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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

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Abstract

We previously demonstrated that the *CDR1* gene, encoding a multidrug transporter in *Candida albicans*, is differentially upregulated by various drugs and steroids. In order to get an insight into the molecular basis of the induction of this gene we analyzed its promoter region. The transcription start site was mapped to 63 nucleotides upstream of the initiating ATG. Reporter assays revealed the presence of four upstream activating and four upstream repressing sequence domains along the entire promoter. Like the native gene, promoter-luciferase recombinants showed enhanced activity in response to various stresses like drugs, human steroid hormones and heavy metals. Mutational analysis demonstrated that while the proximal promoter (−345/+1) contains all the regulatory domains required for its induction by various other stresses, the miconazole response is mediated via the distal promoter (−857/−1147), harboring an AP-1 site. The involvement of the AP-1 element in mediating the latter effect was evident by an increase in AP-1 binding activity following miconazole treatment. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *CDR1*; ABC transporter; Gene expression; Promoter; AP-1; *Candida albicans*

1. Introduction

In spite of the widespread occurrence of the ABC (ATP binding cassette) and MFS (major facilitator superfamily) genes and their involvement in conferring multidrug resistance (MDR) in *Candida albicans*, the molecular mechanisms controlling their expression is poorly understood [1]. In contrast, the regulatory network controlling the expression of the MDR genes in *Saccharomyces cerevisiae* is relatively well elucidated [2]. For example, it is known that the expression of *PDR5*, the functional homologue of *CDR1*, is under the control of two transcription factors, viz. *PDR1* and *PDR3* [3]. Recently, Raymond and coworkers have identified a family of transcription regulators named *FCR*s in *C. albicans*,

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which appear to be the functional homologues of *Pdr1*/*Pdr3*. Among them, at least one, viz. *FCR1*, functions as a negative regulator of MDRs [4]. However, the plausible targets of *FCR* genes remain to be identified. *CAP1*, a b-Zip transcription factor and a homologue of *YAP1* in *S. cerevisiae*, has also been implicated in conferring resistance to multiple drugs and to oxidative stress in *C. albicans* [5].

We have earlier demonstrated that at least two of the *C. albicans* MDR genes, *CDR1* and *CaMDR1*, are transcriptionally activated to various extents by different environmental stresses [6,7]. However, the molecular basis of their activation by those agents is yet to be established [4,8]. As a long-term goal to unravel the regulatory mechanism(s) which controls the expression of MDR genes in *C. albicans*, we have characterized for the first time the 5′ flanking sequence of the *CDR1* gene. Our results demonstrate that *CDR1* gene expression is under the complex control of multiple positively and negatively acting *cis*-regulatory elements. We also demonstrate that the regulatory domains responding to the induction by various drugs are clustered in the proximal region (−345/+1) except for the miconazole-responsive domain that is located in the distal part (−857/−1147) of the promoter.

2. Materials and methods

2.1. Strains

C. albicans ATCC 10261 was used for preparing nuclear extracts and RNA isolation. *C. albicans* strain Red 3/6 (purchased from David Soll, University of Iowa, USA), an *ade2* derivative of strain WO-1, was maintained on YNB medium supplemented with 3 mM adenine sulfate and was used for reporter assay [9].

2.2. Primer extension analysis

Total RNA was isolated from a *C. albicans* strain (ATCC 10261) grown to mid-exponential phase following treatment with 100 μg ml^{-1} miconazole. RNA (50 μg) was hybridized at 50°C overnight with 1.25 pmol of the labeled primer complementary to the region following the first ATG (see Fig. 1A). The hybrid was then precipitated and reverse transcribed using AMV-Reverse transcriptase (Boehringer Mannheim) for 60 min at 42°C according to the manufacturer's protocol. The reaction was stopped, and the pelleted DNA was resuspended in 0.1 N NaOH and 0.5 M EDTA for 25 min at 30°C. The DNA was then denatured and electrophoresed on a 6% sequencing gel in a parallel lane along with the dideoxy sequencing reactions performed with the same primer.

2.3. Construction of 5′ deletion mutants of the CDR1 promoter

The 1147-bp *CDR1* promoter was cloned into pCRW3, a luciferase reporter plasmid, in two steps. First the proximal 331-bp *Dra*I-*Sac*I fragment was cloned into its *Sma*I-*Sac*I site and then the distal 854-bp *Sac*I-*Sac*I fragment was introduced in the right orientation to give the full-length promoter-reporter recombinant. Due to the unavailability of suitable restriction sites for deletions, the 1.14-kb promoter fragment was subcloned into pBluescript (KS), the recombinant was linearized with *Xho*I and *Apa*I and deletions were made using exonuclease III from the *Xho*I site. The deletion points were determined by sequencing the deletion ends, the selected fragments were released by *Pst*I-*Kpn*I digestion and cloned into the reporter vector pCRW3.

2.4. Transformation and in vitro assay of luciferase activity

C. albicans strain Red 3/6 was electroporated according to the method described by Kohler et al. [10]. Transformants appearing after 3–4 days were screened by Southern analysis using the 1.1-kb Rluc fragment (of the *Renilla* luciferase gene) as the probe. The luciferase reporter assay was essentially done as described by Srikantha et al. [9] using a TD 20/20 luminometer (Turner Designs) and the activity was expressed as relative luminescence units (Rlu) per μg protein. Protein was estimated by the Bradford assay [11]. Since all the plasmids used in this study contained an autonomously replicating sequence, we took precautions to avoid discrepancies that might result from varying copy numbers [12]. Transformants were randomly picked up, character-

ized by Southern hybridization, and directly used for the reporter assay without subculturing or storing. Each experiment was repeated several times.

2.5. Gel mobility shift assay

Cell extracts were prepared as described by Srikantha et al. [9]. The protein concentration of the extracts was determined using the Bradford method [11]. The extracts were stored at −80°C in aliquots and were used only once. A synthetic, 30-nucleotide oligonucleotide, harboring the AP-1 element (AATGTTTATGCG**TGACCCA**AACATAATCTC), was procured commercially from Integrated DNA Technologies, Inc. (Iowa, USA) and was used as a probe for gel mobility shift assay. Labeled oligonucleotide was incubated with 5–10 μg cell extracts at room temperature for 20 min and the complexes formed were resolved on a 7% polyacrylamide gel with 0.5×TBE as the running buffer. For competition experiments 50–100-fold molar excess of cold double-stranded oligonucleotide was added to the incubation mixture prior to the addition of the probe.

3. Results and discussion

3.1. Identification of the transcription start site and the TATA element of the CDR1 promoter

The *CDR1* gene, isolated previously [13], contains a 1210-bp sequence upstream of its initiation codon (see Fig. 1A). In order to analyze the regulation of its expression by various agents, we delineated its promoter by first identifying the transcription initiation site. A synthetic 21-bp oligonucleotide complementary to a region spanning the 5′ end of the cod-

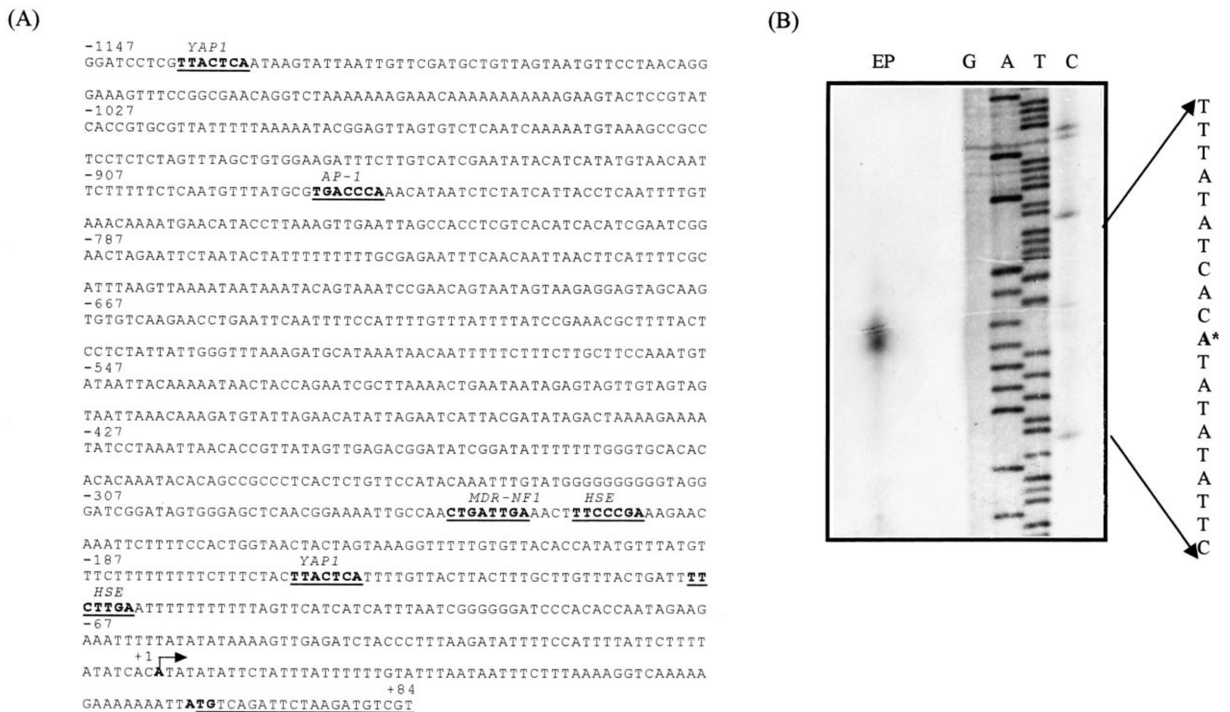

Fig. 1. A: Nucleotide sequence of the 1.1-kb upstream region of the *CDR1* gene (reproduced from Prasad et al. [13]). The putative TATA element along with a number of other *cis*-regulatory elements are identified. YAP-1: yeast AP-1; AP-1: mammalian AP-1; HSE: heat shock element; MDR-NF-1: MDR nuclear factor 1; +1: transcription start site. B: Mapping of the transcription start point of *CDR1* promoter was carried out by extending a 21-mer synthetic oligonucleotide primer complementary to the underlined sequence in A. The transcription start site at 63 nucleotides upstream of the first ATG is identified by an asterisk. EP: extension product.

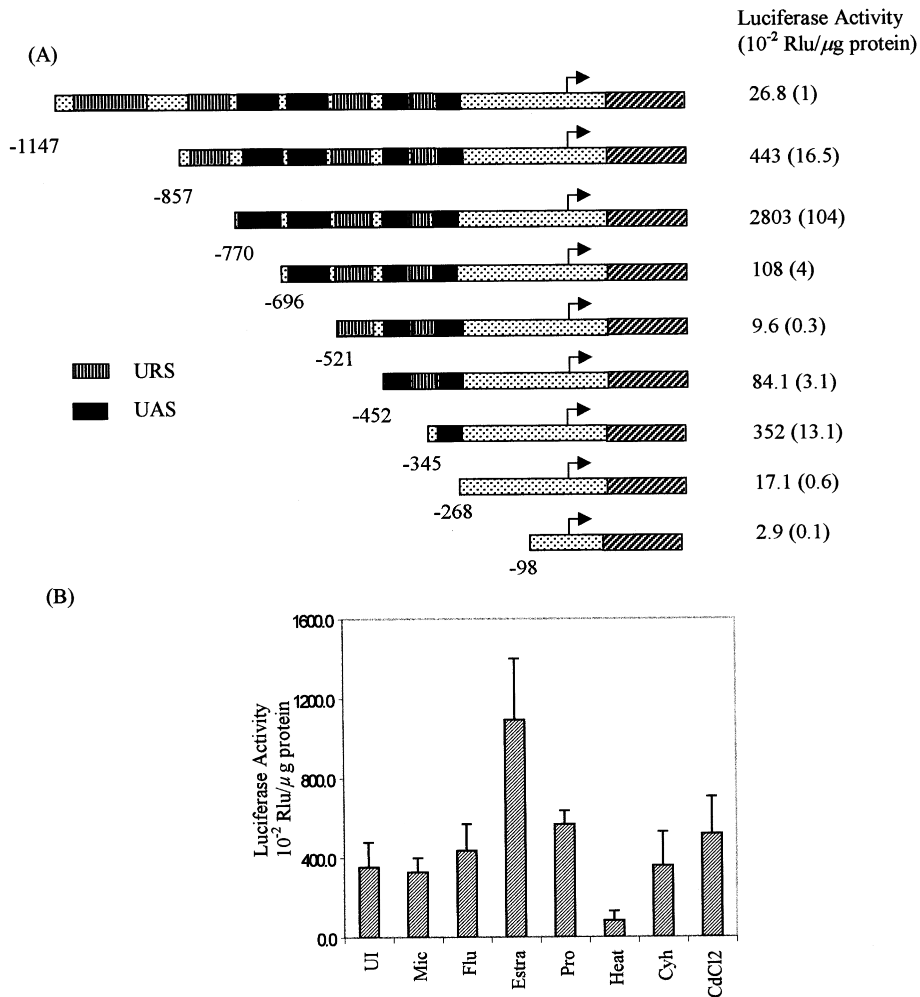

Fig. 2. The *CDR1* promoter consists of multiple regulatory domains. A: The 5′ deleted promoter-Rluc recombinants were transformed in *C. albicans* cells and assayed for luciferase expression. Promoter activity expressed as luminescence (Rlu μg^{-1} protein) are an average of six independent experiments done in duplicate. The relative promoter activity, taking that of the full-length promoter as 1, is shown in parentheses. Each number shown in the diagram denotes the end point of deletion. B: The drug-responsive regions are clustered in the proximal promoter. The Δ345 bp promoter-luciferase recombinant was tested for its responsiveness to various stresses as shown above. Mic: miconazole (100 μg ml^{-1}; 1 h); Flu: fluconazole (50 μg ml^{-1}; 1 h); Estra: β-estradiol (1 mM; 15 min); Pro: progesterone (1 mM; 15 min); Heat: 42°C exposure (15 min); Cyh: cycloheximide (1 μg ml^{-1}; 1 h); $CdCl_2$: cadmium chloride (0.5 mM; 1 h); UI stands for uninduced cells.

ing sequence (see Fig. 1A) was used to perform a primer extension analysis using total RNA isolated from *C. albicans* cells treated with miconazole, a potent inducer of *CDR1*. The radiolabeled primer was extended to a 63-nucleotide product (Fig. 1B). The same product in a much lower amount was also obtained when cells were not treated with the drug (data not shown). Therefore, it established that the transcription of the *CDR1* gene is initiated 63 nucleotides upstream of the coding sequence. In yeast, the transcription start site is generally located at 40–120 bp downstream of the TATA sequence [14] and in conformity with this pattern, the *CDR1* transcription start site is also preceded by a TATA sequence 52 bp upstream (see Fig. 1A). We thus conclude that this TATA sequence is the functional

TATA element of the *CDR1* promoter. As shown in Fig. 1A, sequence analysis of this 1.14-kb promoter fragment using the TRANSFAC program (BCM Search Launcher) revealed the presence of a number of *cis*-regulatory elements such as AP-1, YAP-1 (yeast AP-1), HSE (heat shock element), MDR-NF1 (MDR nuclear factor-1, also known as drug response element, DRE).

3.2. The CDR1 promoter is composed of multiple upstream activating (UAS) and upstream repressing (URS) sequence domains and the drug-responsive domains are clustered in the proximal promoter

We have earlier reported the upregulation of the *CDR1* transcript in response to various stresses including drugs and steroids [6]. In order to identify the *cis*-regulatory domains involved in its regulation, a series of 5′ deletion mutants were constructed (see Section 2 for details). Each mutant was then tested for its relative level of expression as against that of the full-length promoter. As shown in Fig. 2A, deletion of the region spanning nucleotides −1147 to −857 resulted in a more than 15-fold increase in *CDR1* promoter-driven luciferase activity indicating the presence of a strong negative regulatory domain (URS1) within this region. Upon further deletion to position −770, the activity was further increased by five-fold (100-fold compared to full-length promoter activity), indicating the presence of another potent negative regulatory domain (URS2) causing a substantial repression in the activity of the native promoter. Upon further deletion to −521 bp, the activity was drastically reduced to one third of the original. This reduction in activity was due to the loss of two domains, one located upstream of −696 and the other between −696 ad −521 bp (see Fig. 2A), both presumably harboring upstream activation sequences (UAS1 and UAS2). Subsequent deletions also identified the presence of two more negative (URS3 and URS4) and one more positive regulatory domains (UAS3) located between −521 and −345 bp. Another positive regulatory domain (UAS4) was also located between −345 and −98. It appears from the reporter analyses of the sequentially deleted promoter fragments that the *CDR1* promoter is highly modular in nature, containing at least four potent positive (UAS) and four negative (URS) regulatory domains evenly distributed along the entire promoter fragment. Such a modular nature of promoters has earlier been shown in *S. cerevisiae*. Although, based on sequence homology to their counterparts in *S. cerevisiae*, UAS-like elements have earlier been suggested in *C. albicans*, this is the first experimental evidence of the existence of such regulatory elements in a *C. albicans* promoter. Nevertheless, the physiological role of these regulatory domains is not apparent yet and is likely to be a component of its responsiveness to various stresses.

In order to identify the functionality of these domains in the context of upregulation of *CDR1* by various drugs, the promoter recombinants were then tested for their response to a number of drugs. As shown in Fig. 2B, the proximal promoter, carrying a deletion to position −345 bp, was inducible by steroids (β-estradiol and progesterone), a heavy metal (Cd^{2+}) and several other drugs except miconazole. It is pertinent to mention here that the full-length promoter also responded to all these agents in a similar manner (data not shown).

3.3. Miconazole induction of CDR1 is mediated by an upstream domain harboring an AP-1 element

While the full-length *CDR1* promoter (−1147) is responsive to miconazole, the truncated (−345) promoter is independent of miconazole induction (Fig. 2B). Therefore, in order to locate the putative miconazole-responsive domain in the *CDR1* promoter, the promoter-luciferase recombinants, as described above, were tested for their activity in the absence and presence of miconazole. As shown in Fig. 3A, the full-length promoter (−1147) was induced by miconazole to the extent of 3.5-fold. However, deletion of about 250 bp from its 5′ end (to −857) resulted in the complete loss of the miconazole response. All the subsequent deletion mutants failed to be induced by the drug and the response by one of them, Δ452, is shown in Fig. 3A as a representative. The sequence analysis of this region (−1147 to −857) of the promoter revealed the presence of one YAP-1 and an AP-1 like element (see Fig. 1A). While the YAP-1 sequence shows a considerable degree of degeneracy [15], the mammalian AP-1 (TGACTCA) is highly conserved and the AP-1 se-

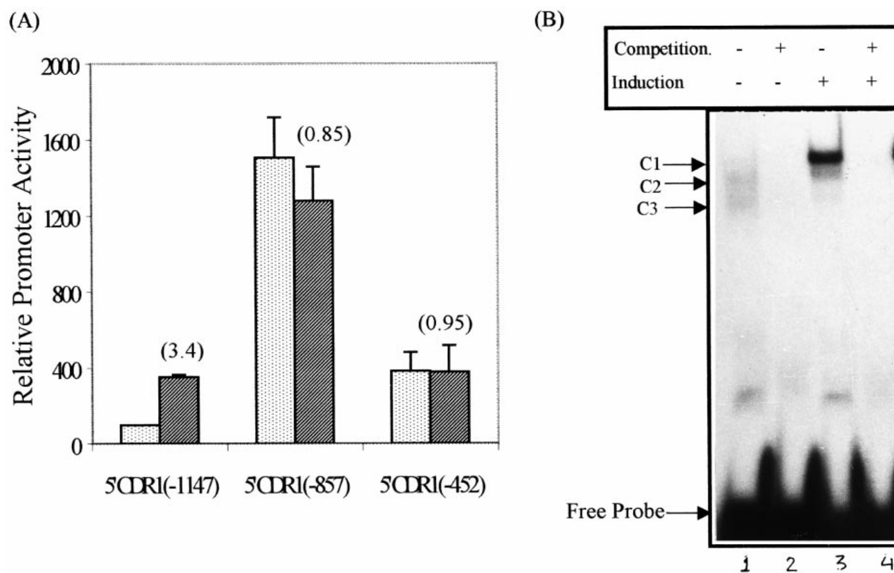

Fig. 3. Identification of a miconazole-responsive domain in the *CDR1* promoter. A: The deletion constructs as described in Fig. 2A were assayed before and after miconazole induction (100 μg ml^{-1} for 1 h). For comparison, the luciferase activity shown by the uninduced full-length promoter was taken as 100 and that shown by others was expressed accordingly. The fold change as against the corresponding control (uninduced) is mentioned on each bar. B: Stimulation of AP-1 binding activity following miconazole induction. A synthetic oligonucleotide harboring the upstream AP-1 sequence (see Fig. 1A for details) was used as the probe to perform gel mobility shift assay as described in Section 2. Among three complexes formed, only the C1 complex was strongly induced by miconazole treatment and it was competed out by a 50-fold molar excess of unlabeled oligonucleotide.

quence in the miconazole response region (TGACCCA) has six out of seven bases identical to the mammalian AP-1. Nonetheless, the yeast AP-1(s) binds to both YAP and mammalian AP-1 sequences albeit with different efficiency [15]. Since the yeast homologues of AP-1 (YAPs/CAP) have already been implicated in stress response [15,16], we designed a double-stranded oligonucleotide harboring the AP-1-like sequence (TGACCCA) in the miconazole response domain and used it as a probe for gel mobility shift analysis using extracts from miconazole-treated cells. As shown in Fig. 3B, following miconazole induction for 1 h, one of the AP-1 binding complexes (C1) was strongly induced (compare lane 1 with lane 3) whereas the other two complexes (C2 and C3) remained unaffected. This complex (C1) was competitively inhibited when a 50-fold molar excess of the unlabeled oligonucleotide was used. These results confirm the specificity of the DNA-protein interaction involving the AP-1-like factor. In order to test the nature of this binding activity, we also challenged it with an excess of unlabeled mammalian AP-1 binding sites and that resulted in substantial disappearance of C1 (data not shown). Thus it is anticipated that the miconazole response to the *CDR1* promoter is mediated by the AP-1 element present in the upstream region of the promoter.

Yeast AP-1 factors, like their mammalian counterparts, have been shown to activate gene expression in response to a variety of extracellular stimuli. In particular, these factors are required for the response to oxidative stress and for surviving exposure to a variety of cytotoxic agents, viz. heavy metal ions [17]. The involvement of AP-1 in drug resistance has already been shown in *S. cerevisiae* [16], *Schizosaccharomyces pombe* [18], *Histoplasma capsulatum* [19] and *C. albicans* [5,8]. Since another AP-1-like binding site (YAP-1) is also present in the proximal region of the promoter (see Fig. 1A), the basis of the specificity of action of the distal AP-1-like element is not clear yet. It is likely that some additional elements in conjunction with the respective AP-1 sites might also contribute to its specificity.

Our data unambiguously establish that the 1.14-kb

upstream fragment of the *CDR1* gene represents its promoter that mimics its response to a wide spectrum of agents such as drugs, human steroid hormones and cadmium ion. Further dissection of the promoter revealed a complex array of regulatory elements distributed evenly along the entire regulatory region. It appears from the reporter analysis of the sequentially deleted promoter fragments that the *CDR1* promoter is highly modular in nature. The detailed analysis of these regulatory elements is the theme of our current research.

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