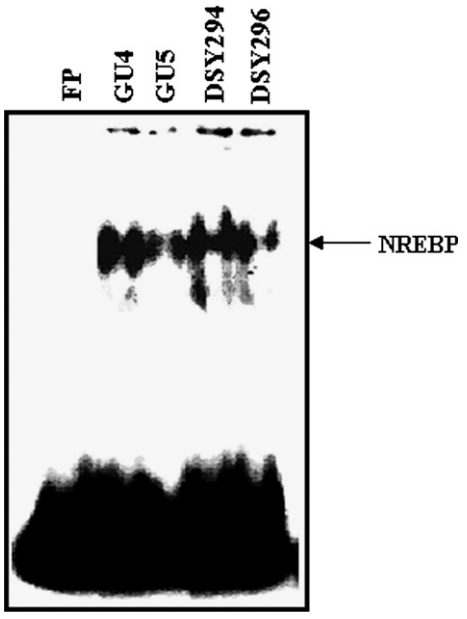

Fig. 4. Differential interaction of NREBP with NRE of *CDR1* in clinical isolates. EMSAs were performed using a synthetic double stranded oligonucleotide (annealed) harboring the NRE as [γ-^{32}P]ATP labeled probe and 30 μg of nuclear extract isolated from each drug-susceptible (Gu4 and DSY294) and resistant (Gu5 and DSY296) isolates. FP: free probe (without protein).

earlier identified in the *CDR1* promoter region [14]. For this purpose, two different reporter genes, *GFP* and *lacZ*, were placed under the control of the *CDR1* promoter from an azole-susceptible *C. albicans* strain and integrated at an ectopic locus in the genome of two pairs of matched clinical *C. albicans* isolates in which stable *CDR1* overexpression coincided with azole resistance. We observed that the reporter genes were expressed at higher levels in the azole-resistant strains as compared with the corresponding sensitive strains, although the *CDR1* promoter fragment controlling their expression was the same in all cases. This result demonstrated that in both resistant isolates the *CDR1* promoter was upregulated by alterations in *trans*-regulatory factors. In agreement with this, sequence analysis of the *CDR1* upstream region (1196 bp from ATG) did not reveal any differences in isolates Gu4 and Gu5 (data not shown), and no mutations in the regulatory region of *CDR1* had been detected by others in the second pair of isolates used in our present study, DSY294/DSY296 [9]. Coste et al. recently reported that a mutated allele of the transcriptional activator of *CDR* genes (*TAC1*) is responsible for *CDR1* and *CDR2* overexpression in the azole-resistant isolate DSY296 [22].

Mutation and deletion of the NRE gave further insight into its role in the control of *CDR1* expression in the clinical *C. albicans* isolates. Deletion or mutation of the NRE resulted in activation of the *CDR1* promoter in the azole-susceptible isolates, but did not further enhance the constitutively elevated *CDR1* promoter activity in the azole-resistant isolates. From these results we conclude that the NRE is involved in maintaining *CDR1* expression at basal levels in the absence of inducing conditions and that the NRE-mediated repression is overcome in azole-resistant clinical *C. albicans* isolates, resulting in constitutive *CDR1* overexpression. A ~55 kDa protein from nuclear extracts was previously found to specifically bind to and protect the NRE from DNaseI digestion in in vitro experiments. In the present study, we observed reduced binding to a *CDR1* promoter fragment comprising the NRE of nuclear extracts from the azole-resistant isolates as compared with extracts from the azole-sensitive isolates. Furthermore, DNaseI footprinting revealed no protection of the NRE by nuclear extracts from resistant isolates (data not shown). Taken together, these results suggest that in azole-sensitive *C. albicans* strains the NREBP is bound to the NRE and functions as a transcriptional repressor to keep *CDR1* expression at basal levels. In azole-resistant isolates binding of the NREBP to the *CDR1* promoter is impaired, resulting in activation of *CDR1* expression. Whether the reduced binding of the NREBP to the NRE is caused by decreased NREBP levels, altered cellular localization, or protein modification remains to be established. However, the recent findings by Coste et al. [22] imply that at least in the clinical isolate DSY296 the transcriptional activator Tac1p may be involved in overcoming the NRE-mediated repression.

In silico analysis revealed that the NRE (5′-CTGA TTGA-3′) of *CDR1* harbors ATTGA and shows significant homology with an inverted CCAAT element (ICE), also known as the Y-box (ATTGA), which is present in the promoter region of several genes including human *MDR1* and topoisomerase IIα (topo IIα) that have been shown to be involved in the development of drug resistance in human cancer cells [23,24]. Interestingly, point mutation in the ICE of the topo IIα promoter resulted in a significant increase in its transcriptional activity in drug-sensitive CEM cells (a human leukemia cell line) which was not the case in drug-resistant cells [23]. In addition, in EMSAs the element was recognized by a protein complex that was present at reduced levels in drug-resistant cell lines, suggesting a negative regulatory role of the ICE in transcriptional activation of human topo IIα [23], similar to the proposed role of the NRE in the *C. albicans CDR1* promoter.

Of note, the transcriptional activation of *PDR5* of *S. cerevisiae*, a close homologue of Cdr1p, is simultaneously controlled by both transcriptional activators (*PDR1* and *PDR3*) and a repressor (*RDR1*) [25–28]. *PDR1* and *PDR3* are considered as the master regulators in *S. cerevesiae*, because mutations in their activation domain generated hyperactive alleles [29,30]. Interestingly, the activators (*PDR1* and *PDR3*) and repressor (*RDR1*) in *S. cerevesiae* recognize a common

cis-regulatory element (PDRE) and contribute to the expression of *PDR5* [25,31]. Transcriptional regulation of *CDR1* also appears to be the result of a combinatorial effect of positive and negative regulatory factors. Coste et al. [22] also suggested that in addition to *TAC1* other regulators of *CDR1* may exist. One such regulator, *CaNDT80*, was recently identified by Chen et al. [32]. Inactivation of *CaNDT80* did not completely abolish the drug-induced upregulation of *CDR1*, implying the involvement of other regulators which may contribute to the overexpression of *CDR1*. The *CDR1* regulatory region contains multiple upstream activating (UAS) and repressing sequences (URS), which may interact with *trans*-regulatory factors and control *CDR1* expression [7,8]. That *CDR1* expression is also under negative control is supported by transcription profiling studies with strains deleted for the global repressors *TUP1*, *MIG1*, and *NRG1*, in which *CDR1* expression was increased [33]. Our present study suggests that the NREBP is a transcriptional repressor that is involved in the control of *CDR1* expression.

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