Identification of promoter elements responsible for the regulation of *MDR1* from *Candida albicans*, a major facilitator transporter involved in azole resistance

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Upregulation of the *MDR1* (multidrug resistance 1) gene is involved in the development of resistance to antifungal agents in clinical isolates of the pathogen *Candida albicans*. To better understand the molecular mechanisms underlying the phenomenon, the *cis*-acting regulatory elements present in the *MDR1* promoter were characterized using a β -galactosidase reporter system. In an azole-susceptible strain, transcription of this reporter is transiently upregulated in response to either benomyl or H₂O₂, whereas its expression is constitutively high in an azole-resistant strain (FR2). Two *cis*-acting regulatory elements within the *MDR1* promoter were identified that are necessary and sufficient to confer the same transcriptional responses on a heterologous promoter (*CDR2*). One, a benomyl response element (BRE), is situated at position –296 to –260 with respect to the ATG start codon. It is required for benomyl-dependent *MDR1* upregulation and is also necessary for constitutive high expression of *MDR1*. A second element, termed H₂O₂ response element (HRE), is situated at position –561 to –520. The HRE is required for H₂O₂-dependent *MDR1* upregulation, but dispensable for constitutive high expression. Two potential binding sites (TTAG/CTAA) for the bZip transcription factor Cap1p (*Candida* AP-1 protein) lie within the HRE. Moreover, inactivation of *CAP1* abolished the transient response to H₂O₂. Cap1p, which has been previously implicated in cellular responses to oxidative stress, may thus play a *trans*-acting and positive regulatory role in the H₂O₂-dependent transcription of *MDR1*. A minimal BRE (–290 to –273) that is sufficient to detect *in vitro* sequence-specific binding of protein complexes in crude extracts prepared from *C. albicans* was also defined. Interestingly, the sequence includes a perfect match to the consensus binding sequence of Mcm1p, raising the possibility that *MDR1* may be a direct target of this MADS box transcriptional activator. In conclusion, while the identity of the *trans*-acting factors that bind to the BRE and HRE remains to be confirmed, the tools developed during this characterization of the *cis*-acting elements of the *MDR1* promoter should now serve to elucidate the nature of the components that modulate its activity.

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INTRODUCTION

The yeast *Candida albicans* is an opportunistic pathogen that causes oral, vaginal or systemic infections in immunocompromised individuals and immunocompetent women

Abbreviations: BRE, benomyl response element; bZip, basic leucine zipper; DRE, drug-responsive element; EMSA, electrophoretic mobility shift assay; HRE, H₂O₂ response element; MADS, minichromosome maintenance, agamous deficiens and serum response factor; MF, major facilitator; 4-NQO, 4-nitroquinoline-*N*-oxide; RLU, *Renilla* luciferase units; RLUC, *Renilla* luciferase; T-BHP, *tert*-butyl hydrogen peroxide.

(Coleman *et al.*, 1993). The azole class of antifungals (for example fluconazole) is widely used to treat *C. albicans* infections. Fluconazole-resistant strains have emerged, especially in HIV-positive patients with recurrent candidiasis who have undergone prolonged fluconazole therapy (Ghannoum *et al.*, 1996; Law *et al.*, 1994; Marr *et al.*, 2000; Rex *et al.*, 1995). The molecular mechanisms proposed to explain azole resistance are diverse: (i) the cellular content of the azole target Erg11p (ergosterol biosynthesis), a 14 α -lanosterol demethylase, can be elevated, (ii) the affinity of Erg11p for azole antifungal agents can be decreased, (iii) the

ergosterol biosynthetic pathway can be altered by inactivation of the sterol $\Delta^{5,6}$ -desaturase and (iv) cells can fail to accumulate these agents due to the upregulation of multidrug transporters belonging to the ABC transporters and the major facilitator (MF) families. Some azole-resistant strains upregulate the ABC transporters *CDR1* (*Candida* drug resistance) and *CDR2*, while others upregulate MF *MDR1* (multidrug resistance 1) (Sanglard & Odds, 2002; White *et al.*, 1998).

Overexpression and gene inactivation studies have shown that *MDR1* confers specific resistance to fluconazole, benomyl, cycloheximide, benzotriazole, methotrexate, 4-nitroquinoline-*N*-oxide (4-NQO) and sulfomethuron methyl (Ben-Yaacov *et al.*, 1994; Fling *et al.*, 1991; Goldway *et al.*, 1995; Wirsching *et al.*, 2000b). Disruption of *MDR1* causes an increased susceptibility to methotrexate, cycloheximide and 4-NQO (Goldway *et al.*, 1995; Sanglard *et al.*, 1996). However, its inactivation does not increase susceptibility to benomyl, suggesting that other mechanisms are involved in this resistance (Goldway *et al.*, 1995). *MDR1* is usually not expressed at detectable levels in fluconazole-susceptible *C. albicans* isolates but its constitutive upregulation in some fluconazole-resistant isolates reveals a correlation between azole resistance and overexpression of *MDR1* (Franz *et al.*, 1998; Sanglard *et al.*, 1995; White, 1997; White *et al.*, 1997).

Little is known about the transcriptional regulation of *MDR1* in *C. albicans*. However, a dramatic transient upregulation of *MDR1* can be observed in azole-susceptible strains after treatment with toxic hydrophobic agents (benomyl, methotrexate, 4-NQO, *o*-phenanthroline), oxidizing agents [diamide, H_2O_2 , *tert*-butyl hydrogen peroxide (T-BHP) and diethylmaleate] and the alkylating agent methyl methanesulfonate (Gupta *et al.*, 1998; Harry *et al.*, 2005). Clinical isolates with increased *MDR1* expression have not undergone amplification of the *MDR1* gene (Wirsching *et al.*, 2000a). Sequence analysis of *MDR1* promoters from clinical isolates compared to wild-type promoters did not reveal *cis*-acting mutations (Harry *et al.*, 2005; Wirsching *et al.*, 2000a). Thus *MDR1* upregulation in clinical strains is most likely due to transcriptional activation resulting from mutations of factors acting *in trans* (Wirsching *et al.*, 2000a). Deletion analysis of the *MDR1* promoter, performed with a luciferase reporter gene, suggested that regulatory functions could be assigned to two separate regions: an element required for induction by benomyl was assigned to the region between -399 and -299 upstream of the start codon and another region located between -601 and -500 was responsible for *MDR1* induction by the oxidative agent T-BHP.

In *Saccharomyces cerevisiae*, drug resistance is also mediated by membrane transport proteins belonging to the ABC transporter and major facilitator superfamilies and the different mechanisms controlling the expression of these multidrug resistant genes are relatively well understood (Sa-Correia & Tenreiro, 2002; Taglicht & Michaelis, 1998). The

homologue of *MDR1*, *FLR1*, confers resistance to multiple drugs such as fluconazole, 4-NQO, cycloheximide, methotrexate and benomyl (Alarco *et al.*, 1997; Broco *et al.*, 1999; Nguyen *et al.*, 2001; Tenreiro *et al.*, 2001). Induction of *FLR1* expression by the oxidizing agents H_2O_2 , diamide, T-BHP and diethylmaleate, as well by the alkylating agent methyl methanesulfonate (Broco *et al.*, 1999; Jelinsky & Samson, 1999; Nguyen *et al.*, 2001), is mediated by Yap1p (also called Pdr4p or Snq3p), a basic leucine zipper (bZip) transcription factor (Nguyen *et al.*, 2001; Tenreiro *et al.*, 2001). The direct targets of Yap1p, principally genes involved in oxidative stress, have a common promoter element, the palindromic YRE (Yap1p response element: TTAC/GTAA) (Fernandes *et al.*, 1997; Kuge & Jones, 1994; Toone & Jones, 1999).

In *C. albicans*, Cap1p, a bZip transcription factor structurally and functionally related to *S. cerevisiae* Yap1p, has been implicated in multidrug resistance and oxidative stress resistance (Alarco & Raymond, 1999; Wang *et al.*, 2006; Zhang *et al.*, 2000). It was also shown to confer fluconazole resistance in *S. cerevisiae* by transcriptionally activating *FLR1* (Alarco *et al.*, 1997), suggesting it has the same sequence specificity as Yap1p. Conversely, the *C. albicans* *MDR1* promoter harbours the putative Cap1p-binding site (TTAGTAA at -532). Together these data indicate that mechanisms regulating *MDR1* and its *S. cerevisiae* orthologue may be grossly similar. However, while in *S. cerevisiae* inactivation of Yap1p abolished *FLR1* transcription (Tenreiro *et al.*, 2001), Alarco & Raymond (1999) showed that disruption of *CAP1* in the azole-resistant strain FR2 did not suppress but rather increased the level of *MDR1* expression. In *C. albicans* Cap1p may act as both a repressor and an activator of *MDR1* transcription, since experiments performed by Harry *et al.* (2005) demonstrating the importance of the YRE in the T-BHP response suggest that Cap1p is an activator of *MDR1* expression in response to oxidative stress.

In this study, we used a reporter system to explore the transcriptional regulation of *MDR1* in azole-resistant and azole-susceptible strains. The system has identified two different *cis*-acting regulatory elements and has permitted us to locate them at high resolution. The first, located between -296 and -260, was responsible for dramatic *MDR1* upregulation in an azole-susceptible strain exposed to benomyl as well as for high constitutive expression in an azole-resistant strain. The second, which harbours two putative binding sites for Cap1p, is located between -561 and -520. Like the *trans*-acting Cap1p, this *cis*-acting element was not required for high constitutive expression of *MDR1* in an azole-resistant strain. However, the response of *MDR1* to H_2O_2 was dependent upon the presence of both Cap1p and its binding site within the *MDR1* promoter. The benomyl-dependent response was also partially dependent upon the presence of Cap1p, raising the possibility that interactions may occur between complexes bound at the two regulatory elements.

METHODS

Strains and media. The *C. albicans* strains used in this study are listed in Tables 1 and 2. Strains were grown either in complete medium YEPD [1 % Bacto peptone (Difco), 0.5 % yeast extract (Difco) and 2 % glucose (Fluka)] or in minimal medium YNB [Yeast Nitrogen Base (Difco) and 2 % glucose (Fluka)]. For solid media, 2 % agar (Difco) was added to both media. *Escherichia coli* DH5 α (Hanahan, 1985) was used as a host for plasmid construction and propagation. DH5 α was grown in Luria–Bertani (LB) broth or LB agar plates supplemented with ampicillin (0.1 mg ml⁻¹) when required.

Yeast transformation. Cells from 0.2 ml of a stationary-phase culture were resuspended in 0.1 ml of a solution containing 200 mM lithium acetate pH 7.5, 40 % (w/v) polyethylene glycol 8000, 15 mg DTT ml⁻¹ and 250 μ g denatured salmon sperm DNA ml⁻¹. Linearized transforming DNA (0.5–4 μ g) was added to the yeast suspension, which was incubated at 44 °C for 60 min. Transformation mixtures were plated directly onto selective medium and incubated for 2–3 days at 30 °C.

Construction of promoter–*lacZ* fusions. To generate *MDR1*–*lacZ* and *CDR2*–*lacZ* fusions used in drug-induction assays, the 999 bp *MDR1* and 903 bp *CDR2* promoters were first amplified by PCR using the *Taq* DNA polymerase (Roche), with primers *MDR1*-5'-999-KPN and *MDR1*-3'-PST or primers *CDR2*-KPN and *CDR2*-PST (Table 3). The generated fragments were cloned into pAU36 (Uhl & Johnson, 2001) previously digested with *KpnI* and *PstI*. The resulting plasmids (pBR1 and pDS295, respectively) were digested with either *NruI* for the *MDR1*–*lacZ* fusion or *SnaBI* for the *CDR2*–*lacZ* fusion prior to transformation into *C. albicans*, resulting in their integration at the *MDR1* and *CDR2* loci, respectively.

For analysis of *MDR1* promoter deletions, a cassette containing the 5' and 3' flanking regions of *MDR1* was inserted into the *Bam*HI site of pAU36. This cassette was designed to contain a restriction site (*NheI*) to separate the 5' and 3' flanking regions of *MDR1*, therefore allowing linearization of promoter–*lacZ* fusions in pAU36-derived plasmids and integration at the *MDR1* genomic site (see also Fig. 1a). The cassette was prepared in a two-step process. *C. albicans* genomic DNA was used as a PCR template to amplify two distinct 500 bp fragments that overlap by the presence of the *NheI* restriction site. In this step, the external primers UTR3-5' and UTR5-3' were used with internal primers UTR3-3'bis and UTR5-5'bis (Table 3) with the Expand High Fidelity system (Roche). The two purified PCR fragments were then used as a PCR template with external primers (UTR3-5' and UTR5-3'). The resulting product was next introduced into the *Bam*HI site of pAU36 to generate pBR4. The 5' deletions of the promoter region were generated by PCR using the reverse primer *MDR1*-3'-PST and the different forward primers listed in Table 3. The resulting fragments were inserted at the *KpnI* and *PstI* sites of pBR4 to generate the plasmids listed in Table 3. For the construction of internal deletions

and transversions within the *MDR1* promoter, a similar two-step PCR strategy was used. First, external primers *MDR1*-5'-598-KPN and *MDR1*-3'-PST were used with variable primers designed for specific deletions and transversions to generate fragment pairs with a 20 bp overlap region (Table 4). After purification, the overlapping fragment pairs were used as templates with external primers to reconstitute *MDR1* promoter derivatives. The fragments obtained were cloned into the *KpnI* and *PstI* sites of pBR4 to generate the plasmids listed in Table 4. All plasmids generated were linearized by *NheI* and their correct integration as a single copy at the *MDR1* locus was verified by Southern blotting (see also Fig. 1b).

***Renilla* luciferase reporter system.** Chimeric *CDR2* promoters containing the BRE (50 bp, –309 to –260) or the HRE (41 bp, –556 to –516) fused to the *Renilla* luciferase (RLUC) reporter system were constructed by the following procedures. The plasmid pAC66, containing the *HIS3* ORF under the control of the BRE (50 bp) *CDR2* promoter (198 bp) (D. Sanglard, unpublished), was used as a template for PCR with primer LUCCDR23 and BRE50F-*CDR2*-X or YRE50F*CDR2*p-XBA. The primer BRE50F-*CDR2*-X adds an *XbaI* restriction site to the 5' end of the BRE whereas the primer YRE50F*CDR2*p-XBA overlaps with the 5' end of *CDR2* promoter and contains 41 bp of the HRE. The constant primer LUCCDR23 overlaps the 3' end of the *CDR2* promoter and adds a *PstI* restriction site at the 3' end of the BRE- or HRE-fused *CDR2* promoter (see Table 5). The resulting PCR fragments were digested with *XbaI* and *PstI*, then inserted into compatible sites of pMM43 to generate pBR101 and pBR108. The plasmid pMM43 (de Micheli *et al.*, 2002) contains the RLUC reporter system and 198 bp of the *CDR2* promoter but lacks the drug-responsive element (DRE), an element required for *CDR2* upregulation. This plasmid was used as a negative control in luciferase reporter assays. As a positive control, the promoter of *MDR1*, 598 bp, was first amplified by PCR from plasmid pBR6 using primers *MDR1*-5'-598-KPN and *MDR1*-3'-PST. Digestion of the PCR product by *XbaI* and *PstI* generated a fragment of 592bp (–592 to +1) due to the presence of a *XbaI* restriction site at position –592 of the promoter. This fragment was inserted into the compatible sites of pMM43 to obtain pBR99.

To insert additional BRE or HRE, complementary primers (X-BRE50F-X and X-BRE50R-X for the BRE, and Xba-YRE.F-XBA and Xba-YRE.R-XBA for the HRE) with *XbaI* site extensions were annealed by progressive diminution of temperature from 95 °C to room temperature. Annealed fragments were phosphorylated with T4 polynucleotide kinase (Roche) and placed upstream of the BRE *CDR2* promoter cloned in pBR101 previously digested by *XbaI*. All plasmids containing the RLUC reporter system were inserted at the *C. albicans* *LEU2* genomic locus after digestion by *Sall*, which cuts at a single site in the *LEU2* gene in these plasmids.

DNA amplification by PCR. DNA fragments used in cloning steps or as probes were generated by PCR with the *Taq* DNA polymerase (Roche) following the instructions of the manufacturer. The reaction

Table 1. *C. albicans* strains used as hosts for plasmid transformation

Strain	Parental strain	Genotype	Reference
CAF2-1	SC5314	<i>ura3Δ::imm434/URA3</i>	Fonzi & Irwin (1993)
CAF4-2	CAF2-1	<i>ura3Δ::imm434/ura3Δ::imm434</i>	Fonzi & Irwin (1993)
DSY449	CAF4-2	<i>cdr1Δ::hisG/cdr1Δ::hisG CAP1/CAP1</i>	Sanglard <i>et al.</i> (1996)
MMY411	DSY449	<i>cap1Δ::hisG/cap1Δ::hisG</i>	Zhang <i>et al.</i> (2000)
FR2	SGY243	<i>ade2/ade2 ura3Δ::ADE2/ura3Δ::ADE2 CAP1/CAP1</i>	Albertson <i>et al.</i> (1996)
MMY412	FR2	<i>cap1Δ::hisG/cap1Δ::hisG</i>	This study

Table 2. *C. albicans* strains resulting from transformation with the constructs listed in Tables 3 and 4

Strain					Genotype	MDR1 promoter region*
Parental strain:						
CAF4-2	DSY449	MMY411	FR2	MMY412		
DSY2575						
	BRY1				Ura ⁺ [pBR1]	−999 to +1
	BRY8	BRY107			Ura ⁺ [pBR6]	−1459 to +1
	BRY9	BRY333			Ura ⁺ [pBR7]	−1318 to +1
	BRY10	BRY343			Ura ⁺ [pBR8]	−1204 to +1
	BRY11	BRY334			Ura ⁺ [pBR9]	−1100 to +1
	BRY12	BRY335	BRY61	BRY186	Ura ⁺ [pBR10]	−999 to +1
	BRY13	BRY339			Ura ⁺ [pBR11]	−900 to +1
	BRY14	BRY346	BRY63	BRY188	Ura ⁺ [pBR12]	−801 to +1
	BRY269	BRY92			Ura ⁺ [pBR13]	−697 to +1
	BRY16	BRY93	BRY65	BRY192	Ura ⁺ [pBR14]	−598 to +1
	BRY17	BRY94	BRY67	BRY195	Ura ⁺ [pBR15]	−501 to +1
	BRY18	BRY95	BRY70	BRY198	Ura ⁺ [pBR16]	−401 to +1
	BRY19	BRY97	BRY74	BRY199	Ura ⁺ [pBR17]	−302 to +1
	BRY20	BRY98	BRY84	BRY205	Ura ⁺ [pBR18]	−201 to +1
	BRY21	BRY99	BRY79	BRY210	Ura ⁺ [pBR20]	−98 to +1
	BRY51		BRY53		Ura ⁺ [pBR26]	−279 to +1
	BRY49		BRY55		Ura ⁺ [pBR27]	−261 to +1
	BRY47		BRY57		Ura ⁺ [pBR28]	−242 to +1
	BRY45		BRY59		Ura ⁺ [pBR29]	−222 to +1
	BRY134		BRY235		Ura ⁺ [pBR23]	−296 to +1
	BRY137		BRY237		Ura ⁺ [pBR24]	−290 to +1
	BRY140		BRY239		Ura ⁺ [pBR25]	−284 to +1
	BRY108				Ura ⁺ [pBR34]	−580 to +1
	BRY111				Ura ⁺ [pBR35]	−560 to +1
	BRY115				Ura ⁺ [pBR36]	−540 to +1
	BRY117				Ura ⁺ [pBR37]	−520 to +1
	BRY302		BRY308		Ura ⁺ [pBR72]	−598 to +1/t −287 to −266
	BRY351		BRY380		Ura ⁺ [pBR89]	−598 to +1/t −295 to −286
	BRY359		BRY382		Ura ⁺ [pBR143]	−598 to +1/t −282 to −274
	BRY362		BRY384		Ura ⁺ [pBR90]	−598 to +1/t −276 to −267
	BRY481		BRY503		Ura ⁺ [pBR78]	−598 to +1/Δ −295 to −287
	BRY484		BRY506		Ura ⁺ [pBR79]	−598 to +1/Δ −286 to −278
	BRY487		BRY509		Ura ⁺ [pBR80]	−598 to +1/Δ −277 to −269
	BRY518		BRY533		Ura ⁺ [pBR86]	−598 to +1/Δ −291 to −269
	BRY577		BRY558		Ura ⁺ [pBR160]	−598 to +1/Δ −552 to −521
	BRY575		BRY557		Ura ⁺ [pBR165]	−598 to +1/Δ −552 to −521
						Δ −291 to −269
	BRY632		BRY605		Ura ⁺ [pMM43]	−198 to +1 of CDR2 promoter
	BRY635		BRY607		Ura ⁺ [pBR99]	−592 to +1
	BRY640		BRY611		Ura ⁺ [pBR101]	−309 to −260
						−198 to +1 of CDR2 promoter
	BRY645		BRY617		Ura ⁺ [pBR103]	2 copies of −309 to −260
						−198 to +1 of CDR2 promoter
	BRY647		BRY619		Ura ⁺ [pBR105]	−556 to −516
						−309 to −260
						−198 to +1 of CDR2 promoter
	BRY651		BRY627		Ura ⁺ [pBR108]	−556 to −516
						−198 to +1 of CDR2 promoter

*t, transversion.

Table 3. Plasmid constructs used in drug induction assays and deletion promoter analysis

Vector name	Backbone	Primers used to generate the cloned fragments*	Amplified <i>MDR1</i> promoter region	PCR template
pBR1	pAU36	MDR1-5'-999-KPN: GCGCAAAGGTACCAAACAAAAAGGAAATAATACACACAGCCGTG MDR1-3'-PST: GCGCAAAC <u>CTGCAGGTGAAGT</u> TCTATGTAAGTAGATGTATTGCT	−999 to +1	CAF2-1
pDS295	pAU36	CDR2-KPN: GCGCAAAGGTACCGGTTCTCTAAATAAAAACTAGAAGGTTAT CDR2-PST: GCGCAAAC <u>CTGCAGATGTTTT</u> ATTGTATGTGTTAATTAGTGAA	−903 to +1 (<i>CDR2</i> promoter)	CAF2-1
pBR4	pBR1	UTR5-5'bis: GTCTCGTACAAGAACAATAGCTAGCCGTAACACTCCTGTTAGAGA UTR5-3': GCGCAAAGGATCCTGATGAGGTACGCGATATCT UTR3-5': GCGCAAAGGATCCGTATGCTAATTAGGAGGTGTCA UTR3-3'bis: TCTCTAACAGGAGTGTTACGCTAGCTATTGTTCTTGTACGAGAC	−1890 to −1498 +1690 to +2162‡	CAF2-1
pBR6	pBR4	MDR1-5'-1459-KPN: GCGCAAAGGTACCGTGGAACAAACTATTCCACTACTTAGTC MDR1-3'-PST: GCGCAAAC <u>CTGCAGGTGAAGT</u> TCTATGTAAGTAGATGTATTGCT	−1459 to +1	CAF2-1
pBR7†	pBR4	MDR1-5'-1318-KPN: GCGCAAAGGTACCGTTAGAGTCTAGTGCTGGTGTGCGATAAC	−1318 to +1	pBR6
pBR8	pBR4	MDR1-5'-1204-KPN: GCGCAAAGGTACCGAATAACGCTTTTGTACAATTGTATTATTCG	−1204 to +1	pBR6
pBR9	pBR4	MDR1-5'-1101-KPN: GCGCAAAGGTACCTGTAGAAACGGGATCCGTGAAAACTAG	−1100 to +1	pBR6
pBR10	pBR4	MDR1-5'-999-KPN: GCGCAAAGGTACCAAACAAAAAGGAAATAATACACACAGCCGTG	−999 to +1	pBR6
pBR11	pBR4	MDR1-5'-900-KPN: GCGCAAAGGTACCTAAGTTGAGTGACGATCAAATACTTTGAAC	−900 to +1	pBR6
pBR12	pBR4	MDR1-5'-801-KPN: GCGCAAAGGTACCTCCAATTTGTAAGCAAAGTGAAAGTGTATATATC	−801 to +1	pBR6
pBR13	pBR4	MDR1-5'-697-KPN: GCGCAAAGGTACCTATACTGAAGAAATAAGTTGTTCAATAGTGTT	−697 to +1	pBR6
pBR14	pBR4	MDR1-5'-598-KPN: GCGCAAAGGTACCAAAGCATCTAGAAAAAAGCGGATTTACTC	−598 to +1	pBR6
pBR15	pBR4	MDR1-5'-501-KPN: GCGCAAAGGTACCCAATTTTCATTTTAGGAAATTTACCGAGTTT	−501 to +1	pBR6
pBR16	pBR4	MDR1-5'-401-KPN: GCGCAAAGGTACCCATCGCCGTTTTTCCTTGCCGTGGCA	−401 to +1	pBR6
pBR17	pBR4	MDR1-5'-302-KPN: GCGCAAAGGTACCATCACACGGTAAAAATCCTAATTGGGAAAA	−302 to +1	pBR6
pBR18	pBR4	MDR1-5'-201-KPN: GCGCAAAGGTACCAACTTCTATCGCGAAATGAATAATATCCTTAT	−201 to +1	pBR6
pBR20	pBR4	MDR1-5'-99-KPN: GCGCAAAGGTACCCAGTTTCCAACAATTCTACTTTTTTTTATTCCG	−98 to +1	pBR6
pBR26	pBR4	MDR1-5'-279-KPN: GCGCAAAGGTACCGGGAAAAATACCGAGAATGACACAACCTAAG	−279 to +1	pBR6
pBR27	pBR4	MDR1-5'-261-KPN: GCGCAAAGGTACCGACACAACCTAAGATTTTGCACCTCGG	−261 to +1	pBR6
pBR28	pBR4	MDR1-5'-242-KPN: GCGCAAAGGTACCCACTCGGAAATTATATTCTTCATCGC	−242 to +1	pBR6
pBR29	pBR4	MDR1-5'-222-KPN: GCGCAAAGGTACCCTTCATCGCTATTTTCTATAAACTTCTATC	−222 to +1	pBR6
pBR23	pBR4	MDR1-5'-296-KPN: GCGCAAAGGTACCACGGTAAAAATCCTAATTGGGAAAAATACC	−296 to +1	pBR6
pBR24	pBR4	MDR1-5'-290-KPN: GCGCAAAGGTACCAAATCCTAATTGGGAAAAATACCGAGAA	−290 to +1	pBR6
pBR25	pBR4	MDR1-5'-284-KPN: GCGCAAAGGTACCTAATTGGGAAAAATACCGAGAATGACACAAC	−284 to +1	pBR6
pBR34	pBR4	MDR1-5'-580-KPN: GCGCAAAGGTACCGGCGGATTTACTCCTTATACAACCTACTG	−580 to +1	pBR6
pBR35	pBR4	MDR1-5'-560-KPN: GCGCAAAGGTACCAACTCTACTGGTAACTATTGGCGAAAGATTAG	−560 to +1	pBR6
pBR36	pBR4	MDR1-5'-540-KPN: GCGCAAAGGTACCGCGAAAGATTAGTAAATAAAGTTGCTATTTTTG	−540 to +1	pBR6
pBR37	pBR4	MDR1-5'-520-KPN: GCGCAAAGGTACCGTTGCTATTTTGTGTACATCAATTTTCATTTAG	−520 to +1	pBR6

* Restriction sites within primer sequences are underlined.

†Primers from pBR7 to pBR37 were used in combination with the reverse primer MDR1-3'-PST.

‡The amplified region corresponds to the terminator of *MDR1*.

Fig. 1. Insertion of *MDR1-lacZ* fusions at the *C. albicans* *MDR1* genomic locus. (a) Schematic representation of *MDR1-lacZ* fusions at the genomic *MDR1* locus. The plasmids containing the *MDR1* promoters (P) fused to *lacZ* were first digested with *NheI* and were integrated by double crossing over at the *MDR1* genomic locus using sequences (UTR5' and UTR3') flanking *MDR1* inserted at the *Bam*HI restriction site of pAU36. Restriction maps of the *MDR1* genomic locus are shown with and without insertion of the *MDR1-lacZ* fusion constructs. (b) Southern blot analysis of genomic DNA from strains derived from FR2 and transformed with different lengths of promoter. Genomic DNA was digested with *NsiI* and transferred onto a GeneScreen Plus nylon membrane. Hybridization of the restricted genomic DNA with the *MDR1* labelled probe [represented in (a) by a black bar] yielded one fragment of 2.7 kb for the wild type strain and an additional fragment, of between 6.1 and 7.3 kb, for the yeast transformants, depending of the length of the inserted promoter. A double insertion of the plasmid into the *C. albicans* genome yielded an additional signal at approximately 8.4–9.7 kb.

mixture (final volume 100 μ l) was composed of 10 μ l of 10 $\times$ *Taq* DNA polymerase buffer (Roche), 10 μ l of $MgCl_2$ (Roche), 1 μ l of each 100 μ M forward and reverse primer, 4 μ l of 10 mM deoxynucleoside triphosphates (Roche), 10 U of *Taq* DNA polymerase and 1 μ l of 0.5–1 μ g μ l⁻¹ genomic DNA. PCR was performed under the following conditions: one cycle for 4 min at 94 $^{\circ}$ C, 1 min at 54–60 $^{\circ}$ C and 2 min at 72 $^{\circ}$ C; 30 cycles for 30 at 94 $^{\circ}$ C, 1 min at 54–60 $^{\circ}$ C and 2 min at 72 $^{\circ}$ C followed by one terminal cycle for 10 min at 72 $^{\circ}$ C. After analysis of 1/10 vol. of PCR products by electrophoresis on 1% agarose gel, DNA fragments were purified with the QIAquick PCR purification kit (QIAGEN) and resuspended in 50 μ l sterile water.

Southern blotting. Southern blots were carried out as described previously (Sanglard *et al.*, 1999). DNA probes used in this study were generated by PCR using the following pairs of primers: for the verification of genomic insertion of pMM43-derived plasmids at the *LEU2* genomic locus, CALEU2-3 (5'-CACCACCACTAAAACCC-CAACAAATCA-3') and CALEU2-5 (5'-ATGTCTGTAAAACCCA-AACCATTACT-3'); for insertion of pAU36-derived plasmids at the

MDR1 locus, MDR1-5'-501-KPN (5'-GCGCAAAGGTACCCAAT-TTTCATTTTAGGAAATTACCGAGTTT-3') and LacZ-rev (5'-ATGTCTGTAAAACCCAACCACTTACT-3'). Radioactive DNA probes were generated by random priming using the Megaprime DNA labelling kit (Amersham). Radioactive signals were revealed by exposure to Kodak BioMax MR film (Amersham Biosciences).

Electrophoretic mobility shift assays (EMSAs). Protein extracts from *C. albicans* strain CAF2-1 were prepared from 2 l cultures in YEPD grown to mid exponential phase until the density reached 1.5×10^7 cells ml⁻¹. An equal quantity of cells was exposed to benomyl (35 μ g ml⁻¹) for 30 min or left untreated as a control. The benomyl-treated or control cells were centrifuged for 20 min at 5500 r.p.m. and each cell pellet was washed with 10 ml extraction buffer [400 mM (NH₄)₂SO₄, 10 mM $MgCl_2$, 1 mM EDTA, 10% (v/v), glycerol, 7 mM mercaptoethanol, 200 mM Tris/HCl pH 8]. Washed cells were centrifuged for 10 min at 5500 r.p.m. and the pellet obtained was resuspended in 10 ml extraction buffer supplemented with a protease inhibitor mix (Roche). Glass beads (10 g)

Table 4. Plasmid constructs used to analyse the effects of BRE and HRE deletions or transversions

Vector	Backbone	Primers used to generate the cloned fragments*	Amplified promoter region†	PCR template
pBR72	pBR4	MDR1-5'-598-KPN: GCGCAA <u>AGGTACCA</u> AGCATCTAGAAAAAAGCGGATTTACTC MDR1-3-trans: <i>GATTGCGGGGAAACCGGCTTCTTTT</i> ACCGTTGTGATTAATTGGTAG MDR1-5-trans: <i>GAAGCCGTTTCCCCCGCAATCGAATGACACAACCTAAGATTTTC</i> MDR1-3'-PST: GCGCAA <u>ACTGCAGGTGAAGTTCTATGTAAGTAGATGTATTGCT</u> *	-598 to +1 t -287 to -266	pBR6
pBR89‡	pBR4	MDR1-3-trans2: CCAATTAGT <u>CGGGGCAATTTGTG</u> GATTAATTGGTAGCAACACC MDR1-5-trans2: TAATCACAAAT <u>TGCCCCG</u> ACTAATTGGGAAAAATACCGAG	-598 to +1 t -295 to -286	pBR6
pBR143	pBR4	MDR1-3-trans3: CTCGGTATTGGGAAACCGTAGGATTTTACCGTTGTGATTAATTC MDR1-5-trans3: AAATCCTACGGTTTCCAATACCGAGAATGACACAACC	-598 to +1 t -282 to -274	pBR6
pBR90	pBR4	MDR1-3-trans4bis: GTCATTCTATTGCGGGGGCCCAATTAGGATTTTACCGTTG MDR1-5-trans4: CTAATTGGGCCCCCGCAATAGAAATGACACAACCTAAGATTTTG	-598 to +1 t -276 to -267	pBR6
pBR78	pBR4	MDR1promdel2-R: CTCGGTATTTTCCCAATTAGGTTGTGATTAATTGGTAGCAACAC MDR1promdel2-F: GTGTTGCTACCAATTAATCACAACCTAATTGGGAAAAATACCGAG	-598 to +1 Δ-295 to -287	pBR6
pBR79	pBR4	MDR1promdel3-R: GTTGTGTCATTCTCGGTATTTTCATTTTACCGTTGTGATTAATTG- GTAG MDR1promdel3-F: CTACCAATTAATCACAACGGTAAATGAAAAATACCGAGAATGAC- ACAAC	-598 to +1 Δ-286 to -278	pBR6
pBR80	pBR4	MDR1promdel4-R: ATCTTAGGTTGTGTCATTCTCGCCAATTAGGATTTTACCGTTGTG MDR1promdel4-F: CACAACGGTAAATCCTAATTGGCGAGAATGACACAACCTAAGAT	-598 to +1 Δ-277 to -269	pBR6
pBR86	pBR4	MDR1promdel1-R: CTTAGGTTGTGTCATTCTCGACCGTTGTGATTAATTGGTAGC MDR1promdel1-F: GCTACCAATTAATCACAACGGTGCAGAATGACACAACCTAAG	-598 to +1 Δ-291 to -269	pBR6
pBR160	pBR4	MDR1-YRE-532-R: AATTGATGTAAACAAAAATAGCAACGTAGAGTTGTATCAGGAGTA MDR1-YRE-532-F: TACTCCTGATACAACCTACGTTGCTATTTTGTGTACATCAATT	-598 to +1 Δ-552 to -521	pBR6
pBR165	pBR4	MDR1-YRE-532-R: AATTGATGTAAACAAAAATAGCAACGTAGAGTTGTATCAGGAGTA MDR1-YRE-532-F: TACTCCTGATACAACCTACGTTGCTATTTTGTGTACATCAATT	-598 to +1 Δ-552 to -521 Δ-291 to -269	pBR86

**KpnI* and *PstI* restriction sites within primer sequences are underlined.

†t, transversion; transversed nucleotides are in italics.

‡Primers used in combination with the external primers MDR1-5'-598-KPN and MDR1-3'-PST (from pBR89 to pBR165).

were added to the cell suspension and the resulting mixture was disrupted for 5 min at 4 °C using a Macro bead-beater (Biospec Products). The homogenized solution was kept on ice for 30 min before centrifugation at 4 °C for 30 min at 13 000 r.p.m. The supernatant was transferred to a new tube and the cell pellet was reextracted with 5 ml fresh extraction buffer. The two supernatants were combined and the resulting solution was centrifuged for 1 h at 13 000 r.p.m. at 4 °C. The supernatant was recovered and ammonium sulfate was added to a final concentration of 0.3 g ml⁻¹. After dissolution of ammonium sulfate, the protein extract was centrifuged for 10 min at 13 000 r.p.m. and 4 °C. Precipitated proteins were resuspended in 250 µl protein buffer (20 mM HEPES, 5 mM EDTA, 7 mM mercaptoethanol, 1 mM PMSF and 20 % glycerol) supplemented with protease inhibitors (Roche). Protein concentrations of the crude extracts were determined using the Bradford method.

Double-stranded oligonucleotide probes were prepared by annealing 5' end labelled oligonucleotides with their complementary strands. The sequences of the probes are described in Figs 7 and 8. Single-strand oligonucleotides (5 pmol) were incubated at 37 °C for 45 min with 10 U T4 polynucleotide kinase (Roche) and 40 µCi (1.48 MBq) [γ -³²P]ATP (3000 Ci mmol⁻¹, 10 µCi µl⁻¹) in a final volume of 10 µl. The excess of [γ -³²P]ATP was eliminated using the Nucleotide Removal

kit (Qiagen). The last elution step was performed with 100 µl of sterile water. After addition of the unlabelled oligonucleotides, the annealing of the complementary strands was performed by progressive temperature reduction after a 3 min treatment at 95 °C.

EMSA was carried out with 30 µg protein extract to which was added 7 µl binding buffer [20 mM HEPES pH 7.9, 50 mM KCl, 0.5 mM EDTA (Invitrogen), 0.1 % Nonidet P-40, 1 mg bovine serum albumin (Sigma) ml⁻¹ and 5 % glycerol (Invitrogen)], 1 µl poly[d(I-C)] (Roche) (1 mg ml⁻¹), 1 µl 0.2 M PMSF, 1 µl 0.1 M DTT and 10 fmol labelled probe (approx. 10⁶ c.p.m.). The mix was incubated at room temperature for 20 min. In competition experiments, 250-, 500- and 1000-fold excess of unlabelled double-stranded oligonucleotides, corresponding to a promoter region of the *RTA4* gene, were added just before the labelled probes. After addition of 3 µl 40 % glycerol, the reactions were loaded onto a non-denaturing 5 % polyacrylamide gel containing 0.5 × Tris/borate/EDTA. In parallel, 20 µl loading buffer for proteins was loaded to follow probe migration. Gels were run at 150 V in 0.5 × TBE buffer, then transferred to Whatman 3 MM paper and dried under vacuum. Probes were detected by autoradiography.

Measurement of β -galactosidase reporter activities. Cells were grown overnight in 3 ml YEPD at 30 °C under constant agitation. Cultures were then diluted in 5 ml fresh medium to obtain a

Table 5. Plasmids for the study of BRE and HRE activities in the *CDR2* promoter

Vector	Backbone	Primers used to generate the cloned fragments	<i>MDR1</i> promoter region	PCR template
pBR99	pMM43	MDR1-5'-598-KPN: GCGCAAAGGTACCAAAGCATCTAGAAAAAAGCGGATTTACTC MDR1-3'-PST: GCGCAAAGTGCAGGTGAAGTTCTATGTAAGTAGATGTATTGCT	−592 to +1	pBR6
pBR101	pMM43	BRE50F-CDR2-X: GCGCAAATCTAGACCAATTAATCACAACGGTAAAATCCTAATTG LUCCDR23: CGAACTGCAGATGTTTTTATTGTATGTGTTAATTAGT	−309 to −260 −198 to +1 of <i>CDR2p</i>	pAC66
pBR103	pBR101	X-BRE50F-X: CTAGACCAATTAATCACAACGGTAAAATCCTAATTGGGAAAAATACC- GAGAATGAT X-BRE50R-X: CTAGATCATTCTCGGTATTTTCCCAATTAGGATTTTACCGTTGTGAT- TAATTGGT	−309 to −260	
pBR105	pBR101	Xba-YRE.F-XBA: CTAGACTACTGGTAACTATTGGCGAAAGATTAGTAAATAAAGTTGCT Xba-YRE.R-XBA: CTAGAGCAACTTTATTTACTAATCTTTCCGCAATAGTTACCAGTAGT	−556 to −516	
pBR108	pMM43	YRE50FCDR2p-X: GCGCAAATCTAGACTACTGGTAACTATTGGCGAAAGATTAGTAAAT- AAAGTTGCGTTTTCAAAGCCTCTATTATG LUCCDR23: CGAACTGCAGATGTTTTTATTGTATGTGTTAATTAGT	−556 to −516 −198 to +1 of <i>CDR2p</i>	pAC66

cell density of approximately 5×10^6 cells ml^{−1} and were regrown under agitation until the density reached 1.5×10^7 cells ml^{−1}. At this point, 1 ml aliquots from cultures that were not exposed to drugs (azole-resistant strains FR2 or MMY412) were centrifuged at 4 °C for 5 min at 5500 r.p.m. Exposure of other strains (azole-susceptible strains DSY449 and MMY411) to different drugs was carried out for defined time periods at 30 °C with constant agitation. After drug exposure, cell density was measured and cultures were centrifuged at 4 °C and 5500 r.p.m. for 10 min. The cell pellets were washed with 1 vol. Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄) and centrifuged at 4 °C for a further 10 min. The washed cell pellets were resuspended in 150 µl Z buffer supplemented with 38.6 mM mercaptoethanol. After addition of 50 µl chloroform and 20 µl 0.1 % SDS, cells were disrupted by vortexing. To each aliquot was added 700 µl ONPG (1 mg ml^{−1}; Sigma) in Z buffer supplemented with mercaptoethanol and preheated at 30 °C. The mixtures were incubated at 37 °C until they developed a yellow colour (20 min to 3 h). The enzyme was inactivated by the addition of 500 µl 1 M Na₂CO₃. The mixtures were centrifuged at 13 000 r.p.m. for 10 min and the OD₄₂₀ was read. The β -galactosidase activities were calculated using the following equation: β -galactosidase activity (Miller units) = $(1000 \times OD_{420}) / (t \times v \times OD_{600})$, where t is the time of reaction expressed in min and v is the volume of culture used in the assay expressed in ml.

Measurement of *Renilla* luciferase reporter activities. Cells were grown overnight in 3 ml YEPD at 30 °C under constant shaking, then diluted in 5 ml fresh medium to obtain a cell density of approximately 1.5×10^7 cells ml^{−1}. Benomyl (35 µg ml^{−1}) was added to the cell suspensions and cultures were incubated with constant shaking at 30 °C for a further 30 min. After drug exposure, cells were centrifuged for 10 min at 5500 r.p.m. The pellet was washed twice with 1 ml cold sterile water followed by 1 ml cold RLUC buffer (0.5 M NaCl, 0.1 M K₂HPO₄, 1 mM EDTA, 0.6 mM sodium azide). The final pellet was resuspended in 200 µl cold RLUC buffer supplemented with protease inhibitors (Roche) and an equal volume of chilled acid-washed glass beads was added. The cells were disrupted at 4 °C for 30 s using a Minibead beater (Biospec Products) and then centrifuged at 4 °C for 10 min at 13 000 r.p.m. The supernatants containing soluble proteins were stored at −20 °C or used directly for measurement of luciferase activities.

The relative luciferase activities of the different clones were measured using the Dual-Luciferase kit (Promega). Five microlitres of the crude protein extracts was added to 25 µl LAR II buffer according to the recommendations of the manufacturer and firefly luciferase activities were measured using a Lumat LB 9507 luminometer (E. G. and G. Berthold). Twenty-five microlitres of freshly prepared STOP and GLO buffer was then added to the reactions and *Renilla* luciferase activities were measured. Specific luciferase activities, expressed in *Renilla* luciferase units [RLU; i.e. U (µg protein)^{−1}] were calculated after determination of the protein concentration of cell extracts by the Bradford method.

Statistical analysis. For the analysis of promoter deletions, a mathematical model was fitted using non-linear least squares. The choice of the model was based on the observation that, starting from a maximal response (H_1 in the equation), the response decreases to a minimum level (H_0) over a very short interval of length. The centre of this interval (A) represents the critical length for loss of response, while the width of this interval indicates the abruptness of the change (the slope B). The final equation was $y = H_0 + (H_1 - H_0) / \{1 + \exp[(A - x)/B]\}$, where y is the response and x the promoter length. For the mutant strains (*cap1Δ/Δ*), the same equation was used with parameters written in lower case (h_0 , h_1 , a , b). The fit of these equations contained eight parameters. The differences between the parameters determined for the wild type strain and the corresponding mutant were next tested using the generalized F test.

Comparisons between two sets of values (e.g. promoter bearing transversion or deletion versus wild type promoter) were performed using the nonparametric Mann–Whitney test.

RESULTS

The upregulation of *MDR1* occurring in clinical azole-resistant strains is mimicked by exposure to benomyl, H₂O₂ and diamide

To analyse the transcriptional activity of *MDR1*, the proximal 999 bp of the *MDR1* promoter was fused in

frame to the *lacZ* reporter gene from *Streptococcus thermophilus* (Uhl & Johnson, 2001). The promoter fusion (*MDR1-lacZ*) was integrated in the azole-susceptible mutant strain DSY449 (*cdr1Δ/Δ*) at the *MDR1* locus as described in Methods. We chose to use this strain because the absence of *CDR1* may reduce drug efflux and thereby enhance the sensitivity of drug-dependent *MDR1* activation.

Upregulation of *MDR1* can occur in *C. albicans* azole-resistant strains or in azole-susceptible strains exposed to diverse drugs including benomyl and H_2O_2 (Gupta *et al.*, 1998; Harry *et al.*, 2005). Therefore we tested the response of the reporter to a panel of compounds known as inducers of *MDR1*. In order to test the *MDR1* response specificity, we also tested the response to oestradiol and fluphenazine, compounds known to induce *CDR2* (de Micheli *et al.*, 2002). For this purpose, a *CDR2-lacZ* reporter system was integrated in DSY449 in the same fashion as for the *MDR1-lacZ* construct. The relative increase of *MDR1-lacZ* and *CDR2-lacZ* reporter activities after treatment of *C. albicans* with these compounds is shown in Table 6. Upon exposure to benomyl, H_2O_2 or diamide, *MDR1-lacZ* reporter activities increased more than 10-fold as compared to untreated conditions, while no increase was observed for the *CDR2-lacZ* promoter fusion. In contrast, oestradiol and fluphenazine treatment specifically increased *CDR2-lacZ* reporter activities (by approximately 100-fold). Thus, the response of the two reporter genes to five different agents phenocopies the effects on the endogenous *MDR1* and *CDR2* genes. Exposure of *C. albicans* to methotrexate, sulfomethuron methyl and phenanthroline had no influence on transcription of either reporter. Based on these results, responses to the hydrophobic compound benomyl and to the oxidizing agent H_2O_2 were selected as readouts for the functional dissection of the *MDR1* promoter. As the chemical properties of these drugs are different, we reasoned that they might reveal different modes of activation of the *MDR1* promoter.

Identification of a promoter *cis*-acting element responsible for benomyl-dependent *MDR1* upregulation

Systematic 5' deletions of the *MDR1* promoter were performed in order to identify the *cis*-acting regulatory elements involved in the response to benomyl. The *MDR1-lacZ* promoter constructs were introduced into strain DSY449 at the *MDR1* genomic locus by double recombination as described in Methods (see also Fig. 1a). Correct integration was verified by Southern blot analysis (Fig. 1b).

A single transformant containing each deletion construct was chosen for testing of reporter activity in the absence and presence of benomyl. As shown in Fig. 2(a), the reporter under control of the proximal 1459 bp of the *MDR1* promoter exhibited a 26.1 ± 4.4 -fold increase of activity upon exposure to benomyl. Progressive deletions (99 to 141 bp) from -1459 to -302 (with respect to the ATG start codon) did not significantly alter the relative increase of reporter activity due to benomyl exposure. However, deletion of the region between -302 and -201 resulted in a 10-fold decrease in benomyl-dependent reporter activity, indicating that the minimal domain required for dramatic benomyl-dependent upregulation must lie within this region. This element was named BRE (for benomyl response element). To further delimit the BRE, small deletions of the region between nucleotides -302 and -222 were generated (Fig. 2b). Progressive 4 to 7 bp (-302 to -279) and 18 to 20 bp deletions (-279 to -222) revealed that benomyl-dependent *MDR1* transcription was significantly lowered by removal of sequence downstream of -296. The benomyl-dependent increase in reporter activity was 22.9 ± 3.1 -fold for the construct containing the *MDR1* promoter region terminating at -296. It decreased to 4.7 ± 0.7 - and 6.4 ± 1.1 -fold for constructs with promoters terminating at -290 and -286, respectively. A further decline of relative reporter activity to 2.1 ± 0.5 -fold at -279 was also observed. These data indicate that the 5' extremity of the BRE lies between -296 and -279.

Table 6. Effects of several compounds on *MDR1* and *CDR2* expression

Compound*	Fold increase of β -galactosidase activity†	
	<i>MDR1</i> promoter	<i>CDR2</i> promoter
Benomyl (35 $\mu\text{g ml}^{-1}$)	19.5 ± 4.8	1.0 ± 0.2
Oestradiol (10 $\mu\text{g ml}^{-1}$)	1.2 ± 0.3	95.8 ± 12.9
Fluphenazine (510 $\mu\text{g ml}^{-1}$)	1.1 ± 0.1	102.5 ± 9.1
Phenanthroline (20 $\mu\text{g ml}^{-1}$)	1.0 ± 0.2	1.1 ± 0.2
Sulfomethuron methyl (20 $\mu\text{g ml}^{-1}$)	1.1 ± 0.1	1.2 ± 0.4
Methotrexate (100 $\mu\text{g ml}^{-1}$)	1.2 ± 0.2	0.9 ± 0.2
Diamide (172 $\mu\text{g ml}^{-1}$)	12.6 ± 0.7	1.2 ± 0.4
H_2O_2 (0.003 %)	14.2 ± 1.4	0.9 ± 0.2

*Exposure time was 30 min at 30 °C.

†Mean of three separate experiments.

Fig. 2. Identification of a domain in the *MDR1* promoter involved in benomyl response. (a) Deletion analysis of the *MDR1* promoter fused to the *lacZ* reporter system under benomyl exposure. Constructs with 5' deletions in the *MDR1* promoter (shown on the left of the graph) were introduced into DSY449 (white bars) and MMY411 (*cap1Δ/cap1Δ*, black bars). The presence of a region involved in benomyl response (BRE) in both strains is shown by a black box. Results are expressed as relative increase of reporter activity, which corresponds to the ratio between the specific activities measured under exposed and unexposed conditions. The values are the means of single measurements obtained with three independent clones. Values significantly ($P \leq 0.05$) different from those obtained with the 1459 bp full-length promoter in DSY449 are indicated by an asterisk. As outlined in Methods, all reporter activities shown (strain DSY449) were used to model an equation with the following terms: $y = 2.1 + (25.4 - 2.1) / \{1 + \exp[(292.9 - x)/1.7]\}$. From this equation, it could be determined that the critical length for loss of the benomyl response was at -293, meaning that results obtained with promoter lengths below this critical length are significantly different from those obtained with longer lengths. For strain MMY411, the equation was $y = 2.1 + (13.0 - 2.1) / \{1 + \exp[(292.9 - x)/1.7]\}$. The generalized F test established that the maximal responses ($H_1 = 25.4$ and $h_1 = 13.0$) were significantly different ($P \leq 0.05$) between the two strains. (b) Precise delimitation of the BRE. Successive 5' deletions (4 to 7 bp between -302 and -279 or 18 to 20 bp between -279 and -222) were constructed and analysed in DSY449 (white bars). The minimal domain containing the BRE is represented by a black box within each promoter. Asterisks indicate the position of values that differ significantly ($P \leq 0.05$) from those obtained with the promoter of 302 bp in Mann-Whitney tests. The presence of two cytosines at the 3' end of the *KpnI* restriction site gave promoter lengths of 1101 and 286 bp, obtained from PCR products of 1100 and 284 bp, respectively.

A residual twofold increase in benomyl-dependent transcription appears to be independent of the BRE and suggests that the basal *MDR1* promoter can still respond to the drug.

H₂O₂-mediated *MDR1* expression is dependent on a *cis*-acting element

To investigate whether H₂O₂ upregulation of *MDR1* was mediated by the same element that is required for the

benomyl response, strains expressing the *MDR1* promoter deletion constructs were exposed to H₂O₂. The reporter activities of these strains were compared in the presence and absence of H₂O₂. H₂O₂-induced transcriptional activity of the reporter under control of the proximal 1459 bp of the *MDR1* promoter was increased 13.5 ± 3.4 -fold as compared to expression of the same reporter in the absence of H₂O₂. Upon progressive 5' deletion of the promoter in increments of approximately 100 bp, the reporter activity was stable

Fig. 3. The promoter domain responsible for the H_2O_2 response in the *MDR1* promoter is distinct from the BRE. (a) Deletion analysis of the *MDR1* promoter fused to the *lacZ* reporter system under H_2O_2 exposure. The presence of a domain implicated in H_2O_2 response (HRE) in both strains is indicated by a grey box. Results are expressed as relative increase of reporter activity, which corresponds to the ratio between the specific activities measured under exposed and unexposed conditions. The values are the means of single measurements obtained with three independent clones. Values significantly ($P \leq 0.05$) different from those obtained with the 1459 bp full-length promoter in DSY449 are indicated by an asterisk. Results obtained with strains DSY449 and MMY411 exposed to H_2O_2 allowed the formulation of the following equations: $y = 1.3 + (10.7 - 1.3) / \{1 + \exp[(540.4 - x)/0.9]\}$ and $y = 1.3$. As explained in the legend of Fig. 2(a), the critical length for the loss of H_2O_2 response was at -540.4 , meaning that results obtained with promoter lengths below this critical length are different from those obtained with longer lengths. Except for the variables H_0 and h_0 ($= 1.3$) representing the minimal H_2O_2 responses in both strains, all parameters of both equations were significantly different ($P \leq 0.05$) as revealed by the generalized F test. (b) Precise delimitation of the HRE. Successive 20 bp deletions from -598 to -501 were analysed in strain DSY449 exposed to H_2O_2 and used to delimit a domain (-560 to -520) containing the HRE, which is indicated by a grey box. Asterisks indicate the position of values significantly different ($P \leq 0.05$) from those obtained with the promoter of 598 bp in Mann-Whitney tests. The presence of one cytosine at the 3' end of the *KpnI* restriction site gave a promoter length of 561 bp obtained from a PCR product of 560 bp.

until the region between -598 and -501 was removed. In strains expressing a reporter under the control of the proximal -598 nucleotides of the *MDR1* promoter, the relative increase of reporter activity after H_2O_2 exposure was 10.0 ± 2.8 -fold, while the relative increase in H_2O_2 -dependent reporter activity dropped to only 1.5 ± 0.15 -fold when the *MDR1* promoter terminated at -501 (Fig. 3a). Progressive 18 to 21 bp deletions of the promoter region between -598 and -520 (Fig. 3b) located the H_2O_2 -dependent regulatory element between -561 and -520 : the relative increase of reporter activity observed under H_2O_2

exposure decreased from approximately 13.3 ± 1.1 -fold at -561 to 4.8 ± 0.4 - and 2.4 ± 0.2 -fold at -540 and -520 , respectively. We have called the *cis*-acting element thus identified HRE (for H_2O_2 response element). The location of this element clearly distinguishes it from the BRE described above. Interestingly, two sequences matching the consensus of Cap1p-binding sites (TTAG/CTAA) were located within the HRE, raising the possibility that Cap1p, a bZip transcription factor involved in oxidative stress, might regulate H_2O_2 -dependent expression of *MDR1* by binding to this element.

The *cis*-acting BRE, but not the HRE, is involved in *MDR1* upregulation of an azole-resistant strain

To evaluate the role played by the BRE and HRE in the constitutive high expression of *MDR1* that occurs in some azole-resistant strains, promoter deletion constructs were also inserted at the genomic *MDR1* locus of the fluconazole-resistant strain FR2, which constitutively expresses *MDR1* at high levels (Albertson *et al.*, 1996). In general, the specific β -galactosidase activities measured in this strain (Fig. 4) with *MDR1* promoter lengths between -999 and -302 [mean 58.2 ± 5.5 Miller units (U)] were significantly higher than those in strain DSY449 upon its exposure to benomyl (mean 4.7 ± 1.7 U). Moreover, reporter activities were not significantly altered when FR2 cells were exposed to benomyl

(data not shown). As shown in Fig. 4(a), reporter activities were not significantly altered by promoter deletions ranging from -999 to -302 , thus indicating that the *cis*-acting HRE located between -561 and -520 was not required for constitutive high expression of *MDR1* in FR2. In contrast, removal of the promoter sequences between -302 and -201 resulted in a more than 10-fold decrease in reporter activity. Interestingly, upon progressive shortening of the *MDR1* promoter from -296 to -279 (Fig. 4b), the data indicate a stepwise decrease in reporter activities: a first significant decrease between -296 (56.1 ± 3.9 U) and -290 (40.8 ± 4.8 U) and a second significant decrease between -286 (36.7 ± 2.1 U) and -279 (2.6 ± 0.2 U). This stepwise decline in reporter activities is reminiscent of that observed for the same promoter deletions in strain DSY449 exposed to benomyl (Fig. 2b), suggesting that both

Fig. 4. The BRE is responsible for the high level of expression of *MDR1* in the azole-resistant strain FR2. (a) Deletion analysis of the *MDR1* promoter fused to the *lacZ* reporter in FR2 (white bars) and MMY412 (*cap1* Δ/Δ , black bars) strains. The domain (BRE) responsible for high reporter activity in both strains is represented by a black box. Specific β -galactosidase activities (expressed in Miller units) are the means of single measurements obtained with three independent clones. Values significantly ($P \leq 0.05$) different from those obtained with the 999 bp full-length promoter in FR2 are indicated by an asterisk. As explained in the legend of Fig. 2(a), two equations could be established for all reporter activities obtained in the azole-resistant strain FR2: $y = 0.3 + (57.2 - 0.3) / [1 + \exp[(285.8 - x) / 3.0]]$, and the *cap1* Δ/Δ -derived mutant MMY412: $y = 0.3 + (52.0 - 0.3) / [1 + \exp[(285.8 - x) / 3.0]]$. From these equations, the critical length for loss of reporter activity was determined to be -286 , meaning that results obtained with promoter lengths below this critical length are different from those obtained with longer lengths. The generalized F test established that all parameters of the two equations in strains FR2 and MMY412 were not significantly different ($P \geq 0.05$). (b) Precise delimitation of the domain responsible for the high reporter activity in strain FR2. Promoters with 5' deletions (4 to 7 bp between -302 and -279 and 18 to 20 bp between -279 and -222) between -302 and -222 were analysed. The domain containing the BRE is represented by a black box. Asterisks indicate values significantly different ($P \leq 0.05$) from those obtained with the promoter of 302 bp in Mann-Whitney tests.

benomyl-induced transcription and constitutive high expression of *MDR1* may be under control of the same functional subelements within the BRE.

Transversions and deletions within the BRE

Deletions and transversions (G to A, C to T, A to G and T to C) within the region containing the *cis*-acting BRE identified by promoter deletion analysis (–296 to –279) were undertaken to verify the involvement of this element both in the benomyl-dependent response and in the constitutively high expression of *MDR1* observed in the azole-resistant strain FR2. The results obtained further establish the minimal sequence element required for the expression of *MDR1* in both conditions. Transversions within the putative BRE (T1: –287 to –266) or of smaller regions within this element (T2: –295 to –286; T3: –282 to –274; and T4: –276 to –267) had a dramatic effect on the response to

benomyl in strain DSY449 (Fig. 5a). For transversions T1 to T3, the residual increase in reporter activity in benomyl-treated cells as compared to the full-length promoter (22.1 ± 2.6 fold) was between 1.4 ± 0.02 - and 2.0 ± 0.3 -fold, similar to that of constructs under control of the basal promoter (Fig. 2, promoter deletions –201 and –98). The effect of transversion T4 was also significant but less pronounced. Benomyl treatment still caused a 6.4 ± 2.2 -fold increase in the activity of this reporter, suggesting that the BRE retained some functional capacity. Consistent with these results, the deletion of the region containing the BRE (D1: –291 to –269) also abolished the increase of reporter activity in response to benomyl (Fig. 5b). The relative increase of reporter activity was 1.3 ± 0.2 -fold with internal deletion D1 compared with 29.0 ± 2.7 -fold with the full-length *MDR1* promoter. Smaller internal deletions within this region (D2: –295 to –287; D3: –286 to –278; and D4: –277 to –269) also decreased benomyl-induced reporter

Fig. 5. Effects of nucleotide transversions and deletions within the BRE of the *MDR1* promoter. (a) Effects of nucleotide transversions within the BRE. The full-length *MDR1* promoters with transversions T1 to T4 within the BRE were fused to the *lacZ* reporter gene and constructs were inserted into strain DSY449, which was exposed to benomyl, and into FR2 in single copies as outlined in Fig. 1(a). Hatched boxes representing transversions T1 to T4 are shown at the corresponding positions (left). Results are given as relative increase of reporter activity after benomyl exposure in strain DSY449 (middle) or as specific β -galactosidase activities (expressed in Miller units) for reporter expression in strain FR2 (right). The values are the means of single measurements obtained with three independent clones. Values significantly different ($P \leq 0.05$) from those obtained with the wild type promoter in Mann–Whitney tests are indicated by an asterisk. (b) Effects of deletions within the BRE. Constructs with deletions D1 to D4 were fused to the *lacZ* reporter gene and analysed either in strain DSY449 exposed to benomyl (middle) or in strain FR2 (right). Deleted sequences and corresponding positions are represented by the crossed boxes for each promoter (left).

activities as compared to the full-length promoter, but did not completely abolish the response. The promoter carrying deletion D2 (−295 to −287) still showed a 17.1 ± 5.5 -fold increase in relative reporter activity in response to benomyl treatment, while the promoters carrying deletions D3 and D4 retained a less pronounced response to benomyl treatment (10.0 ± 2.5 -fold and 10.7 ± 1.5 -fold increase, respectively). The results confirmed that the BRE delimited by successive 5' promoter deletion analysis (−296 to −276) is essential for the benomyl-dependent response of *MDR1*. It is however interesting to note that transversions T2 and T3 had significantly more severe effects on benomyl-dependent transcriptional activity than did the deletions D2 and D3.

The effects of the same transversions and deletions upon constitutively high expression of the *MDR1* promoter were investigated in the background of the azole-resistant strain FR2. Transversions T1 to T4 each diminished reporter activities but none of them completely abolished the increase over activity measured in the azole-susceptible strain. Relative to the unaltered *MDR1* promoter, the constitutive activities of *MDR1* promoters carrying transversions T1, T2, T3 and T4 were respectively 3.7-, 2-, 11.6- and 8.7-fold lower (Fig. 5a). The constitutive activities of *MDR1* promoters bearing internal deletions D1, D2, D3 and D4 were respectively 3.5-, 1.7-, 1.7- and 2.5-fold lower than for the *MDR1* promoter that had no internal deletion (Fig. 5b). As previously noted for benomyl-dependent reporter activity, partial transversions of the BRE tended to have more severe effects than the corresponding deletions. However in this case, it was transversions T3 and T4 that had significantly stronger effects than deletions D3 and D4. These results demonstrate that the region containing the BRE also plays a role in the high constitutive expression of *MDR1*.

The partial retention of reporter activity in constructs lacking parts of the BRE may suggest that this regulatory region is composed of several discrete functional domains but we cannot rule out the involvement of other compensatory mechanisms. Moreover, while 5' deletions only delimit the 5' extremity of regulatory elements (i.e. −296, see Fig. 2b), the results presented above aid the localization of central elements of the BRE. Deletions D2 (−295 to −287), D3 (−286 to −278) and D4 (−277 to −269) all led to a decrease of benomyl-dependent *MDR1* response and of reporter activity in strain FR2, thus indicating that they all contain nucleotides important for the functionality of the BRE. Since these three deleted regions are sequential in the *MDR1* promoter, it can be concluded that at least all the nucleotides of the central deleted region D3 (−286 to −278) are part of the BRE.

Cap1p, a bZip transcription factor, acts as a *trans*-acting factor of *MDR1* regulation

The presence of two Cap1p-like binding sites within the *cis*-acting HRE (TTAG/CTAA; at −549 and −532) suggested that Cap1p, a bZip transcription factor involved in oxidative

stress, could play a role in regulating H₂O₂-dependent *MDR1* expression by binding to these sequences. To further explore this hypothesis, promoter deletion analysis was undertaken in MMY411 (*cap1Δ/Δ*), a strain derived from DSY449 that lacks *CAP1*. For all promoter fragments tested, the reporter activities obtained in the absence or presence of H₂O₂ treatment were not significantly different in this strain. The relative levels of reporter activity after H₂O₂ exposure were between 0.8 ± 0.1 - and 1.7 ± 0.6 -fold higher than that recorded when the strain was not treated with H₂O₂ (Fig. 3a, black columns). Thus, H₂O₂-dependent expression is essentially completely lost in the *cap1Δ/Δ* strain irrespective of whether the HRE is present. Therefore Cap1p is likely to be the main positive regulator involved in the response of the *MDR1* promoter to H₂O₂. We also examined the benomyl-dependent response of reporters under control of *MDR1* promoter fragments in the *cap1Δ/Δ* strain. Inactivation of *CAP1* did not prevent the different reporter constructs from responding to benomyl. Reporter constructs carrying at least 302 nucleotides of the proximal *MDR1* promoter showed a benomyl-dependent increase in activity (13.0 ± 5.1 -fold, black columns Fig. 2a). However, since the level of the responses was generally twofold lower than that recorded in the corresponding wild-type strain (25.9 ± 5.3 -fold, white columns Fig. 2a), we cannot exclude the possibility that Cap1p plays a minor role in mediating the transient response to benomyl. Finally, we observed no significant differences between the specific reporter activities measured in the azole-resistant strain FR2 (58.2 ± 5.5 U) and the corresponding *cap1Δ/Δ* mutant MMY412 (51.3 ± 7.5 U, Fig. 4a), indicating that Cap1p is not necessary for constitutive high expression of *MDR1* in FR2.

Importance of the *cis*-acting HRE for *MDR1* regulation

As the response of *MDR1* to benomyl was partially reduced in the strain lacking *CAP1*, the deletion of a region (−552 to −521) within the HRE (delimited between −561 and −520) was performed in the full-length *MDR1* promoter and reporter activities were measured under benomyl exposure in DSY449. As shown in Fig. 6(a), deletion of the region between −552 and −521 within the full-length *MDR1* promoter did not alter the response to benomyl: the increase in reporter activity after benomyl exposure (25.7 ± 5.0 -fold) was not significantly different from that in the control (30.1 ± 3.1 -fold). The simultaneous deletion of the BRE and HRE resulted in a similar effect to the deletion of the BRE alone (2.1 ± 0.5 -fold versus 1.3 ± 0.2 -fold increase in reporter activity). Consistent with the results shown in Fig. 2(a), these results indicate that the upregulation of *MDR1* under benomyl exposure is not dependent on the HRE. The HRE was also not involved in high constitutive expression of *MDR1* in strain FR2. In this strain, reporter activities were identical in the presence (66.4 ± 5.2 U) or absence (73.6 ± 3.7 U) of the HRE (Fig. 6b). Moreover, reporter activities after the simultaneous deletion of BRE

Fig. 6. Effects of HRE deletions on *MDR1* regulation. Nucleotide deletions within the HRE were generated in the full-length promoter or in the BRE-deleted *MDR1* promoter. Nucleotide coordinates are given for each deletion. Each HRE deletion was analysed either in DSY449 exposed to benomyl (a) or to H_2O_2 (c), or in FR2 (b). Results are given as relative increase of reporter activity after benomyl or H_2O_2 exposure in strain DSY449 or as specific β -galactosidase activities (expressed in Miller units) for reporter expression in strain FR2. The values are the means of single measurements obtained with three independent clones. Asterisks indicate the position of values significantly different ($P \leq 0.05$) from those obtained with the full-length promoter in Mann-Whitney tests.

and HRE (23.6 ± 2.6 U) were also similar to those observed in the absence of the BRE (16.3 ± 0.7 U) (Fig. 6b).

In contrast, the effects of the deletion of the HRE and BRE suggested that both elements were involved in H_2O_2 -dependent response of *MDR1*. Under H_2O_2 exposure (Fig. 6c), the increase in reporter activity measured with the promoter lacking the BRE (5.6 ± 1.0 -fold) was approximately twofold lower than that with the full-length promoter (9.4 ± 2.0 -fold). In the absence of HRE, as well as with the simultaneous deletion of HRE and BRE, the relative increase of reporter activity in response to H_2O_2 exposure was 2.9 ± 0.1 -fold and 2.0 ± 0.7 -fold, respectively, which represents a threefold and 4.6-fold lower response than that recorded for reporters under control of the full-length promoter. This suggests that not only the HRE but also the BRE could mediate H_2O_2 -dependent responses of *MDR1*.

In summary, our results show that the HRE is not essential for *MDR1* upregulation by benomyl or in an azole-resistant isolate but is required for H_2O_2 -dependent regulation of this gene. In the presence of H_2O_2 , Cap1p is likely to act *in trans* on the *MDR1* promoter through the HRE. While we cannot exclude an indirect role for Cap1p in the benomyl response, it does not act through either of the *cis*-acting elements that we have identified in *MDR1*.

The BRE and HRE are functional in a heterologous promoter

To further assess the *cis*-acting role of the BRE in response to benomyl, the element was placed in a minimal 198 bp *CDR2* promoter lacking its own regulatory element (DRE). The *CDR2* promoter is not responsive to benomyl and is therefore convenient for addressing the involvement of the BRE in this process. A BRE-*CDR2* chimeric promoter was

constructed and used with a *Renilla* luciferase reporter system. Because the sequences immediately flanking the BRE may modulate its function, a 50 bp *MDR1* promoter sequence was used which spanned nucleotides -309 to -260. The reporter system was inserted at the *LEU2* genomic locus of strains DSY449 and FR2. As shown in Fig. 7(a), the presence of the BRE conferred benomyl responsiveness to the minimal *CDR2* promoter. Up to 12.0 ± 2.1 -fold increase in reporter activity could be measured in benomyl-treated cells as compared to the control, untreated cells. Activity was reduced however as compared to the native 592 bp *MDR1* promoter (72.8 ± 5.6 -fold). When two copies of the BRE were fused to the *CDR2* promoter, the increase in reporter activity (96.8 ± 46.4 -fold) was comparable to that of the *MDR1* promoter. To assess the role of the HRE in the benomyl-dependent response, a 41 bp *MDR1* promoter sequence spanning nucleotides -556 to -516, and containing the HRE with Cap1p-binding sites, was placed upstream of the BRE-*CDR2* promoter. The increase in reporter activity induced by benomyl (17.6 ± 1.6 -fold) was not significantly different from that observed with the BRE-*CDR2* promoter (12 ± 2.1 -fold). When the HRE alone was fused to the *CDR2* promoter, almost no increase in reporter activity could be detected (1.6 ± 0.5 -fold), indicating that the HRE is not involved in the benomyl response of *MDR1*. In FR2, the BRE-*CDR2* chimeric promoter mediated high reporter activity. As shown in Fig. 7(b), the specific luciferase activity obtained with the BRE-*CDR2* fusion ($1.9 \pm 0.45 \times 10^6$ RLU) was only 2.5-fold lower than that obtained with the *MDR1* promoter ($4.9 \pm 1.0 \times 10^6$ RLU). Insertion of two copies of the BRE upstream of the minimal *CDR2* promoter gave reporter activities ($5.8 \pm 1.6 \times 10^6$ RLU) similar to those measured with the *MDR1* promoter. Since the specific reporter activities measured with the HRE-BRE-*CDR2* and the BRE-*CDR2* fusions ($1.7 \pm 0.3 \times 10^6$ and

Fig. 7. Insertion of the BRE and HRE into the *CDR2* promoter. BRE- (black rectangle) and HRE- (grey rectangle) containing sequences were first cloned upstream of the first 198 bp of the promoter of *CDR2* fused to the *Renilla* luciferase reporter gene. This minimal promoter is lacking the DRE but still contains elements required for efficient transcription. The effects of addition of HRE and/or BRE to the *CDR2* promoter were analysed either in DSY449 exposed to benomyl (a) or to H₂O₂ (c), or in FR2 (b). The results are given as relative increase of reporter activity after benomyl or H₂O₂ exposure in strain DSY449 or as specific activities for reporter expression, expressed in RLU, in strain FR2. The values are the means of single measurements obtained with three independent clones. Asterisks indicate the position of values that differ significantly ($P \leq 0.05$) from those obtained with the minimal *CDR2* promoter in Mann–Whitney tests.

$1.9 \pm 0.5 \times 10^6$ RLU, respectively) were similar and only background reporter activity was detected with the HRE alone ($< 10^4$ RLU), the HRE was not considered to be involved in the high levels of expression of *MDR1* in FR2. The data presented in Fig. 7 also contribute to a more precise localization of the functional BRE. Placing the 5' extremity at position -296 as previously determined, the 3' extremity can be proposed to be at position -260, since this corresponds to the position identified in the testing of BRE functionality.

We investigated whether the HRE could confer an H₂O₂-dependent response to the minimal *CDR2* promoter in strain DSY449 (Fig. 7c). However, when the HRE alone was fused to the minimal *CDR2* promoter, the relative increase in reporter activity (1.9 ± 0.7 -fold) was not different from the background activity increase measured with the basal *CDR2* promoter (2.0 ± 1.0 -fold). The relative increase in reporter activities after H₂O₂ exposure obtained with the BRE-*CDR2* promoter was similar (2.9 ± 1.8 -fold) to that with the HRE-*CDR2* promoter. However, when the HRE was placed upstream of the BRE-*CDR2* promoter, a 43.0 ± 3.1 -fold increase in reporter activity generated by H₂O₂ exposure was measured, which was 1.6-fold higher than with the *MDR1* promoter (27.3 ± 3.7 -fold). Thus, consistent with our observation that deletion of either element reduced the H₂O₂-dependent response of the *MDR1* promoter, both elements are required to convey significant H₂O₂-dependent upregulation to a heterologous promoter.

Trans-acting factors bind to the BRE

To assess the possible interaction between the BRE and putative transcription factors present in *C. albicans* protein

extracts, EMSAs were performed with double-stranded probes corresponding to several regions of the BRE. In this experiment, it was possible to observe the formation of a DNA–protein complex with labelled probe 1 (-296 to -259; Fig. 8a), which contained the BRE delimited by 5' promoter deletion analysis and insertion into another promoter context (between -296 and -260). This complex was present at higher levels, as revealed by the intensity of the band, when a protein extract from benomyl-exposed cells was used in comparison to that observed with proteins from untreated cells. The protein–DNA complex was specific for the BRE, since competition with increasing amounts of unlabelled BRE resulted in the disappearance of the complex (Fig. 8a). Furthermore, in the presence of a molar excess of an unlabelled probe, corresponding to a promoter region of the *RTA4* gene, the formation of the protein–DNA complex could be still observed. Finally, using this labelled *RTA4* as a probe (probe 2), formation of a protein–DNA complex with a similar migration pattern was not observed.

Labelled probes harbouring transversions identical to those used in promoter analysis (T1 to T4; probes 3–6) were also incubated with *C. albicans* protein extracts. However, as illustrated in Fig. 8(b), no specific band could be detected, thus corroborating the negative effects of these transversions upon reporter activities. Moreover, EMSAs carried out with labelled probes harbouring internal deletions (deletion D5: probe 7, -282 to -277 and deletion D6: probe 8, -285 to -274) showed no specific binding (Fig. 8b). This result is consistent with the finding that deletions within this region strongly reduced reporter activities (Fig. 5b). These results

Fig. 8. Specific protein complexes are formed with the BRE. (a) EMSA of the BRE with protein extracts from CAF2-1 cells exposed or not exposed to benomyl. Labelled probes 1 and 2 correspond to the sequence of the BRE (–296 to –259), which is longer than the BRE-containing domain delimited by promoter deletion analysis, and to the promoter sequence of *RTA4* (–165 to –123), respectively. Labelled probes were separated as described in Methods. Arrows indicate the position of specific and non-specific DNA–protein complexes and of free probes. Specificity of binding was assayed by competition with varying amounts of unlabelled probes 1 and 2. (b) EMSA of the BRE with transversed or deleted oligonucleotide probes. Cell extracts were obtained from CAF2-1 exposed to benomyl. The sequences of probes are shown at the bottom. Transversions are underlined whereas deleted nucleotides are outlined in grey. The BRE probe (probe 1) served as a positive control for DNA–protein complex formation.

show that the promoter regulatory region previously delimited (–296 to –260) corresponds to that which can bind sequence specific protein complexes *in vitro*.

To further delimit the BRE sequence necessary for the binding *in trans* of specific protein complexes, band-shift assays were performed with probe lengths reduced at both the 5' and 3' extremities of probe 1 (–296 to –259) containing the BRE. Removal of 5 and 6 bp at the 5' extremity of probe 1 (respectively probes 9 and 10) and of 5 to 13 bp at its 3' extremity (probes 15–17) did not abolish the formation of a DNA–protein complex (Fig. 9a). The intensity of bands for protein complexes with probes lacking 7 bp at the 5' extremity (probe 11) or 14 bp at the 3' extremity (probe 18) was slightly reduced as compared to the full-length probe 1. Finally, removal of at least 8 bp at the 5' extremity (probes 12–14) or at least 15 bp at the 3'

extremity (probes 19–22) resulted in the absence of protein complex formation (Fig. 9a). The results show that the minimal protein-binding region of the BRE is likely to be from –290 to –273. As expected, a specific DNA protein complex could be revealed (Fig. 9b) by using a labelled probe covering this region (probe 23).

DISCUSSION

Cis-acting regulatory elements of the *MDR1* promoter

In this study, we report the identification of two *cis*-acting promoter elements, the HRE and the BRE, important for the regulation of *MDR1*. The first element, the HRE, was localized to between –561 and –520 by promoter deletion

Fig. 9. Precise delimitation of the BRE required for the formation of DNA–protein complexes. (a) Progressive nucleotide deletions at the 5' and 3' extremities of the BRE probe. Probe sequences are given at the bottom. Cell extracts were obtained from CAF2-1 exposed to benomyl. (b) EMSA with the minimal BRE delimited by 5' and 3' deletions. Competition assays with the minimal BRE sequence (–290 and –273) and proteins from CAF2-1 cells exposed or not exposed to benomyl. The sequence of the probe used is given at the bottom. A sequence from the *RTA4* promoter was used as a non-specific competitor (see Fig. 8).

analysis and was responsible for H₂O₂-dependent *MDR1* regulation. A transcriptional response of the *MDR1* promoter to the oxidizing agent T-BHP, previously delimited by Harry *et al.* (2005) to lie within the region (–601 to –500), might also be attributable to the HRE. The HRE contains two YRE-like sequences, one that perfectly matches the consensus sequence (–532 to –526: TTAGTAA) and a second that differs from the consensus by two nucleotides (–549 to –543: TAACTAT). Deletion of the HRE almost abolished the H₂O₂-dependent response of the *MDR1* promoter (Fig. 3), suggesting strongly that the YRE-like sequences play a central role in this induction. The HRE within the *MDR1* promoter may be a downstream target of Cap1p, the functional homologue of the *S. cerevisiae* Yap1p, a bZip transcription factor binding to the YRE consensus sequence (Zhang *et al.*, 2000). In accordance with this hypothesis, the deletion of *CAP1* abolished the H₂O₂ response of *MDR1*. However, the

absence of the HRE did not alter the response of the *MDR1* promoter when *C. albicans* was exposed to benomyl. In contrast, the promoter of *FLR1* from *S. cerevisiae* (a functional homologue of *MDR1*) was shown to be activated by benomyl via a YRE (Nguyen *et al.*, 2001). Such species-specific differences serve to illustrate that the signal transduction pathways regulated by *CAP1* and *YAP1* are likely to have evolved significant differences despite the evidence for functional conservation of some of their downstream targets.

Surprisingly, the HRE had no impact on high constitutive expression of *MDR1*, since absence of this element did not alter *MDR1* expression (Fig. 6). Moreover, when fused to the promoter *CDR2*, the HRE was unable to confer a high reporter activity in FR2. In the background of an FR2-derived mutant lacking *CAP1*, *MDR1*–*lacZ* reporter activities were also unchanged as compared to the parent strain. These

results highlight the existence of distinct transcriptional programmes responsible for *MDR1* upregulation. While the influence of the HRE appears restricted to the H_2O_2 response, the deletion of *CAP1* also decreased, but did not eliminate, the benomyl-dependent response of *MDR1* (Fig. 2). Thus, our work reveals that there is some degree of crosstalk between the elements that mediate distinct responses. As the benomyl response of *MDR1* is dependent on the BRE, we speculate that Cap1p may cooperate with unknown factor(s) acting on the BRE.

The second *cis*-acting element of the *MDR1* promoter, the BRE, was responsible both for the benomyl-dependent response of *MDR1* and for high constitutive expression of this gene in the azole-resistant strain FR2. The delimitation of the minimal regulatory element that constitutes the BRE requires still further promoter analysis. The promoter truncations we examined have only delimited the 5' extremity of the regulatory elements (i.e., -296, see Fig. 2); internal deletions and transversions (Fig. 5) demonstrated that nucleotides between -286 and -278 are part of the BRE, but do not give a precise position of the 3' extremity of the regulatory element. Nevertheless, the 3' functional extremity of the BRE can be deduced from results obtained with the BRE-*CDR2* promoter fusion. They suggest that the position of the 3' extremity must lie upstream of position -260, therefore defining the functional BRE between -296 and -260. In the light of results obtained by EMSA with oligonucleotides corresponding to the sequence of the BRE, the minimal oligonucleotide length still enabling sequence-protein binding was determined to be located between -290 and -273 (Figs. 8 and 9) and thus lies fully within the functionally defined BRE. The convergence of both methods to this specific region of the promoter strengthens its implication as a regulatory element. *In vivo* and *in vitro* footprinting analysis of the BRE can now be applied to aid precise delimitation of this regulatory element and the further characterization of the proteins that bind to it.

The functional *cis*-acting BRE identified in our work (-296 to -260) lies close to a *cis*-acting benomyl-responsive region, as delimited by a separate study (Harry *et al.*, 2005). In that study, removal of the region between -399 and -299 led to a 20-fold diminution of the benomyl-dependent increase in reporter gene activities, whereas a weaker diminution (two- to fourfold) was observed when the region between -299 and -200 was absent from the *MDR1* promoter. Thus, while both studies indicate that the BRE-containing sequences are important, we have not seen evidence of a second, more distal benomyl-responsive element. This discrepancy could be the result of several factors, including the strain background or the type of gene reporter system used to assess promoter activity. Above all, the genomic site of reporter gene insertion might contribute to such differences. In the present study, the *MDR1-lacZ* reporter system was integrated at the *MDR1* genomic locus, whereas the *Renilla* luciferase reporter system used in the

study of Harry *et al.* (2005) was inserted at the *ADE2* genomic locus. A very recent study by Hiller *et al.* (2006) also shows the existence of several *cis*-acting domains in the *MDR1* promoter. This promoter analysis was performed with a GFP reporter system integrated at the *ACT1* locus in an azole-susceptible strain exposed to benomyl (3 h at $50 \mu\text{g ml}^{-1}$) and in an azole-resistant isolate expressing *MDR1* at high levels. This experimental context is similar to the one used here, except that the drug exposure time was longer and the gene reporter system was different and not integrated at the *MDR1* locus. The authors identified three different regulatory regions (region 1: -397 to -301; region 2: -588 to -500; region 3: -287 to -209). In their study, region 2 was implicated in the benomyl-dependent *MDR1* response and regions 1 and 3 were required for constitutive high expression of *MDR1*. Thus while there is some consensus of agreement on the delimitation of regulatory regions, there are also significant discrepancies between the findings of Hiller *et al.* (2006) and the other two studies, this work and Harry *et al.* (2005). Most notably, our results clearly implicate the HRE in the H_2O_2 response of *MDR1*, while Hiller *et al.* (2006) found that the region encompassing this element (region 2) was responsible for benomyl-dependent *MDR1* upregulation, a feature not confirmed in our study. The discrepancies between these different studies could have several origins, as mentioned above. The details of which regulatory elements respond to particular signals may also depend on chromatin-mediated factors and the gene's global context within the genome. Thus we believe it is of importance to have integrated the reporter constructs at the *MDR1* locus. Moreover, the demonstration that we can recapitulate the functions of the HRE and BRE in the context of another promoter further validates the transcriptional *cis*-acting roles that we assign to them. Furthermore, we demonstrated protein binding to the BRE, thus confirming that this *cis*-acting element may interact with as yet unknown factor(s).

A BRE-like sequence is present in the promoter of genes co-regulated with *MDR1* and contains a Mcm1p-binding site

In a previous study, we determined, by transcript profiling experiments, that *MDR1* was co-regulated with other genes in an azole-resistant strain and in a susceptible strain exposed to benomyl (Karababa *et al.*, 2004). We therefore expected to identify a common regulatory *cis*-acting motif resembling the BRE in the promoters of these genes. Alignment of promoter sequences revealed that nine of the 16 commonly upregulated genes indeed contain one or two sequences that match the signature of the BRE (Fig. 10a). Conserved sequence 1 (-287 to -267) in the *MDR1* promoter matches the previously delimited BRE (-296 to -260) and differs by 4 nucleotides from the consensus established after promoter sequences alignment (5'-TCCMMWTTWGKAAAKTYHCCG-3'). While the presence of the BRE signature in these promoters is therefore consistent with their co-regulation, additional work will be

(a)

Gene name	Strand	Position	p value	conserved sequence
<i>IFD1</i>	+	-235	1.14E-10	ACCCCATTAGGAAATCCCCCG
<i>IFD5</i>	+	-235	1.14E-10	ACCCCATTAGGAAATCCCCCG
<i>IPF17186</i>	-	-341	8.91E-10	TCTCCATTGGGAAAATCTCCG
<i>GRP2 (1)</i>	-	-407	1.12E-09	TCTCCATTAGGAAAGTAACCG
<i>GPX1</i>	-	-244	3.96E-09	TACAAATTAGGAAAGTTTCCG
<i>IFD4</i>	-	215	9.23E-09	TCCCAATTTGTTAATATTCCG
<i>MDR1 (1)</i>	+	-287	1.10E-08	TCCTAATTGGGAAAAATACCG
<i>IPF5987</i>	+	268	2.76E-08	GCCAGATTTGGCGAGTATCCG
<i>GRP2 (2)</i>	+	217	3.78E-08	TCCCTTTTTGTGACTTTCCCG
<i>IFD7</i>	-	268	4.40E-08	TCCTATTTTGTATAGTTTCCG
<i>MDR1 (2)</i>	+	496	6.35E-08	TTCATTTTAGGAAATTTACCG
<i>IPF7817</i>	-	-679	2.23E-07	TCCCGATTTGCCAATTTTACT
consensus				TCCMMWTTWGKAAAKTYHCCG
<i>MCM1</i>				
				CCYWWWWNGN

(b)

<i>C. albicans MDR1</i>	-300	CACAACGGTAAAAT CCTAATTGGG AAAAATACCGAGAATGACACAACCTAAGATTT	-245
<i>C. dubliniensis MDR1</i>	-320	TATCACGGTAAA- TCCACATTAGG AAAAATACCGAGAATGGCACAACCTTAGATTT	-266

Fig. 10. BRE-like consensus in genes from *Candida* species. (a) Identification of a potential consensus regulatory sequence in the promoters of genes co-regulated with *MDR1* under benomyl-exposed conditions and in an azole-resistant strain. The co-regulated genes were described previously (Karababa *et al.*, 2004). Alignment of promoter sequences (815 to 1000 bp) was performed with the software MEME, available at <http://meme.sdsc.edu/meme/meme.html>, with the following parameters: number of motifs, 5; minimum number of sites, 2; maximum number of sites, 50; minimum width of motif, 6; maximum width of motif, 30. The number of copies of the consensus sequence present within the same promoter is indicated in parentheses. *IFD1* and *IFD5* correspond to two alleles of the same gene. Only nucleotides with probabilities of 0.2 or higher at that position in the motif appear in the consensus. The *P* value represents the probability of a random string (generated from the background nucleotide frequencies) having the same match score or higher within the promoter. Nucleotide symbols are as follows: M=A/C, W=A/T, Y=T/C, K=A/T, H=T/C/A. (b) Alignment of *MDR1* promoter sequences from *C. albicans* and *C. dubliniensis*. Bases matching the Mcm1p-binding site are indicated in bold. Nucleotide sequences were obtained from the Pasteur database at <http://genolist.pasteur.fr/CandidaDB/index.html> and from the *C. dubliniensis* database at <http://www.sanger.ac.uk/sequencing/Candida/dubliniensis/>.

required to confirm that the BRE is important for the regulation of these genes. A second BRE-like sequence (−496 to −476) was identified in the *MDR1* promoter (see Fig. 10a); however, promoter deletion analysis revealed that this sequence is not involved in *MDR1* regulation either in DSY449 exposed to benomyl or in FR2. Furthermore, EMSA with a probe corresponding to this second sequence failed to reveal protein-binding activity (data not shown).

When inspecting the BRE sequence (−296 to −260) for known transcription factor binding sites, using the TESS Master software (<http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ>), a binding site for Mcm1p (CCTAATTGGG; −288 to −277) matching the consensus binding site (CCYWWWWNGN) (Chang *et al.*, 2003; Passmore *et al.*, 1989; Spellman *et al.*, 1998; Wynne & Treisman, 1992) was

localized within this sequence. A similar promoter context exists in the *MDR1* promoter of *Candida dubliniensis* (Fig. 10b), a species related to *C. albicans* in which *MDR1* upregulation has been demonstrated (Moran *et al.*, 1998). The presence of the putative Mcm1p-binding site in the BRE suggests that Mcm1p could act as a *trans*-acting factor on the BRE and thus be involved in the regulation of *MDR1* via the BRE. Interestingly, this sequence overlaps the region deleted in Fig. 5 (D3: −286 to −278), which was considered to be a central part of the BRE. In *S. cerevisiae*, *MCM1* is an essential gene of the MADS box transcription factor family involved in a variety of cellular processes, including chromatin remodelling, arginine response and mating as well as cell cycle regulation (Keleher *et al.*, 1988; Passmore *et al.*, 1988). In *C. albicans*, *MCM1* is crucial for the determination of cell morphology and is autoregulated by a feedback mechanism.

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