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Regulation of Transcription Factor Pdr1p Function by an Hsp70 Protein in *Saccharomyces cerevisiae*

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Multiple or pleiotropic drug resistance in the yeast *Saccharomyces cerevisiae* requires the expression of several ATP binding cassette transporter-encoding genes under the control of the zinc finger-containing transcription factor Pdr1p. The ATP binding cassette transporter-encoding genes regulated by Pdr1p include *PDR5* and *YOR1*, which are required for normal cycloheximide and oligomycin tolerances, respectively. We have isolated a new member of the *PDR* gene family that encodes a member of the Hsp70 family of proteins found in this organism. This gene has been designated *PDR13* and is required for normal growth. Overexpression of Pdr13p leads to an increase in both the expression of *PDR5* and *YOR1* and a corresponding enhancement in drug resistance. Pdr13p requires the presence of both the *PDR1* structural gene and the Pdr1p binding sites in target promoters to mediate its effect on drug resistance and gene expression. A dominant, gain-of-function mutant allele of *PDR13* was isolated and shown to have the same phenotypic effects as when the gene is present on a 2μm plasmid. Genetic and Western blotting experiments indicated that Pdr13p exerts its effect on Pdr1p at a posttranslational step. These data support the view that Pdr13p influences pleiotropic drug resistance by enhancing the function of the transcriptional regulatory protein Pdr1p.

Multiple drug resistance refers to a limited number of genetic alterations giving rise to a complex spectrum of tolerance to cytotoxic compounds having different intracellular targets (32). In human tumor cells, multiple drug resistance is often associated with overproduction of ATP binding cassette (ABC) transporter-encoding genes, such as *MDR1* (22) or *MRP* (11). The overproduction of these gene products leads to enhanced efflux of toxic drugs across the plasma membrane and permits tolerance of otherwise lethal dosages.

In the yeast *Saccharomyces cerevisiae*, a similar multidrug tolerance phenotype can be observed and is referred to as pleiotropic drug resistance. The first *PDR* gene identified and cloned was designated *PDR1* (2). DNA sequence analysis of this locus indicated that Pdr1p was a zinc finger-containing protein that showed strong sequence similarity to other fungal transcriptional regulatory proteins (2). Strains lacking *PDR1* were hypersensitive to a broad range of drugs, including cycloheximide and oligomycin. Sequence analysis of *S. cerevisiae* chromosome II indicated that a homolog of *PDR1*, *PDR3* (16), was present at this location and encoded a protein showing 36% amino acid identity with Pdr1p (15). Genetic and biochemical experiments demonstrated that Pdr1p and Pdr3p act to influence pleiotropic drug resistance (15, 33). However, mutants lacking *PDR3* were not observed to have a pronounced defect in drug resistance, unlike Δ*pdr1* strains, suggesting that Pdr1p was the major contributor of drug tolerance (15, 33).

PDR1 was originally identified on the basis of semidominant mutant alleles that produced high-level resistance to cycloheximide and oligomycin, among other compounds (reviewed in reference 3). Epistasis and Northern blot experiments demonstrated that *PDR1* conferred cycloheximide resistance through the transcriptional activation of the *PDR5* gene (43). *PDR5* was shown to encode an ABC transporter protein (5, 6, 28) that is located in the plasma membrane (5, 17) and that can act as a drug efflux pump (36, 38). Deletion mapping and DNase I footprinting analysis indicated that both Pdr1p and Pdr3p bound to several sites upstream of the *PDR5* transcription start site and activated the expression of this gene (33, 34). These binding sites were named Pdr1p/Pdr3p response elements (PDREs).

While semidominant *PDR1* mutants required *PDR5* to mediate cycloheximide resistance, the loss of *PDR5* did not affect *PDR1*-mediated oligomycin resistance. We screened a high-copy-number plasmid library for sequences that would elevate oligomycin resistance and recovered several different genes (35). One of these loci was found to encode an ABC transporter protein resembling the *MRP* gene product and was designated *YOR1* (35). The loss of *YOR1* leads to a large decrease in Pdr1p-mediated oligomycin resistance but has no effect on cycloheximide resistance. A second gene identified in this screen elevated both oligomycin and cycloheximide tolerance. We refer to this gene as *PDR13.*

In this study, we demonstrate that *PDR13* encodes an Hsp70 homolog that acts to elevate the function of Pdr1p, leading to increased expression of PDRE-containing genes and drug resistance. Strains lacking *PDR13* are compromised for growth and induce the expression of stress-responsive genes. A gain-of-function mutant form of Pdr13p is able to complement the growth defect of a Δ*pdr13* strain and elevates both PDRE-containing gene expression and drug resistance. Taken together, these data implicate Pdr13p as an upstream modulator of the expression of *PDR* genes through control of the transcription factor Pdr1p.

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MATERIALS AND METHODS

Yeast strains and media. The genotypes of the yeast strains used in this study are listed in Table 1. Yeast transformations were performed with the lithium acetate procedure of Ito et al. (29) or a high-efficiency technique (21). Standard

TABLE 1. *S. cerevisiae* strains used

Strain designation	Description	Source or reference
SEY6210	*MAT*α *leu2-3,112 ura3-52 lys2-801 trp1-Δ901 his3-Δ200 suc2-Δ9* Mel$^-$	Scott Emr
PB2	*MAT*α *leu2-3,112 ura3-52 lys2-801 trp1-Δ901 his3-Δ200 suc2-Δ9* Mel$^-$ *pdr3-Δ1::hisG*	33
PB3	*MAT*α *leu2-3,112 ura3-52 lys2-801 trp1-Δ901 his3-Δ200 suc2-Δ9* Mel$^-$ *pdr1-Δ2::hisG*	33
PB4	*MAT*α *leu2-3,112 ura3-52 lys2-801 trp1-Δ901 his3-Δ200 suc2-Δ9* Mel$^-$ *pdr1-Δ2::hisG pdr3-Δ1::hisG*	33
DKY2.1	*MAT*α *leu2-3,112 ura3-52 lys2-801 trp1-Δ901 his3-Δ200 suc2-Δ9* Mel$^-$ *pdr5-Δ1::hisG*	33
DKY7	*MAT*α *leu2-3,112 ura3-52 lys2-801 trp1-Δ901 his3-Δ200 suc2-Δ9* Mel$^-$ *yor1-1::hisG*	35
YRT9	*MAT*α *leu2-3,112 ura3-52 lys2-801 trp1-Δ901 his3-Δ200 suc2-Δ9* Mel$^-$ *pdr13-Δ1::hisG*	This study
TCH4	*MAT*α *leu2-3,112 ura3-52 lys2-801 trp1-Δ901 his3-Δ200 suc2-Δ9* Mel$^-$ *pdr13-Δ1::hisG pdr3-Δ1::hisG PDR5-HIS3-PDR5-lacZ*	This study
TCH1	*MAT*α *leu2-3,112 ura3-52 lys2-801 trp1-Δ901 his3-Δ200 suc2-Δ9* Mel$^-$ *pdr13-Δ1::hisG pdr3-Δ1::hisG*	This study

YPD medium and minimal medium were used for the growth of cells and drug resistance assays (49). Drug resistance assays were performed by spot tests (53). β-Galactosidase activity was measured as previously described (24).

Plasmids. An integrating *PDR5-lacZ* fusion gene was constructed by transferring an *Eco*RI/*Sal*I fragment from pKV2 (33) into pRS303 (50) to form pTH120. Strain TCH4 was generated by transforming TCH1 with *Stu*I-cut pTH120, which directs recombination to *PDR5*. A *Sal*I/*Not*I fragment carrying the wild-type *PDR13* gene was cloned into the 2μm-containing vector pRS424 (10) to form pTH87. The same *PDR13* fragment was also inserted into pRS316 (50) to generate pTH86 and into pRS314 (50) to produce pTH143. The original YEp24-based recombinant carrying *PDR13* was designated pDOC10-2. The *GAL1-PDR1* fusion gene contained a *PDR1* fragment (extending from the ATG to a *Sal*I site located 700 bp downstream of the translation stop codon) cloned downstream of the *GAL1* promoter carried in the low-copy-number plasmid pSEYC68-GAL (44). The resulting construct was named pTH7. The *YOR1-lacZ* fusion plasmid was previously described (26), and a mutated variant of this clone that lacked the PDREs was constructed.

Construction of a *PDR13* mutant library and selection of hyperactive *PDR13* alleles. pTH86 DNA (20 μg) was mutagenized with 100 μl of 45% formic acid for 1 min. The DNA was recovered by ethanol precipitation, and the *PDR13* gene was amplified by PCR from this mutagenized template by use of the flanking T3 and T7 universal primers. The PCR product was cleaved with *Sal*I/*Not*I and cloned into similarly digested pRS314. The library was amplified in bacteria, and plasmid DNA was prepared.

Strain TCH4 was transformed by a high-efficiency method (21) with the mutant *PDR13* library and plated on SD plates (49) containing 0.25 μg of cycloheximide per ml. Survivors were tested for the presence of elevated levels of *PDR5-lacZ* expression, and plasmids were recovered from appropriate transformants. TCH4 cells were retransformed with each plasmid to confirm that the elevated cycloheximide tolerance and *PDR5* expression phenotypes were linked to the plasmid. The sequence of the entire *PDR13* gene carried in each plasmid was determined by the University of Iowa DNA Core Facility by use of a set of custom oligonucleotide primers.

Immunological methods. Rabbit polyclonal antisera were generated by standard immunization techniques (27) against Pdr1p and Pdr13p expressed in bacteria. Both *S. cerevisiae* proteins were produced as fusion proteins with glutathione *S*-transferase (GST) carried in plasmid pGEX-KG (23). The GST-Pdr13p fusion protein was constructed by cloning into *Sac*I/*Not*I-cleaved pGEX-KG full-length Pdr13p downstream of the GST cassette carried in pGEX-KG as a *Sac*I/*Not*I fragment. The GST-Pdr1p fusion protein was generated by cloning an *Eco*RI fragment encoding Pdr1p residues 768 to 1063 into the *Eco*RI site of pGEX4. Both fusion proteins were purified through the use of glutathione-agarose columns as described previously (19).

Protein extraction and analysis. Cells were grown in minimal medium to an A_{600} of 0.5 to 0.7, harvested at 4°C by centrifugation, and resuspended in sorbitol breaking buffer (0.3 M sorbitol, 0.1 M NaCl, 5 mM $MgCl_2$, 10 mM Tris [pH 7.4]), with protease inhibitors. Cells were broken by agitation in the presence of glass beads for 25 min at 4°C and centrifuged (Eppendorf 5415C) at 12,000 rpm for 5 min. Protein concentrations were determined by the method of Lowry et al. (41). Equal amounts of protein were resuspended in Laemmli sodium dodecyl sulfate (SDS) loading buffer (37) and analyzed by Western blotting with the anti-Pdr1p or anti-Pdr13p antisera. Antigen-antibody complexes were visualized with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin secondary antibody and enhanced chemiluminescence reagents (Pierce Supersignal).

Gene disruption. A *PDR13* disruption allele was produced by deletion of a *Bgl*II fragment from the wild-type *PDR13* gene carried as a *Sal*I/*Not*I fragment in pRT5. The deleted *Bgl*II fragment was replaced with the *hisG-URA3-hisG* fragment from pNKY51 (1). The resulting plasmid replaced *PDR13* DNA from 41 bp upstream of the putative ATG to residue 203 with *hisG-URA3-hisG*. This plasmid was designated pRT6 and was cleaved with *Sal*I/*Not*I prior to transformation into wild-type cells (SEY6210). *URA3* transformants were selected, and correct integration was confirmed by Southern blotting (47). The *URA3* gene was removed from a selected disruptant by treatment with 5-fluoro-orotic acid (7), and the resulting *pdr13-Δ1::hisG* strain was designated YRT9.

RESULTS

Identification of *PDR13* as a pleiotropic drug resistance gene. We previously reported a high-copy-number plasmid library screen in which colonies that exhibited elevated oligomycin resistance were identified by replica plating (35). Two different loci that were both capable of consistently elevating oligomycin tolerance were recovered in this screen. Characterization of the *YOR1* structural gene, encoding an ABC transporter protein, has already been reported (35). The second locus found was designated *PDR13.*

High-copy-number plasmids carrying *PDR13* elevated both oligomycin and cycloheximide resistance. This effect was not seen with the 2μm *YOR1* clone, which increased oligomycin tolerance only (35). DNA sequence analysis of the *S. cerevisiae* insert in the *PDR13* clone indicated that this fragment came from chromosome VIII. Subcloning analysis established that the *PDR13* gene corresponded to the *YHR064c* locus that was found to encode an Hsp70 homolog during the sequencing of chromosome VIII (31). Alignment of Pdr13p with other Hsp70 proteins from *S. cerevisiae*, *Schizosaccharomyces pombe*, and bovine sources indicated that Pdr13p showed the highest degree of sequence similarity (40%) to an *S. pombe* Hsp70 protein and lower levels of homology to other Hsp70 proteins (e.g., 27% similarity to Ssa4p). This sequence similarity is relatively low compared to that seen between some other Hsp70 proteins, which can be as high as 81% (Ssa1p and Ssa4p). However, the sequence similarity of the large Hsp70 proteins (Ssi1p/Lhs1p/Cer1p, Sse1p, and Sse2p) to the other family members is at least as low as that exhibited by Pdr13p (45). Based on these alignment data, Pdr13p is a unique member of the Hsp70 family in *S. cerevisiae.*

Having determined that this Hsp70 homolog was able to increase both oligomycin and cycloheximide resistance, we examined the influence of *PDR13* on strains lacking either *PDR5* or *YOR1*. A large body of data implicates *PDR5* as encoding an ABC transporter protein that is required for cycloheximide tolerance (5, 6, 28, 39), while we have shown that *YOR1* is a key determinant of oligomycin resistance (35). To evaluate if *PDR13* acted through these loci, a 2μm plasmid containing *PDR13* was introduced into isogenic wild-type and Δ*pdr5* and *yor1* mutant strains. Cycloheximide resistance and oligomycin resistance were then assayed in each genetic background.

The presence of the Δ*pdr5* allele eliminated the ability of high-copy-number *PDR13* to stimulate cycloheximide tolerance, even at very low concentrations of cycloheximide (Fig. 1). High-copy-number *PDR13* was also unable to increase oligomycin resistance to normal levels in a strain lacking *YOR1*.

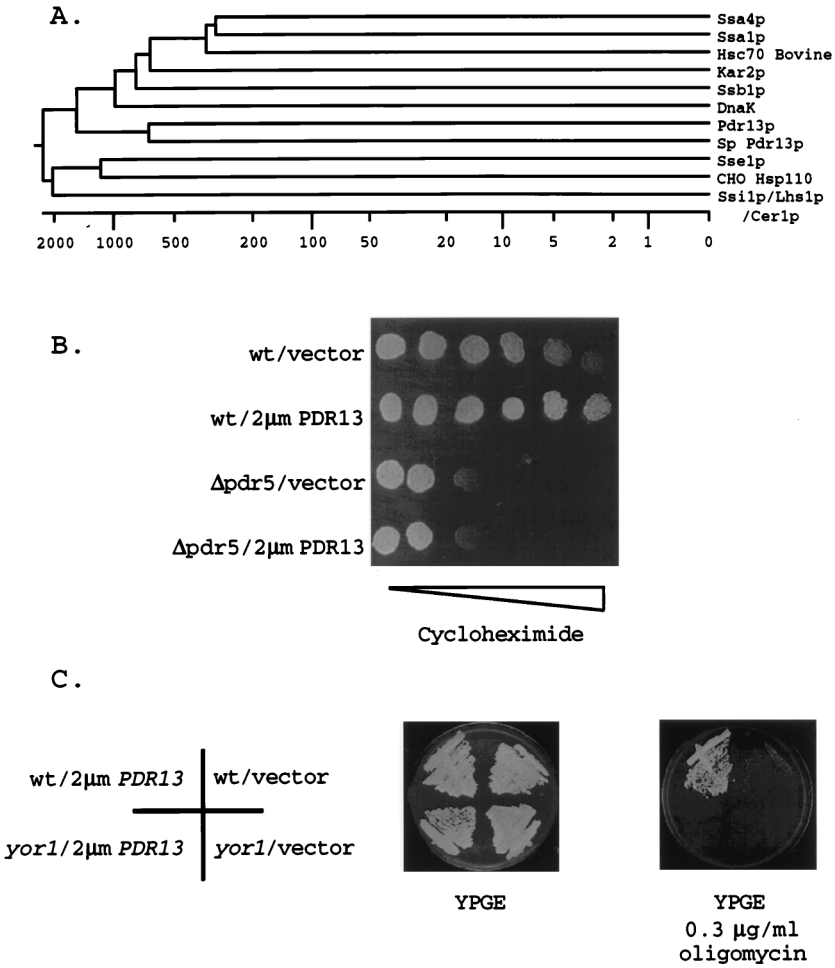

FIG. 1. Drug resistance conferred by the Hsp70 homolog Pdr13p requires the presence of *PDR* genes. (A) A phylogenetic tree showing the relationship of the Pdr13p sequence to sequences of other Hsp70 homologs was generated by use of the Megalign routine of Lasergene software (DNAstar). Sequences were aligned by use of the Jotun-Hein algorithm. The Hsp70 proteins are from *S. cerevisiae*, with the following four exceptions: Hsc70 (bovine), DnaK (*E. coli*), Sp Pdr13p (*S. pombe* Pdr13p homolog), and CHO Hsp110 (*Cricetulus griseus*). The numbers on the bottom of the panel indicate the evolutionary distances between the sequences. (B) The functional dependence of Pdr13p on the presence of *PDR5* was tested by placing 1,000 cells of the indicated genotype on a YPD (49) plate containing a gradient of cycloheximide (indicated by the bar of increasing width). Isogenic wild-type (wt) or Δ*pdr5* cells were transformed with a high-copy-number vector plasmid (pRS426) or the same plasmid carrying the *PDR13* structural gene. (C) The indicated transformants were streaked on YPGE medium (49) or YPGE medium plus oligomycin at 0.3 μg/ml and incubated at 30°C. Wild-type (wt) cells are SEY6210, while the *yor1* (DKY7) mutant is an isogenic derivative. Plasmids were as in panel B.

These findings strongly suggested that Pdr13p required the presence of *PDR* genes to normally elevate drug resistance and suggested two possible models for the action of this Hsp70 protein. First, perhaps Pdr13p influences the activity of the transcription factors that regulate the expression of *PDR* genes. Second, since Hsp70 proteins are known to influence protein folding and translocation across membranes (12), perhaps Pdr13p influences the insertion of Pdr5p and Yor1p into membranes. Below we provide evidence that Pdr13p acts to stimulate expression of the *PDR5* and *YOR1* loci.

High-copy-number *PDR13* elevates the expression of Pdr1p target genes. To determine if high-copy-number *PDR13* influenced the level of expression of *PDR* loci, we used gene fusions between the *PDR5* and *YOR1* structural genes and *lacZ*. Each of these plasmid-borne fusion genes has been demonstrated to respond to the same transcriptional control signals as its chromosomal counterpart (33, 35). A *TRP5-lacZ* fusion gene was used as a control for a locus unrelated to the *PDR* network. Each fusion gene was introduced into a wild-type cell along with either a high-copy-number plasmid containing *PDR13* or the empty vector alone. β-Galactosidase activities were then determined for transformants carrying these plasmids.

Both the *PDR5-lacZ* and the *YOR1-lacZ* fusion genes produced higher levels of β-galactosidase enzyme activity in the presence of high-copy-number *PDR13* (Table 2). *PDR5*-dependent enzyme activity increased from 36 to 170 U/unit of optical density at 600 nm (OD_{600}) upon introduction of the 2μm plasmid carrying *PDR13*. Similarly, *YOR1*-dependent enzyme activity increased from 3.5 to 14 U/OD_{600} in the presence of high-copy-number *PDR13*. *TRP5-lacZ* expression was not affected by changes in *PDR13* gene dosage.

These data indicated that the most likely mechanism of action of Pdr13p was to stimulate the transcription of the *PDR5* and *YOR1* structural genes, which would result in increased cycloheximide and oligomycin resistances, respectively. Previous studies of the function of the *PDR5* and *YOR1* promoters indicated that the action of the closely related zinc finger-containing transcription factors Pdr1p and Pdr3p was required for normal expression of each of these loci (15, 33, 35). Pdr1p and Pdr3p were shown to bind to DNA elements

TABLE 2. Expression of Pdr1p-regulated genes is elevated in the presence of high-copy-number *PDR13*[a]

Fusion gene	β-Galactosidase activity (U/OD_{600}) in the presence of:	
	Vector	2μm PDR13
PDR5-lacZ	36 ± 13	170 ± 34
mPDR5-lacZ	0.5 ± 0.3	0.6 ± 0.3
YOR1-lacZ	3.5 ± 0.5	14 ± 2
mYOR1-lacZ	2 ± 0.1	3 ± 0.2
SNQ2-lacZ	13 ± 3	40 ± 8
TRP5-lacZ	37 ± 8	35 ± 7.5

[a] A wild-type strain (SEY6210) was transformed with the indicated *lacZ* fusion genes carried on low-copy-number plasmids along with either the 2μm plasmid pRS424 (vector) (10) or pRS424 carrying *PDR13*. Plasmids containing altered versions of the *PDR5* and *YOR1* promoters lacking the normal PDREs are designated *mPDR5-lacZ* and *mYOR1-lacZ*, respectively. These mutant plasmids have been described elsewhere (26, 34). Appropriate transformants were grown in minimal medium with necessary supplements, and β-galactosidase activities were assayed as described previously (24). The values reported represent the averages of at least three independent determinations ± standard errors.

designated PDREs (34). To determine if the PDREs were required for the observed effect of Pdr13p, we used mutant derivatives of the *PDR5-lacZ* and *YOR1-lacZ* fusion genes lacking normal copies of the PDREs. These fusion genes were then evaluated with regard to β-galactosidase levels in response to changes in *PDR13* gene dose as described above for the wild-type *lacZ* fusion genes (Table 2).

Loss of the PDREs from the *PDR5* promoter led to a precipitous decrease in β-galactosidase activity produced by the resulting fusion gene. This low level of expression was unaffected by the presence of high-copy-number *PDR13*. Loss of the single PDRE from the *YOR1* promoter eliminated the ability of the resulting gene fusion to respond to an increase in *PDR13* gene dose. These data suggest that Pdr13p acts through the PDREs in both promoters and further that Pdr1p and/or Pdr3p is a target for the action of Pdr13p. To address this possibility, we examined the activity of Pdr13p in strains lacking *PDR1* and/or *PDR3*.

***PDR13* affects the function of Pdr1p but not Pdr3p.** To test the requirement of Pdr1p and/or Pdr3p for normal Pdr13p activity, we used a series of isogenic strains lacking *PDR1*, *PDR3*, or both genes (33). Each strain was transformed with a 2μm vector plasmid or the same plasmid carrying *PDR13*. Transformants were then tested for their cycloheximide resistance phenotypes by spot test assays (Fig. 2).

High-copy-number *PDR13* was only able to increase cycloheximide resistance if the *PDR1* gene was present. Removal of the *PDR3* gene did not affect Pdr13p-stimulated cycloheximide tolerance. The cycloheximide resistance of mutant strains lacking either *PDR1* or *PDR1* and *PDR3* was not enhanced upon introduction of a 2μm plasmid carrying *PDR13*. We interpret these data as indicating that the action of Pdr13p is specific to Pdr1p and that Pdr3p is dispensable.

To further examine the specificity of Pdr13p for Pdr1p, high-copy-number plasmids carrying either the *PDR1* or the *PDR3* structural gene were introduced into a Δ*pdr1 pdr3* strain, and the gene dosage of *PDR13* was varied as described above. Transformants were again assayed for their ability to respond to cycloheximide challenge by spot test assays (Fig. 2).

A Δ*pdr1 pdr3* strain containing a 2μm plasmid carrying the *PDR3* gene exhibited more growth in the presence of cycloheximide than the same strain transformed with a vector plasmid alone. We believe that this increase in cycloheximide resistance is due to the overproduction of Pdr3p when its structural gene is present in high copy numbers, as described before (33). However, this elevated cycloheximide resistance was unchanged when the *PDR13* gene dose was increased. The presence of a 2μm clone of the *PDR1* gene was highly responsive to increases in *PDR13* copy number and conferred the highest level of cycloheximide-resistant growth. This finding suggests that even elevated Pdr3p levels are not responsive to Pdr13p and provides further evidence for the specificity of Pdr13p for Pdr1p.

To confirm that the observed increases in cycloheximide resistance represented enhanced expression of *PDR5*, we introduced a *PDR5-lacZ* fusion gene into the set of isogenic strains and varied the gene dosages of *PDR1* and/or *PDR3*. A high-copy-number plasmid carrying *PDR13* or the empty vector alone was also introduced, and *PDR5*-dependent β-galactosidase levels were determined.

Expression of the *PDR5-lacZ* fusion gene responded to the presence of high-copy-number *PDR13* only if the *PDR1* gene was intact (Table 3). Both the wild-type and the Δ*pdr3* strains exhibited an approximately threefold increase in *PDR5-lacZ* expression when the high-copy-number *PDR13*-containing plasmid was present. The Δ*pdr1* strain expressed *PDR5*-dependent β-galactosidase activity at 22.5 U/OD_{600}; this level was not increased upon introduction of the high-copy-number *PDR13*-containing plasmid. The Δ*pdr1 pdr3* strain produced very low levels of enzyme activity that were not increased by the high-copy-number *PDR13*-containing plasmid.

These data are consistent with the hypothesis that Pdr13p acts as an upstream regulator of Pdr1p. However, these experiments evaluated the effect of *PDR13* when this gene was present on a high-copy-number plasmid. To examine the role of the chromosomal *PDR13* gene, we prepared a strain containing a disrupted allele of this gene.

Growth defects of cells lacking *PDR13*. A gene disruption allele of *PDR13* was prepared by replacing genomic DNA from 41 bp upstream of the potential *PDR13* ATG to amino acid 203 of the coding sequence with *hisG*. The resulting allele, *pdr13-Δ1::hisG*, has a deletion of 203 amino acids from the conserved Hsp70 ATPase domain of Pdr13p. The *PDR13* locus was dis-

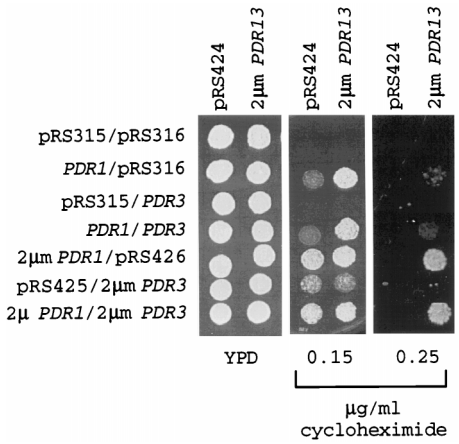

FIG. 2. Elevation of cycloheximide resistance by high-copy-number *PDR13* requires the presence of *PDR1*. A strain lacking both the *PDR1* and the *PDR3* structural genes (PB4) was transformed with the indicated plasmids, resulting in low- or high-copy-number forms of these genes. Along with the plasmids expressing *PDR1* and/or *PDR3*, a 2μm clone of *PDR13* or a high-copy-number vector was introduced into the cells. Transformants were grown in media to select for the presence of each plasmid and then assayed for growth on rich medium (YPD) or this same medium containing the concentrations of cycloheximide shown.

TABLE 3. Elevation of *PDR5* expression by high-copy-number *PDR13* requires the presence of *PDR1*[a]

Fusion gene	β-Galactosidase activity (U/OD_{600}) in the following strains in the presence of the indicated plasmid:							
	Wild type		Δ*pdr1*		Δ*pdr3*		Δ*pdr1 pdr3*	
	Vector	2μm PDR13	Vector	2μm PDR13	Vector	2μm PDR13	Vector	2μm PDR13
PDR5-lacZ	39 ± 12	147 ± 33	22.5 ± 5	22.5 ± 6	44 ± 11	186 ± 45	2 ± 0.7	2 ± 0.5
TRP5-lacZ	29 ± 2	30 ± 4	31 ± 6	32.5 ± 6	35.5 ± 2	36 ± 10	41 ± 3	47 ± 8

[a] A set of isogenic strains containing the indicated alleles of *PDR1* and *PDR3* were transformed with *PDR5-lacZ* and *TRP5-lacZ* fusion genes on low-copy-number plasmids. In addition, either the 2μm plasmid pRS424 (vector) or pRS424 carrying the wild-type *PDR13* locus was introduced into each transformant. Transformants were grown and assayed for β-galactosidase activities as described in Table 2, footnote *a*.

rupted in both haploid and diploid cells. As we found that *PDR13* is not an essential gene (see below), we focused our analysis on the isogenic wild-type and *pdr13-Δ1::hisG* strains.

Introduction of the *pdr13-Δ1::hisG* allele produced a cell that was viable but unable to grow at a normal rate (Fig. 3). The Δ*pdr13* strain exhibited a cold-sensitive growth phenotype. The doubling times of wild-type cells in YPD medium were 94, 85, and 81 min when measured at 21, 30, and 37°C, respectively. The isogenic Δ*pdr13* strain showed doubling times of 320, 189, and 122 min at the same three temperatures in the same medium.

The temperature-dependent ability of the Δ*pdr13* strain to tolerate cycloheximide was also evaluated by spot test assays. Δ*pdr13* cells were defective for growth in the presence of cycloheximide, and the reduction in growth in the presence of this drug closely paralleled the general growth defect of the Δ*pdr13* strain. We were not able to demonstrate a convincing drug resistance-specific defect in Δ*pdr13* cells and attribute this inability to the general poor growth of these cells.

Expression of several different *lacZ* fusion genes was also assayed in the Δ*pdr13* background. A *PDR5-lacZ*, *TRP5-lacZ*, *CUP1-lacZ*, *HSP12-lacZ*, or *CTT1-lacZ* fusion plasmid was introduced into wild-type and Δ*pdr13* cells, and β-galactosidase activities were determined. The *CTT1* and *HSP12* genes are stress-responsive loci that are regulated by the stress response element-binding proteins Msn2p and Msn4p (42, 46, 48, 51). *CUP1* is regulated by the heat shock transcription factor in response to heat shock and oxidative stress (40). These reporter genes were assayed to evaluate the possibility that the loss of the *PDR13* gene would elicit a generalized stress response mediated through either the stress response element- or the heat shock transcription factor-dependent systems. Consistent with this interpretation, *CTT1-lacZ* expression increased by 300% in comparison with that in isogenic Δ*pdr13* and wild-type cells. *HSP12-lacZ* expression and *CUP1-lacZ* expression both increased by 200% in the absence of the *PDR13* locus. *PDR5-lacZ* expression was not significantly affected but *TRP5-lacZ* expression was decreased to 50% normal in Δ*pdr13* cells.

One interpretation consistent with the data given above is that Δ*pdr13* cells are constitutively stressed due to the absence of Pdr13p. This idea is reflected in the diminished rate of growth of these cells as well as the enhanced expression of *CTT1*, *HSP12*, and *CUP1*. The generalized stress caused by the loss of *PDR13* made the analysis of specific defects in *PDR* gene regulation impractical in this genetic background. Previous experiments showed that the loss of multiple *PDR* genes, including *PDR1*, does not detectably influence cell growth rate (see reference 4 for a review). This result argues that Pdr13p has other target proteins that contribute to normal cell growth. We explored this possibility by isolating mutant forms of Pdr13p that support normal growth of cells but are hyperresistant to drugs.

Isolation of a hyper-drug-resistant form of Pdr13p. To dissect the action of Pdr13p on the pleiotropic drug resistance and growth phenotypes, we selected mutant forms of this protein that were able to confer elevated drug resistance but still provided normal growth. This was accomplished by constructing a mutant library of low-copy-number plasmids carrying the *PDR13* gene. This mutant library was then transformed into Δ*pdr13 pdr3* cells containing an integrated *PDR5-lacZ* fusion gene. We selected transformants that grew normally, were hyperresistant to cycloheximide, and exhibited elevated levels of β-galactosidase activity. Plasmids were recovered from these

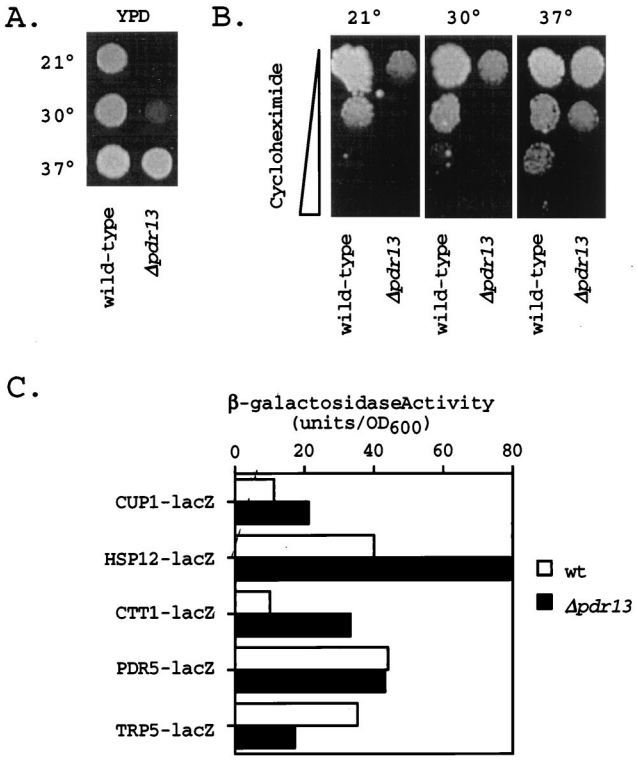

FIG. 3. Cells lacking *PDR13* are defective in growth. (A) One thousand cells each of isogenic wild-type and Δ*pdr13* strains were placed on YPD medium and incubated at the indicated temperatures (Celsius) for 2 days. (B) Isogenic wild-type and Δ*pdr13* cells were tested as in panel A for growth on a YPD plate containing a gradient of cycloheximide increasing from 0 to 0.25 μg/ml (indicated by the width of the bar on the left). Plates were incubated at 21, 30, or 37°C for 4 days. (C) Cells of the wild type (wt) and the isogenic Δ*pdr13* derivative were transformed with the indicated *lacZ* fusion genes carried on low-copy-number plasmids. Transformants were grown in minimal media at 30°C, and β-galactosidase activities were determined. Values are the averages of at least two determinations.

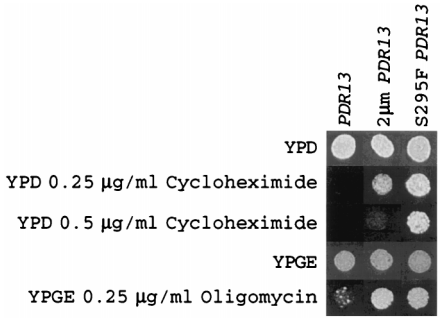

FIG. 4. Enhanced drug resistance of cells expressing S295F Pdr13p. A strain lacking the *PDR13* gene (TCH1) was transformed with low-copy-number plasmids expressing the wild type (PDR13) or the S295F (S295F PDR13) form of *PDR13* or a high-copy-number plasmid carrying *PDR13* (2μm PDR13). Appropriate transformants were tested for their relative resistance to the presence of the indicated concentrations of cycloheximide or oligomycin by spot test assays.

TABLE 4. The S295F allele of *PDR13* elevates Pdr1p target gene expression[a]

PDR13 allele	β-Galactosidase (U/OD_{600}) activity in the presence of:		
	PDR5-lacZ	*TRP5-lacZ*	*CTT1-lacZ*
pdr13-Δ1	70 ± 17	16 ± 2	23 ± 3
PDR13	58 ± 16.5	49 ± 5.5	9 ± 2
2μm *PDR13*	299 ± 84	50 ± 2	5 ± 1
S295F *PDR13*	182 ± 38	51 ± 7	7 ± 1

[a] A Δ*pdr13* strain was transformed with a low-copy-number vector plasmid (pRS314), pRS314 carrying the wild-type or S295F form of the *PDR13* gene, or a high-copy-number plasmid containing the wild-type *PDR13* locus (2μm *PDR13*). Along with these plasmids giving rise to the different *PDR13* alleles, low-copy-number plasmids carrying *PDR5-lacZ*, *TRP5-lacZ*, or *CTT1-lacZ* fusion genes were introduced into the cells. Transformants of interest were grown in minimal medium, and β-galactosidase activities were determined as described previously (24).

transformants and retested to confirm that the gene responsible for the mutant phenotypes was linked to the plasmid. The DNA sequence alteration of the *PDR13* gene was then determined for several plasmids that exhibited linkage to the mutant phenotypes.

The sequences of eight isolates indicated a single amino acid change: serine at position 295 was replaced with phenylalanine (S295F). The relative drug resistances of a Δ*pdr13 pdr3* strain transformed with a high-copy-number *PDR13*-containing plasmid or with low-copy-number plasmids expressing the wild-type or S295F forms of Pdr13p were evaluated by spot test assays (Fig. 4).

The defective growth of the Δ*pdr13 pdr3* strain was restored to normal by transformation with all of these versions of the *PDR13* gene (data not shown). However, differences in drug resistance were clearly apparent. The presence of either high-copy-number *PDR13* or low-copy-number S295F *PDR13* produced a large increase in tolerance to cycloheximide and oligomycin compared to that in low-copy-number wild-type *PDR13* transformants. The single amino acid change in S295F Pdr13p was able to elevate cycloheximide and oligomycin resistance to a level at least equal to that conferred by a high-copy-number clone of the wild-type locus. Other experiments demonstrated that the S295F allele was dominant over the wild-type *PDR13* locus (data not shown).

To confirm that S295F Pdr13p also acted to elevate the expression of Pdr1p target genes, the Δ*pdr13 pdr3* strain was transformed with low-copy-number plasmids expressing either the wild-type or the S295F form of Pdr13p as well as a high-copy-number plasmid carrying wild-type *PDR13*. In addition to these plasmids expressing Pdr13p, a *TRP5-lacZ*, *CTT1-lacZ*, or *PDR5-lacZ* fusion gene was introduced into each background. The expression of each fusion gene was assayed in these three genetic backgrounds (Table 4).

The presence of the S295F allele of *PDR13* elevated the expression of *PDR5-lacZ* to levels similar to those produced by introduction of the 2μm plasmid carrying *PDR13*. Transformants containing any of the three versions of the *PDR13* gene produced equivalent levels of *TRP5-lacZ* expression, indicating that Pdr13p did not globally enhance gene expression. Finally, the presence of the S295F allele of *PDR13* returned *CTT1-lacZ* expression to wild-type levels. These data indicated that the gain-of-function S295F mutant form of Pdr13p reproduced what was previously seen with the high-copy-number wild-type gene. The S295F form of Pdr13p appeared to be a more effective activator of drug resistance than the high-copy-number wild-type protein, although both produced similar levels of *PDR5*-dependent β-galactosidase activity. The reason for this effect is unknown but may be related to the different replication properties of the two plasmid vectors used in this experiment.

To further explore the nature of Pdr13p control of Pdr1p function, we produced a polyclonal antiserum directed against Pdr13p. This antiserum was used in Western blot analysis of protein extracts prepared from Δ*pdr13* cells and transformants carrying the low-copy-number wild-type or S295F form of *PDR13* or a 2μm plasmid expressing wild-type Pdr13p (Fig. 5). The anti-Pdr13p antiserum detected a single polypeptide with a molecular mass of 81 kDa in cells transformed with plasmids expressing the three different *PDR13* alleles. The 81-kDa protein was absent from protein extracts prepared from the Δ*pdr13* cells. Importantly, both the wild-type and the S295F forms of Pdr13p were expressed at equivalent levels, while the high-copy-number plasmid carrying *PDR13* produced approximately threefold more protein than either of the low-copy-number plasmids. This result confirms that the elevated Pdr1p function seen in the presence of the S295F Pdr13p derivative is due to a change in the activity of Pdr13p rather than to a change in its steady-state level.

Pdr13p acts posttranslationally to regulate Pdr1p. A central issue that emerges from these studies concerns the details of the activation of Pdr1p by Pdr13p. Pdr13p may change the

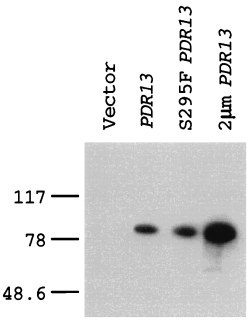

FIG. 5. Equivalent expression of wild-type and S295F forms of Pdr13p. Whole-cell protein extracts were prepared from a Δ*pdr13* mutant strain carrying a vector plasmid (pRS314), a low-copy-number plasmid containing either wild-type *PDR13* or the S295F allele of *PDR13*, and a high-copy-number plasmid (2μm PDR13) bearing the wild-type *PDR13* gene. Protein (25 μg) was resolved by SDS-PAGE and analyzed by Western blotting with anti-Pdr13p antiserum. Numbers on the left are in kilodaltons.

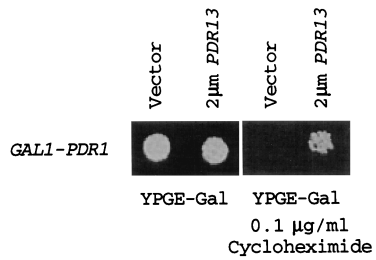

FIG. 6. Pdr13p does not act on the *PDR1* promoter region. A wild-type strain was transformed with a low-copy-number plasmid containing a *GAL1-PDR1* fusion gene. Along with this fusion gene, a high-copy-number plasmid containing the *PDR13* gene (2μm PDR13) or the plasmid vector alone (vector) was introduced. Transformants containing each pair of plasmids were tested for their ability to grow on YPGE-galactose medium (YPGE-Gal) (49) lacking or containing cycloxheximide.

level of expression of Pdr1p or, alternatively, may regulate the activity of Pdr1p. To examine which of these explanations was likely to be correct, we carried out two different experiments. First, the *PDR1* coding sequence was placed under the control of the *GAL1* promoter, and the resulting *GAL1-PDR1* chimera was examined for its response to different *PDR13* gene dosages. Second, an antiserum was produced against Pdr1p and used to directly examine the steady-state levels of Pdr1p in strains containing different *PDR13* gene doses.

The *GAL1-PDR1* fusion gene was constructed by placing a fragment of *PDR1* (extending from its ATG to the end of the coding sequence) downstream of the *GAL1* promoter carried on a low-copy-number vector. This plasmid was introduced into a wild-type strain along with a high-copy-number plasmid expressing Pdr13p or the empty vector. Transformants were then tested for cycloheximide tolerance by spot test assays (Fig. 6).

Pdr1p, produced from the *GAL1* promoter, still responded to the presence of elevated *PDR13* gene dose. Transformants carrying the *GAL1-PDR1* fusion gene were more resistant to cycloheximide in the presence of multiple copies of *PDR13* than in the presence of a single copy of this gene. This observation strongly argues that the effect of Pdr13p on Pdr1p function is not a consequence of control of *PDR1* promoter function.

To directly examine the steady-state levels of Pdr1p in cells with various *PDR13* gene doses, a polyclonal antiserum directed against Pdr1p was produced. Protein extracts were prepared from Δ*pdr1 pdr3* cells transformed with high-copy-number plasmids carrying *PDR1* and *PDR13* or carrying *PDR1* alone or with the empty vector. The steady-state level of Pdr1p in each transformant was then evaluated by Western blot analysis of equal amounts of protein extracts resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 7). The anti-Pdr1p antiserum recognized a single polypeptide species of approximately 121 kDa in transformants carrying the *PDR1* gene. The levels of Pdr1p were not altered by changes in *PDR13* gene doses. This observation provides strong support for the hypothesis that Pdr13p control of Pdr1p occurs through posttranslational modulation of the ability of the zinc finger-containing transcription factor Pdr1p to activate gene expression.

DISCUSSION

While the role of Hsp70 proteins in polypeptide metabolism has long been appreciated, recent work has indicated that Hsp70 proteins can act as regulatory factors that modulate the activity of transcription factors (20). One of the earliest examples of Hsp70 proteins influencing the activity of a transcription factor came from analysis of expression of the *SSA4* gene in *S. cerevisiae* (8). *S. cerevisiae* strains lacking the Hsp70-encoding loci *SSA1* and *SSA2* overproduce the *SSA4* transcript and protein through activation of the heat shock transcription factor (25). Detailed analysis of the maturation of the progesterone receptor has shown that Hsp70 proteins must act on this factor so that it can achieve its active conformation (9). *Escherichia coli* Hsp70 DnaK acts to negatively regulate its own transcription (reviewed in reference 13).

The data presented here argue that Pdr13p is a positive regulator of Pdr1p. Increases in the level of Pdr13p lead to an enhancement in the activity of Pdr1p. While the action of Pdr13p is clearly not restricted to its effect on Pdr1p (see below), Pdr13p does not affect the function of Pdr3p. Pdr3p shows 36% sequence identity to Pdr1p, binds to the same DNA element as Pdr1p, and shows extensive functional overlap with Pdr1p (15, 33). Δ*pdr1* cells containing a 2μm clone of *PDR3* exhibit higher cycloheximide resistance than wild-type cells (Fig. 2). However, the function of even this presumably elevated level of Pdr3p cannot be regulated by Pdr13p, further supporting the notion that this Hsp70 protein controls Pdr1p but not Pdr3p activity.

This observation is important in light of what is known about the phenotype of cells lacking either the *PDR1* or the *PDR3* gene. Loss of *PDR1* from the cell has a pronounced phenotypic effect on cycloheximide resistance, with a Δ*pdr1* cell exhibiting hypersensitivity to a broad range of compounds (2, 15, 33). In opposition to the effect of *PDR1* removal, a Δ*pdr3* cell is not markedly different from a wild-type cell in terms of cycloheximide tolerance (15, 33). Surprisingly, expression studies with *PDR5*, the principal Pdr1p-Pdr3p target gene for cycloheximide resistance, indicate that both mRNA and *PDR5-lacZ* expression levels are similar in Δ*pdr1* and Δ*pdr3* strains (33). A key difference in these two experiments is that one is done in the presence of cycloheximide (phenotype testing), while one is performed in its absence (expression measurements). A hypothesis to explain these data is that drug exposure acts through Pdr13p to stimulate Pdr1p activity, and this activation of Pdr1p is required for normal drug resistance. The fact that Pdr1p but not Pdr3p can respond to Pdr13p would explain the observed phenotypes of strains individually lacking either of these transcription factors. This hypothesis is currently being evaluated.

While Pdr13p does not affect the function of Pdr3p, there are clearly targets of Pdr13p action other than Pdr1p. The reduction in growth rate exhibited by Δ*pdr13* cells indicates that Pdr1p cannot be the only downstream target of Pdr13p. Strains lacking Pdr1p, Pdr1p and Pdr3p, and downstream

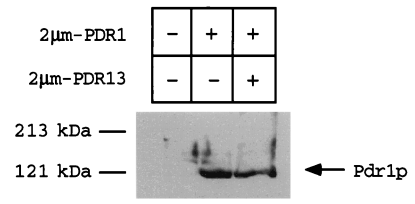

FIG. 7. Pdr13p regulates Pdr1p function at a posttranslational step. Whole-cell protein extracts (100 μg) were electrophoresed by SDS-PAGE. Protein extracts were made from Δ*pdr1 pdr3* cells containing (+) or lacking (−) the indicated high-copy-number plasmids. After electrophoresis, the polypeptides were transferred to nitrocellulose and analyzed by blotting with anti-Pdr1p antiserum.

Pdr1p-Pdr3p target genes have not been found to exhibit growth phenotypes in the absence of drugs (reviewed in reference 4). In contrast, a Δ*pdr13* strain displays a growth phenotype that is partially suppressed at 37°C (Fig. 3). This finding suggests the possibility that some other Hsp70 protein expressed at 37°C is able to provide some degree of Pdr13p function. There is precedence for Hsp70 gene disruptions leading to cold-sensitive phenotypes, as mutant strains lacking both *SSB1* and *SSB2* exhibit a cold-sensitive phenotype that can be suppressed by elevating the growth temperature (14).

An intriguing possibility suggested by the appearance of a growth defect in Δ*pdr13* cells is that Pdr13p may act as a link between essential cellular functions and the *PDR* pathway. Perhaps Pdr13p coordinates the activity of the *PDR* genes with fundamental cell metabolism and activates Pdr1p function in response to an as-yet-unknown signal. It is important to bear in mind that drugs such as cycloheximide and oligomycin are unlikely to represent the natural substrates of the *PDR* genes, making the true function of these genes a still-unresolved question.

The identification of the S295F form of Pdr13p as a hyperactive regulator of Pdr1p function is interesting for three different reasons. First, this particular mutation provides a new class of Hsp70 mutants with altered function. Mutant Hsp70 derivatives with increased function have not been reported before. Isolation of this class of mutant Hsp70 proteins likely has been slowed by the complex, pleiotropic nature of the roles of these proteins. Our ability to mutationally separate the essential function(s) of Pdr13p from its role in the control of Pdr1p activity will allow genetics to be used to analyze the molecular details underlying this regulatory interaction. Second, the S295F mutation is located in the most highly conserved segment of Hsp70 proteins, the ATPase domain (18). This idea is consistent with the work of James et al. (30), who showed that at least part of the specificity of action of *S. cerevisiae* Hsp70 proteins was determined by this N-terminal ATPase domain. *dnaK* mutants with a partial loss of function also mapped to this domain (52). Finally, the change from serine to phenylalanine suggests the possibility that phosphorylation is involved. Alternatively, the large change in amino acid identity between serine and phenylalanine may lead to the observed phenotype. Discrimination between these models requires direct biochemical experiments aimed at addressing the regulation and activity of Pdr13p.

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