Multiple Pdr1p/Pdr3p Binding Sites Are Essential for Normal Expression of the ATP Binding Cassette Transporter Protein-encoding Gene PDR5*

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Saccharomyces cerevisiae has large number of genes that can be genetically altered to produce a multiple or pleiotropic drug resistance phenotype. The homologous zinc finger transcription factors Pdr1p and Pdr3p both elevate resistance to many drugs, including cycloheximide. This elevation in cycloheximide tolerance only occurs in the presence of an intact copy of the PDR5 gene that encodes a plasma membrane-localized ATP binding cassette transporter protein. Previously, we have found that a single binding site for Pdr3p present in the PDR5 promoter is sufficient to provide Pdr3p-responsive gene expression. In this study, we have found that there are three sites in the PDR5 5′-noncoding region that are closely related to one another and are bound by both Pdr1p and Pdr3p. These elements have been designated Pdr1p/Pdr3p response elements (PDREs), and their role in the maintenance of normal PDR5 expression has been analyzed. Mutations have been constructed in each PDRE and shown to eliminate Pdr1p/Pdr3p binding in vitro. Analysis of the effect of these mutant PDREs on normal PDR5 promoter function indicates that each element is required for wild-type expression and drug resistance. A single PDRE placed upstream of a yeast gene lacking its normal upstream activation sequence is sufficient to confer Pdr1p responsiveness to this heterologous promoter.

Multidrug resistance has been defined as broad range resistance to chemotherapeutic agents associated with human tumors (1). Although there are several different mechanisms that can contribute to multidrug resistance, one of the best understood involves overexpression of certain members of the ATP binding cassette transporter family of proteins: MDR1 (2–4) and the multidrug resistance-associated protein (5). Elevated levels of these transporter proteins lead to an enhanced rate of drug efflux from tumor cells with subsequent multidrug resistance (6–8). The resulting cross-resistance to varied cytotoxic agents represents a major impediment to chemotherapy.

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*S. cerevisiae displays an analogous phenotype to mammalian multidrug resistance, termed pleiotropic drug resistance (Pdr). The genes involved in the Pdr phenotype have thus far fallen into two general categories: membrane transporter proteins and their cognate transcriptional regulatory proteins (9). As in higher eukaryotes, several of these membrane transporter proteins are members of the ATP binding cassette transporter superfamily of proteins, including PDR5, SNQ2, and YOR1. PDR5 has been shown to play a role in resistance to a number of cytotoxic agents, including cycloheximide (10–12). Loss of function PDR5 mutants are sensitive to drugs due to an inability to efficiently efflux drugs, suggesting that PDR5 is directly involved in the efflux of these substances (13).

Two homologous zinc finger transcription factors, encoded by PDR1 and PDR3, have previously been demonstrated to be key effectors of pleiotropic drug resistance, including cycloheximide tolerance (14, 15). Epistasis analyses have demonstrated that cycloheximide resistance mediated by Pdr1p or Pdr3p requires the presence of PDR5 (14, 16). Direct measurement of PDR5 gene expression indicates that both Pdr1p and Pdr3p can modulate transcription of PDR5. Deletion analysis of the PDR5 promoter indicates that the presence of a single DNA element, located at −187 in the 5′-noncoding region of the gene, is sufficient to maintain Pdr3p-responsive gene expression (14).

We have assessed the action of Pdr1p on the PDR5 promoter. PDR1 is the major contributor to drug resistance of the PDR1/PDR3 gene pair, presumably due to its importance as a transactivator (14). We show here that the same segment of the PDR5 promoter required for Pdr3p-responsive expression is also required for Pdr1p-responsive expression. Analysis of the PDR5 sequence has shown the presence of two other elements related by primary sequence to the Pdr1p/Pdr3p response element (PDRE) at −187. We have shown by DNase I footprinting analysis that both Pdr1p and Pdr3p are able to bind to these PDREs in vitro. Using site-directed mutagenesis, we have introduced mutations into the PDR5 PDREs that block the ability of both Pdr1p and Pdr3p to bind to these sequence elements. The various combinations of mutant PDREs were placed back in the context of a PDR5-lacZ fusion gene or the native PDR5 locus and analyzed in S. cerevisiae cells. These experiments revealed that all three sites are required for wild-type expression of PDR5, with each site contributing approximately equally. Furthermore, an oligonucleotide corresponding to one of these sites is capable of conferring Pdr1p responsiveness on a heterologous reporter system. From these experiments, we
conclude that the PDRE is both necessary and sufficient to function as the in vivo site of action of Pdr1p and is likely to fulfill this role for Pdr3p as well.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The isogenic set of pdr mutant strains used in the present study has been described elsewhere (14) and consists of: 156S210 (MATα, leu2–3, –112, ura3–52, his3–Δ200, trp1–Δ901, lys2–801, suc2–ΔΔ, Mcl–1), PB2 (SEY210 pdr5–Δ::hisG), PB4 (SEY210 pdr1–Δ::hisG, pdr3–Δ::hisG), and DKY1 (SEY210 pdr5–Δ::hisG). Yeast transformations were performed by the lithium acetate procedure of Ito et al. (17). Standard yeast media were used for growth of cells and drug resistance assays (18). Drug resistance assays were performed by spot test (19). β-galactosidase activity was determined as described in Ref. 20.

Plasmids—The translational fusion constructs between the PDR5 5′-promotor deletions and Escherichia coli lacZ have been described (14). The high copy PDR1 plasmid pRS425-PDR1 was constructed by insertion of a BamHI fragment carrying PDR1 into the unique BamHI site of pRS425 (21). The low-copy plasmid carrying the PDR5 gene was constructed in two steps. First, a Bsp106I/SalI fragment of PDR5 was cloned into the ClaI/SalI sites of pRS314 to form pSM86. The PDR5 gene was reinserted by a SalI/blunt-ended HindIII fragment containing the 3′-end of the PDR5 gene into pSM86 that was cleaved with XhoI, treated with dNTPs and the Klenow fragment, and then cut with SalI. This recombinant was designated pSM89. 1148 bp upstream of the PDR5 transcription start site are contained in pSM89. To produce an appropriate control plasmid for analysis of the mutant PDRE-containing PDR5 promoters, a NotI/SalI fragment containing 360 bp upstream of the transcription start site was transferred into pSM89, producing pDK17. The template used for PCR mutagenesis was the p2-5 subclone that contained a PDR5 promoter fragment extending from an EcoRI linker placed at position –360 to a HindIII site at position –91 of PDR5. A series of EcoRI/HindIII-digested pd5K2 and pDK5, respectively. These two plasmids contain either the wild-type or the mutant version of the site 3 PDRE, placed as the upstream activation sequence (UAS) for a CYC1-lacZ fusion gene in a centromere-containing vector.

Site-directed Mutagenesis—Clustered base substitution mutations were introduced into the three PDREs in the PDR5 promoter using a PCR-based strategy (23). Briefly, a mutagenic primer was annealed to p2-5 template DNA along with the M13 reverse primer. The mutagenic primers were: site 1, TCT TCC TCG AGA TCG CTC ATG GC; site 2, CGT GAT TCC GTC GAG ACG TCA GAT CTG; site 3, TGT CTC GTC GAG ACT CTT CTA CGG CG. PCR and performed and the resulting product was purified from an agarose gel. This PCR product was then mixed with a PCR product prepared from amplification of the same template using the T7 primer and a PDR5-promoter specific primer (AGC GAA CAG CCA TAA GGT TAT GCA C). These two PCR products overlapped by at least 100 bp and were annealed and subjected to PCR in the presence of the T7 and M13 reverse primers. Each single PCR product was gel purified, cleaved with EcoRI and EcoRV, and cloned into pBlueScript. Clones containing mutated PDREs were identified by restriction mapping, and the sequence of the entire amplified region was determined to check for PCR errors. Each single PDRE mutant was then introduced as an EcoRI/EcoRV fragment back into the context of the PDR5 promoter carried on p2-5. The double and triple PDRE mutant promoters were constructed by using the unique BglII and BstXI sites that were unique to the single mutants and form the multiply mutant PDR5 promoters. The PDRE mutant promoters were transfected back into the PDR5-lacZ fusion gene as EcoRI/HindIII fragments and back into the PDR5 gene as NotI/SalI fragments.

DNA Binding Methods—The DNase I protection assay was performed as described previously (24). Two different pOTs-Neo2 (25) subclones expressing the N-terminal 248 amino acids of Pdr1p and the N-terminal 213 residues of Pdr3p were used to produce these proteins in bacteria as before (14).

RESULTS

Pdr1p Activates PDR5 Gene Expression through the Same Region of the PDR5 Promoter as Pdr3p—We have previously demonstrated that wild-type expression of the PDR5 gene required the presence of at least one of the homologous zinc finger-containing transcription factors Pdr1p or Pdr3p (14). Mutagenesis of a PDR5-lacZ fusion gene indicated that a DNA element located at position –140 in the PDR5 promoter was required for Pdr3p responsiveness of this locus (14). Several studies implicate PDR1 as the major contributor to drug resistance of the PDR1/PDR3 gene pair (14, 15). Since Pdr1p and Pdr3p are homologous transcriptional regulators, it is reasonable to suggest that their sites of action on the PDR5 promoter would also be related. To directly test this suggestion, we assessed the ability of a series of PDR5 promoter mutations to respond to the presence of PDR1 on a high copy plasmid.

Low-copy plasmids containing PDR5-lacZ fusion genes with variable amounts of PDR5 5′-noncoding DNA were introduced into wild-type S. cerevisiae cells along with a high copy plasmid containing the PDR1 gene or the vector plasmid alone (Fig. 1). The levels of PDR5-dependent β-galactosidase were then determined in the presence of these two different gene dosages of PDR1. A PDR5 5′-flanking region containing 1093 bp upstream of transcription start produced 29 units/A600 of β-galactosidase in the presence of single-copy PDR1. Introduction of the high copy PDR1 plasmid increased PDR5-dependent enzyme activity to 72 units/A600. Truncation of the PDR5 5′-flanking DNA present in the PDR5-lacZ plasmid to 360 bp upstream of the transcription start site had no effect on the expression or the response of the fusion gene to high copy PDR1. Further removal of PDR5 5′-flanking DNA to –316 caused a large reduction in enzyme activity produced in the presence of single-copy PDR1 but maintained the response to high copy PDR1. A truncation of the PDR5 5′-flanking sequence to –187 produced a fusion gene that was essentially inactive except in the presence of high copy PDR1. Either a site-directed mutation in the previously described Pdr3p binding site or truncation to –112 abolished the ability of the resulting PDR5-lacZ fusion gene to respond to high copy PDR1.

From this analysis, we concluded that the same DNA element required to maintain the Pdr3p responsiveness of PDR5 was also necessary for Pdr1p control of expression. The dramatic loss of function exhibited upon deletion of the –316 to –187 region indicated that an important element for control of PDR5 expression had been lost in the –187 construct. Previous experiments have shown that either PDR1 or PDR3 must be present for significant expression of PDR5 (14). We next investigated the possible existence and relative importance of other Pdr1p/Pdr3p binding sites in the PDR5 promoter.

The PDR5 Promoter Contains Three Sites That Are Bound by Both Bacterially Expressed Pdr1p and Pdr3p—Both Pdr1p and Pdr3p are members of the C6 zinc cluster family of DNA-binding proteins (26). These factors have been found to recognize rotationally symmetric, GC-rich DNA elements related to CGCG, where x can vary from 0 to 11 (27). We inspected the DNA sequence of the PDR5 promoter from –360 to –112 and found two sites in addition to the previously described element (143 to –125) that were candidates for PDR5 binding. The sequence of each site, along with its position in the PDR5 promoter, is shown in Fig. 2. We have designated these PDR5 elements as sites 1, 2, and 3, with site 1 being the 5′-most of the three. Comparison of these three sequences led to the prediction that TCCCGCGGAAA might be the consensus sequence for binding of Pdr1p and Pdr3p.

To determine whether these related DNA sequences were able to be recognized by Pdr1p and/or Pdr3p, we carried out
DNase I protection assays with bacterially produced Pdr1p and Pdr3p (Fig. 3). Each of these three sites can be specifically recognized by bacterially produced Pdr1p and Pdr3p. The DNase I protection pattern on each wild-type template is identical, irrespective of whether Pdr1p or Pdr3p is used.

Having established that three PDREs exist in the \(-360\) to \(-112\) interval of the PDR5 promoter, we prepared mutant versions of each binding site (Fig. 2). This was done in preparation for a functional analysis of the role of each PDRE in PDR5 promoter action. Clustered base substitution mutations were introduced in each PDRE, changing two or three positions of each binding site. The ability of bacterially generated Pdr1p and Pdr3p to bind to each mutant PDRE was next assayed by DNase I protection analysis (Fig. 3). This assay demonstrated that none of the three mutant PDREs could be recognized in vitro by either Pdr1p or Pdr3p. These mutant PDR5 promoters were then used to analyze the effect of loss of a given PDRE on PDR5 expression and drug resistance.

The Three PDREs in the PDR5 Promoter Are Required for Wild-type Expression of PDR5—To assess the quantitative contribution of each PDRE to PDR5 expression, each mutated PDRE was introduced into a PDR5-lacZ fusion plasmid containing \(-360\) bp of 5'-noncoding DNA. The PDR5-lacZ fusion gene was used to simplify measurement of PDR5 expression. Additionally, each mutated PDRE was placed back into the context of the wild-type PDR5 gene. These constructs allowed the physiological consequences of loss of a given PDRE to be evaluated in terms of Pdr5p-mediated cycloheximide resistance. All seven possible combinations of the three mutant PDREs were constructed upstream of both the PDR5-lacZ and wild-type PDR5 genes. This resulted in the generation of two different plasmids for each type of promoter mutation. The PDR5-lacZ and PDR5 versions of each type of promoter mutation were then transformed together into a \(\Delta pdr5\) strain and assayed for \(\beta\)-galactosidase activity as well as cycloheximide resistance (Figs. 4 and 5).

As described above, a PDR5-lacZ fusion gene with \(-360\) bp upstream of the transcription start site produces wild-type...
levels of \( PDR5 \)-dependent \( \beta \)-galactosidase activity. Removal of any one of the PDREs from this fusion gene led to an approximately 50% reduction in the level of expression. Loss of any two of the three PDREs resulted in a much more dramatic loss of \( PDR5 \) expression. The three double \( PDR5 \) promoters were only able to drive 3–6% of wild-type \( PDR5 \) expression. The mutant promoter lacking all three PDREs was essentially inactive.

Along with the \( \beta \)-galactosidase assays, cycloheximide resistance assays were performed. Approximately 5000 cells transformed with one of the \( PDR5 \) promoter mutants driving the expression of \( PDR5 \) were spotted on media containing various concentrations of the drug cycloheximide. The \( D_{pdr5} \) strain transformed with vector only (pRS314) is unable to grow in the presence of 0.1 \( \mu \)g/ml cycloheximide (Fig. 5). Transformants containing the \( PDR5 \) gene lacking all three of the PDREs were also unable to tolerate this low level of cycloheximide. The mutant \( PDR5 \) promoters lacking only a single PDRE were able to support growth on 0.2 \( \mu \)g/ml cycloheximide nearly as well as the wild-type promoter. However, the three double \( PDR5 \) mutants lacked all three of the PDREs and were essentially inactive.

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These experiments demonstrate the importance of the PDREs in the function of the \( PDR5 \) promoter. The ability of one of the site 3 PDREs to confer Pdr1p-responsive expression to a heterologous promoter was evaluated next.

**An Oligonucleotide Corresponding to Site 3 Can Function as a UAS**—The decrease in \( PDR5 \) expression upon removal of each PDRE established that these DNA elements are necessary for normal \( PDR5 \) promoter function. Furthermore, the removal of the site 3 PDRE from the \( PDR5-lacZ \) fusion gene containing...
protein. Loss of function mutations in either of these loci elicit a severe drug hypersensitive phenotype of the resulting strains. Control of PDR gene expression is a key determinant in conferring resistance to toxic agents. Loss of function mutations in either of these loci elicit a severe drug hypersensitive phenotype of the resulting strains. Control of PDR gene expression is a key determinant in conferring resistance to toxic agents.

When the site 1, 2 PDRE mutant promoter was placed upstream of the PDR5 structural gene, the resulting mutant PDR5 gene was capable of complementing the cycloheximide sensitivity of a Δpdr5 mutant strain (Fig. 5). This was not true for a PDR5 gene lacking all three PDREs (Fig. 4), confirming the small but significant functional role of the site 3 PDRE in the absence of the other two elements.

**DISCUSSION**

Both PDR1 and PDR5 are major contributors to the ability of wild-type *S. cerevisiae* cells to tolerate a large variety of cytotoxic agents. Loss of function mutations in either of these loci elicit a severe drug hypersensitive phenotype of the resulting strains. Control of PDR5 expression is a key determinant in setting the level of drug tolerance of cells. In the current study, we have identified cis-acting elements that are required for wild-type PDR5 expression and drug resistance. These elements are encoded in *in vitro* by both Pdr1p and Pdr3p and have been designated PDREs. The PDREs in the PDR5 promoter show the typical sequence composition of recognition sequences for *C. elegans* zinc cluster proteins with sequence comparison suggest-

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**Multiple Pdr1p/Pdr3p Binding Sites in the PDR5 Promoter**

187 bp of 5′-flanking DNA abolished the Pdr1p (and Pdr3p) responsiveness of this promoter. To determine whether a single PDRE was sufficient to serve as a Pdr1p-dependent UAS, an oligonucleotide corresponding this DNA element was synthesized. This site 3 PDRE oligonucleotide was cloned upstream of a CYC1-lacZ fusion gene that lacked intrinsic UAS function. The mutant form of this site 3 PDRE was also synthesized as an oligonucleotide and was likewise cloned upstream of CYC1-lacZ. Each of these PDRE-CYC1-lacZ fusion plasmids, along with PDR5-lacZ control plasmids, was introduced into a Δpdr1 pdr3 strain containing different alleles of the PDR1 gene. The three different groups of transformants contained either no functional PDR1 gene, the wild-type PDR1 locus, or a mutant version of PDR1 (PDR1-6) that causes overproduction of PDR5 transcript and enhanced drug resistance (28, 29). β-Galactosidase activity was determined from appropriate transformants.

The wild-type site 3 PDRE cloned upstream of CYC1-lacZ (PDRE-CYC1-lacZ) was strongly responsive to the PDR1 allele present (Table I). The PDRE-CYC1-lacZ-dependent β-galactosidase activity increased from 0.5 units/A600 in the absence of PDR1 to 2 units/A600 when PDR1 was restored. The presence of the PDR1-6 allele elevated site 3 PDRE-driven enzyme activity to 21 units/A600. By comparison, the mutant version of the site 3 PDRE (mPDRE-CYC1-lacZ) was unable to respond to the changes in the PDR1 allele and produced from 0.3 to 0.5 units/A600 in all genetic backgrounds assayed. The PDR5-lacZ fusion gene with −360 bp of 5′ upstream DNA was assayed as a control for the behavior of the differing PDR1 backgrounds. This fusion plasmid showed the previously described strong response of PDR5 expression to these different PDR1 alleles (28, 29). A derivative of this PDR5-lacZ fusion containing only a functional site 3 PDRE was assayed in parallel as a control for the function of site 3 PDRE in its native PDR5 environment. The site 1, 2 PDRE mutant PDR5-lacZ fusion gene produced extremely low levels of β-galactosidase activity that were not detectably influenced by the presence or absence of the wild-type PDR1 gene. However, this mutant form of the PDR5 promoter still maintained its Pdr1p responsiveness since the PDR5-dependent β-galactosidase activity could be strongly enhanced by introduction of the PDR1-6 allele. Additionally,

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ing that the consensus PDRE consists of TTCCGCGGAA. The same element has been found in other Pdr1p target genes, such as \textit{PDR3} (30), \textit{SNQ2} (28, 31), \textit{D4405}, and \textit{YOR1} (29). In the case of \textit{PDR3} (30) and now \textit{PDR5} (this study), the PDREs have been shown to be required for Pdr1p transcriptional control of the gene in question. We conclude that the PDRE is the \textit{in vivo} site of action for Pdr1p and likely for Pdr3p.

Maintenance of wild-type \textit{PDR5} transcription requires the presence of either the \textit{PDR1} or \textit{PDR3} gene (14). This finding indicated that \textit{PDR5} transcription was strictly \textit{PDR1/PDR3}-dependent. We have extended this observation by producing a \textit{PDR5} promoter that is unable to be bound by bacterially produced Pdr1p or Pdr3p. This triple PDRE mutant promoter is not able to drive \textit{PDR5} expression as measured by either the \textit{PDR5}-dependent \( \beta \)-galactosidase activity or drug resistance. These data further support the view that the main, if not the only, source of activation of \textit{PDR5} transcription is supplied by the action of Pdr1p/Pdr3p at the PDREs. This is not true for all Pdr1p/Pdr3p regulated genes since both \textit{YOR1} (29) and \textit{SNQ2} (28) have significant Pdr1p/Pdr3p independent components of expression.

This study of PDRE function in the \textit{PDR5} promoter also indicates that each of these regulatory elements appears to contribute roughly equally to overall \textit{PDR5} expression levels. As more PDREs are mutated, expression of the \textit{PDR5-lacZ} gene is progressively diminished until all three sites are removed. The same trend is seen when the mutant \textit{PDR5} promoters are placed upstream of the \textit{PDR5} structural gene, with the possible exception of the site 2, 3 double PDRE mutant promoter. This mutant version of the \textit{PDR5} promoter was found to be reproducibly weaker, in terms of conferring cycloheximide tolerance, than the other two double PDRE mutant promoters, even though comparison of these three double mutant PDRE mutant promoters using the \textit{PDR5-lacZ} fusion gene did not reveal any significant differences. One potential explanation for this observation is provided by the possible cycloheximide inducibility of \textit{PDR5} that has been described (12, 30). Since our \( \beta \)-galactosidase assays were performed on cells grown in the absence of cycloheximide, a defect in inducibility would not have been detected. We were unable to demonstrate induction of our wild-type \textit{PDR5-lacZ} fusion by cycloheximide (data not shown), but this finding is complicated by the need for increased synthesis of \( \beta \)-galactosidase in the presence of the translation inhibitor cycloheximide. Direct RNA measurements must be carried out to examine the possible role of cycloheximide inducibility in the function of the \textit{PDR5} promoter.

Mutant forms of either \textit{PDR1} or \textit{PDR3} have been identified that lead to overproduction of \textit{PDR5} transcript (16, 32). The basis for the alteration of function in these dominant, drug resistant mutant transcription factors is not yet well defined. In this study, we show that a single PDRE placed upstream of a \textit{CYC1-lacZ} fusion gene confers Pdr1p responsive expression on this heterologous promoter and is strongly stimulated by the presence of the \textit{PDR1}-6 dominant allele of \textit{PDR1}. These data demonstrate two important facts about Pdr1p-responsive transcrip-

\textsuperscript{3} L. Lambert, J. L. Jonniaux, W. S. Moyer-Rowley, S. Goffeau, and E. Balzi, submitted for publication.

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