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Mutations in the Yeast *PDR3*, *PDR4*, *PDR7* and *PDR9* Pleiotropic (Multiple) Drug Resistance Loci Affect the Transcript Level of an ATP Binding Cassette Transporter Encoding Gene, *PDR5*

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ABSTRACT

The yeast pleiotropic (multiple drug) resistance gene *PDR5* encodes a product with homology to a large number of membrane transport proteins including the mammalian multiple drug resistance family. In this study, we identified four genes on chromosome *II* that affect the steady-state level of *PDR5* transcript in addition to a previously identified positive regulator, *PDR1*. The genes in question are *PDR3*, *PDR4*, *PDR7* and *PDR9*. We also analyzed the interaction between *PDR5* and *YAP1*. *YAP1* encodes a positive regulator with a leucine zipper motif that causes pleiotropic drug resistance when overproduced. *YAP1*-mediated pleiotropic drug resistance is not dependent on the presence of *PDR5* and must act through other genes.

PLEIOTROPIC drug resistance in Saccharomyces is brought about by alteration or amplification of no fewer than seven different genes. These include *PDR1* and *YAP1* (formerly *PDR4*), which encode putative transcriptional regulators (Balzi *et al.* 1987; Moye-Rowley *et al.* 1989), and *PDR5*, the sequence of which encodes a 1511-amino acid protein with multiple transmembrane segments (E. Balzi and A. Goffeau, unpublished results). The *PDR5* gene product is related to a large family of membrane transporters. These include the cystic fibrosis transmembrane conductance regulator protein, the yeast *STE6* gene product and the mammalian *MDR1* membrane pump.

The inter-relationship of the various *PDR* genes is important if one is to ultimately understand the different pathways involved in multiple drug resistance. In previous work (Meyers *et al.* 1992) we presented genetic evidence that *PDR1* is a positive regulator of the *PDR5* locus. Thus, deletion of *PDR1* results in a depression in the steady state level of *PDR5* transcript. We observed, however, that while a deletion of *PDR1* results in mild drug hypersensitivity to cycloheximide (and other inhibitors), the phenotype of a *PDR5* insertion mutation is much more severe. This observation suggested that other genes might share in the regulation of *PDR5*. To identify other *PDR5* regulatory genes, we screened second site pleiotropic drug resistant revertants of a *pdr1* deletion mutation for those revertants that restored or increased the steady-state level of *PDR5* transcript. Three nuclear genes were identified by this procedure. All three map to chromosome *II*. One of the mutants is allelic to the previously identified *PDR4* locus (Preston *et al.* 1987). Two new loci *PDR7* and *PDR9* were also identified. We also showed that the previously identified *PDR3* gene, which is also located on chromosome *II* (Subik *et al.* 1986), overproduces *PDR5* transcript.

We also examined the effect of *YAP1* (formerly called *PDR4*: see Leppert *et al.* 1990) on *PDR*-mediated resistance. The *YAP1* gene product is a positive transcriptional regulatory protein related to the Jun family of transcriptional factors (Moye-Rowley *et al.* 1989). It is known that amplification of *YAP1* results in pleiotropic drug resistance (Leppert *et al.* 1990). Interestingly, we find no evidence that *YAP1* is usually involved in *PDR5* regulation. Rather, it seems to activate some other multiple drug-resistance pathway.

MATERIALS AND METHODS

Yeast strains: The yeast strains used in this study are listed in Table 1. Strains BJ5690, 5691 and 6673 were generously provided by Elizabeth Jones. STX84-5A, STX145-13D, X4119-19C, X4126-6D and STX445-2A come from the Yeast Genetics Stock Center (Berkeley, California). US50-18C, IL125-2B and D1-3 were generously provided to us by our collaborators, Elisabetta Balzi and Andre Goffeau. Sue Klapholz provided strains K398-4D (JG282), K399-7D (JG283) and K396-11A (JG284).

There have been changes in the designation of some yeast *PDR* genes. In particular, the *YAP1* locus (Moye-Rowley *et al.* 1989) was also called *PDR4* (Leppert *et al.* 1990). *PDR4* now represents a gene tightly linked to the chromosome *II* centromere (Preston *et al.* 1991). The current *PDR4* (Preston *et al.* 1987) gene was also designated as *PDR7* (Balzi and Goffeau 1991), although *PDR7* is a new gene defined in the present study.

Plasmids: The plasmid pDR3.3, which contains *PDR5*, was previously described (Leppert *et al.* 1990). pSEY18-R 2.5 (Moye-Rowley *et al.* 1989) which contains *YAP1*, is a 2

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TABLE 1

Yeast strains

Strain	Genotype	Reference/Comments
RW2802	*MATa leu2 ura3 met5 PDR1 PDR5*	Reed Wickner
JG436	*MATa leu2 ura3 met5 PDR1 pdr5*::Tn5	Leppert *et al.* (1990)
JG365-5C	*MATα met5 ura3 PDR1-3 pdr5*::Tn5	Meyers *et al.* (1992)
IL125-2B[a]	*MATα his1 PDR1 PDR5*	Balzi *et al.* (1987)
US50-18C[a]	*MATα his1 PDR1-3 PDR5 ura3*	Balzi *et al.* (1987)
D1-3[a]	*MATα his1 pdr1Δ1:URA3 PDR5*	Balzi *et al.* (1987)
JG200	*MATa gal1 PDR1 PDR5 his3 leu2*	George Sprague
JG225	*MATa lys2-1 tyr1-1 his7-2 met13d trp5-d leu1-12 ade1*	Michael Esposito
JG282 (K398-4D)	*MATa spo11 ura3 ade6 arg4 aro7 asp5 lys2 met14 trp1 pet17*	Sue Klapholz
JG283 (K399-7D)	*MATa spo11 ura3 his2 leu1 met4 pet8*	Sue Klapholz
X4126-6D	*MATa his4 leu2 his5 ilv3 CUP1 gal2 ura1 ade3 rad52 Mal-*	Yeast Genetic Stock Center
STX84-5A	*MATa ade1 rad57 cdc4 ura3 arg4 gal2 pet8 aro7*	Yeast Genetic Stock Center
STX145-13D	*MATa cdc19 tyr1 gal1,5 trp1 rad4 met14 ura1 lys9 pet8 ade2*	Yeast Genetic Stock Center
X4119-19C	*MATa his7 tyr1 cdc9 trp4 aro1 hom2 rad2 thr1 lys11 gal2 ade2*	Yeast Genetic Stock Center
BJ5690	*MATα gal1 lys2 his1 pdr4-1*	Elizabeth Jones
BJ5691	*MATα gal1 lys2 ura3 trp1 pdr4-1*	Elizabeth Jones
BJ6673	*MATα ade1 lys2 ura3 pdr3-1 chyr*	Elizabeth Jones
STX445-2A	*MATα ade6 gal1 PDR1 PDR5*	Yeast Genetics Stock Center
JG284 (K396-11A)	*MATa spo11 ura3 ade1 his1 leu2 lys7 met3 trp5*	Sue Klapholz
JG396-12A	*MATa ura3 ade1* D-1-3-mutant 8D (*pdr9-1*)	This study
JG391-2B	*MATa ura3 leu2 his1* D-1-3-mutant 4A (*pdr4-2*)	This study
JG422-4D	*MATa* D1-3-mutant 16B (*pdr7-1*) *ade2 met13*	This study
JG385-19A	*MATa ura3 ade1,2 lys7 Met- PDR1 pdr5*::Tn5	This study
JG415-5B	*MATa pdr5*::Tn5 *ade2 leu2 met5*	This study
JG349-9B	*MATα pdr5*::Tn5 *ade2 met13 ura3 met5*	Leppert *et al.* (1990)
JG406-3C[b]	*MATα pdr7-1 pdr5*::Tn5	This study
JG406-13B[b]	*MATα pdr7-1 pdr5*::Tn5	This study
JG410-38A	*MATα pdr7-1 ura3 trp1*	This study
RW2802 + pKV-2	RW2802 transformed with plasmid pKV-2	This study
JG423	RW2802 + pKV-2 × JG410-38A	This study
JG423-7A	*MATα met5*	This study
JG424	RW2802 + pKV-2 × BJ6673	This study
JG424-1C	*MATa leu2 lys2 pdr3-1 ura3 pVK-2::URA3*	This study
JG424-3B	*MATa leu2 lys2 ura3 pdr3-1*	This study
JG425	RW2802 + pKV-2 × BJ5691	This study
JG426	JG396-12A × JG423-7A	This study
JG426-8D	*MATα ura3 pdr9-1*	This study
JG429	RW2802 + pKV-2 × JG426-8D	This study
SEY6210	*MATα leu2 his3 ura3 trp1 lys2 YAP1*	This study
SM10	*MATα leu2 his3 ura3 trp1 lys2 YAP1Δ1::HIS3*	This study
JG365-1B	*MATa pdr5*::Tn5 *his1 ura3 leu2*	Meyers *et al.* (1992)
JG393-18C	*MATa YAP1 pdr1Δ1::URA3 ura3 his3*	This study

[a] The D1-3 and US50-18C strains are isogenic. IL125-2B is closely related, but not isogenic.
[b] Full genotype is not known, strains were not retained.

μm-origin plasmid with *URA3* as a selectable marker [see Emr *et al.* (1986) for description of pSEY18]. pKV2 contains a fusion between *Escherichia coli* β-galactosidase gene and the *PDR5* promoter. The latter extends from codon 10 to *ca.* 1200 bases upstream of the transcription start site. This plasmid also contains *CEN4/ARS* sequences and the *URA3* gene.

Media: The recipes for culture and inhibitor media were previously reported (Meyers *et al.* 1992). For the tetrad analysis described in this paper, ascosporal segregants were scored on cycloheximide medium containing 1.0 μg/ml of the inhibitor, unless otherwise indicated, and on sulfometuron methyl medium containing this inhibitor at a concentration of 5 or 8 μg/ml.

Preparation and purification of nucleic acids: RNA was prepared for dot blots or Northern hybridization as previously described (Meyers *et al.* 1992).

Hybridization experiments: Dot blot and Northern hybridization was carried out as described by Sambrook *et al.* (1989). Filters were hybridized at 42° in 50% formamide, 5 × SSPE, 2 × Denhardt's solution, 0.05% SDS and 100 μg/ml heat denatured salmon sperm DNA. Three different post hybridization washes were each performed twice: in 5 × SSPE, 0.1% SDS (42°), 1 × SSPE, 0.1% SDS (room temperature), and 0.1 × SSPE, 0.1% SDS (room temperature). The 4.8-kbp *Pvu*II fragment of the *PDR5* probe (Meyers *et al.* 1992) was purified from a 0.8% agarose gel using a "Gene Clean" kit (Bio 101, La Jolla, California) and nick translated as previously described (Meyers *et al.* 1992). Northern hybridization was performed with the same probes as recently reported (Meyers *et al.* 1992) with the yeast actin gene serving as a control.

Tetrad analysis: Tetrad analysis was performed by conventional means described by Sherman *et al.* (1974).

Isolation of revertants: To find hyperresistant revertants of *pdr1Δ:URA3*, 1 ml YPD (yeast extract, peptone, dextrose) cultures of D1-3 were grown overnight at 30°. The cultures were centrifuged to pellet the cells, washed once with sterile, distilled water and plated in 3 ml of sterile agar overlay (1% agar in water) on YPD medium containing 0.5 μg/ml cycloheximide. Colonies appearing by 72 hr were picked for further testing.

Transformation of yeast: Yeast were transformed using the procedure of HINNEN *et al.* (1978).

Inhibitor testing: The effect of various mutations on drug resistance was assayed either by replica plating or by a spot test. The spot test was performed by growing cells to an absorbance unit $(A.U.)_{600}$ of 1.0 in minimal medium before spotting onto plates containing either 0.25 or 0.5 μg/ml cycloheximide. Assuming an O.D. of 1.0 = 10^7 cells/ml, 1000 cells in 5 μl were spotted on plates and incubated at 30° for 48 hr.

β-Galactosidase assays: β-Galactosidase assays were performed as follows. Approximately 10^7 cells were pelleted and suspended in Z buffer (SAMBROOK *et al.* 1989). Following this, 30 μl of chloroform and 20 μl 0.1% SDS were added along with 0.1 g of glass beads. The cells were vortexed twice at high speed for 10 sec. The lysates were warmed at 30° for 3 min. Following this, 200 μl of a 4 mg/ml *o*-nitrophenyl β-D-galactopyranoside stock solution was added. Reactions were stopped by the addition of 500 μl of 1 M Na_2CO_3. Reaction tubes were microfuged for 5 min at 12,000 × *g* to pellet the debris. Absorption of the supernatant was measured at $A.U._{420}$. Specific activity of β-galactosidase was calculated using the equation: specific activity = 1000 × $A.U._{420}$/ml of cells × $A.U._{600}$ × time of reaction (minutes). β-Galactosidase assays for each segregant were done in triplicate.

RESULTS

Experimental rationale–phenotypes of various mutants: Our experiments were motivated by the cycloheximide resistance phenotypes of mutations in various *PDR* genes. The *pdr5*::Tn*5*mutation used in this study contains an insertion in the promoter region (J. GOLIN, unpublished observations). As a result, there is a very large reduction in the steady state level of *PDR5* transcript (MEYERS *et al.* 1992). The *pdr5*::Tn*5* mutation (LEPPERT *et al.* 1990) also results in extreme hypersensitivity to a large variety of inhibitors including cycloheximide (MEYERS *et al.* 1992). After 72 hours incubation on a low (0.5 μg/ml) dose of cycloheximide, there is little visible growth. This hypersensitivity is also observed in double mutants that have a dominant, hyperresistant *PDR1-3* allele and the *pdr5*::Tn*5* insertion (MEYERS *et al.* 1992). The phenotype of a *PDR1* deletion (*pdr1Δ1::URA3*) is not identical to *pdr5*::Tn*5*. The former is also hypersensitive relative to wild type controls; however growth on cycloheximide plates is readily apparent after 96 hours (data not shown). Although other interpretations are possible, this result suggested that loci in addition to *PDR1* regulate the production of *PDR5* transcript. To identify these other regulatory genes, we isolated second-site suppressors of *pdr1Δ1::URA3* cycloheximide hypersensitivity.

Isolation of putative mutants that suppress the hypersensitivity of a PDR1 deletion (*pdr1Δ1::URA3*): To find other regulatory loci that might interact with *PDR5*, we selected spontaneous revertants of *pdr1Δ1::URA3* on cycloheximide medium as described in MATERIALS AND METHODS. Seventeen independent mutants were identified which were resistant to both cycloheximide and sulfometuron methyl. Since multiple drug resistant suppressors of *pdr1Δ1::URA3* could be due to many different mechanisms, we needed a screen that would quickly identify those genes affecting *PDR5* expression. Because *pdr1Δ1::URA3* causes a marked depression in the production of a steady state level of *PDR5* transcript (MEYERS *et al.* 1992), we sought a subclass of mutants that restored the wild-type level or overproduced it. RNA was extracted from each of the strains and dot blot analysis was performed using a 4.8-kb internal *Pvu*II fragment of pDR3.3 (MEYERS *et al.* 1992) as a probe. Three mutants (D1-3-4A, D1-3-8D, D1-3-16B) clearly overproduced transcript when compared to the *pdr1Δ1::URA3* isogenic strain (data not shown). The initial result was verified by Northern hybridization (Figure 1, compare lane 2 with lanes 5–7). In addition to the mutations identified by hybridization, we also tested the previously identified *PDR2*, *PDR3* and *PDR4* hyperresistant mutants. Significantly, the strains bearing either a *pdr3-1* or *pdr4-1* mutation (lanes 8–10) also have very high steady-state levels of *PDR5* transcript. The *pdr2* mutants do not show increased levels of *PDR5* transcript (data not shown). The newly isolated mutants were tested, along with other *PDR* alleles, for their various drug phenotypes. These are shown in Figure 2 and are summa-

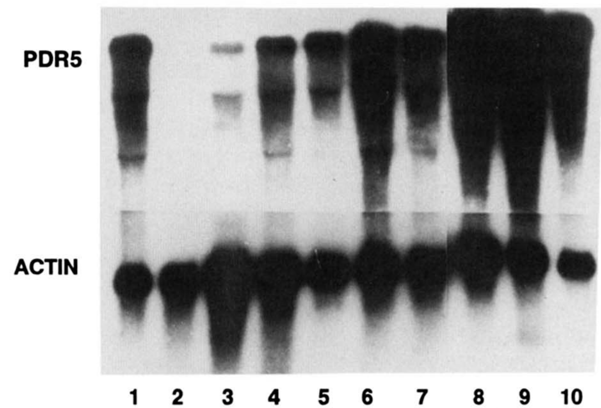

FIGURE 1.—Northern hybridization of various PDR strains. RNA was extracted and subjected to Northern hybridization as previously described (MEYERS *et al.* 1992). The actin message serves to indicate that approximately equal amounts of RNA were loaded into each lane. Lane 1, *PDR1-3* (US50-18C); lane 2, *pdr1Δ1::URA3* (D1-3); lane 3, *PDR1* (JG200); lane 4, *PDR1-7* (JG204); lane 5, D1-3-4A; lane 6, D1-3-8D; lane 7, D1-3-16B; lane 8, *pdr4-1* (BJ5690), lane 9, *pdr4-1* (BJ5691), lane 10, *pdr3-1* (BJ6673). Though the *pdr3-1* and *pdr4-1* strains used in this study do not have corresponding isogenic controls, they clearly overproduce *PDR5* transcript when compared to wild-type controls.

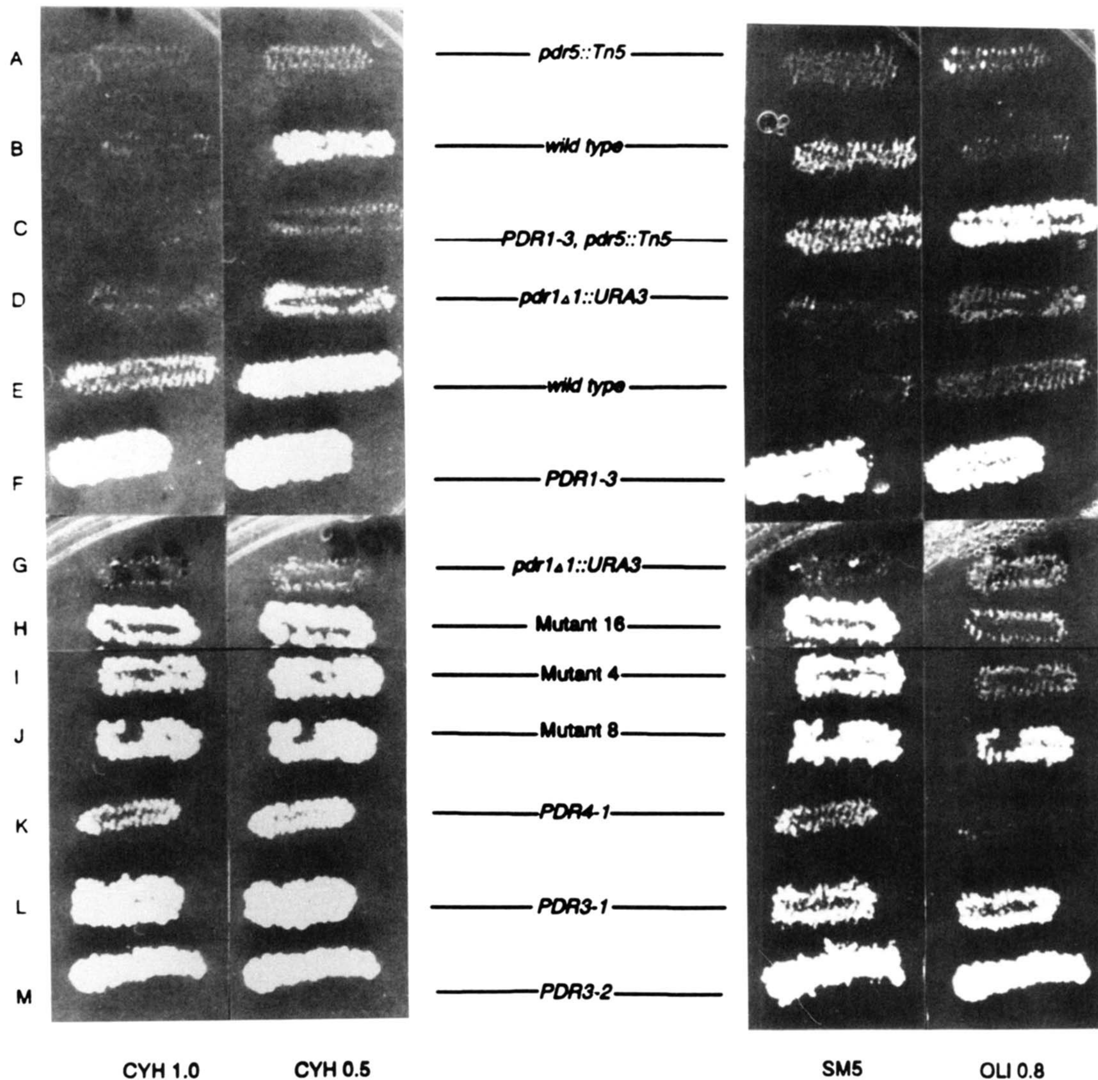

FIGURE 2.—Phenotypes of various strains. The phenotypes of various strains are shown at 72 hr for two doses of cycloheximide (CYH, 0.5 and 1.0 μg/ml) and for a single dose of oligomycin (OLI, 0.8 μg/ml) and sulfometuron methyl (SM5, μg/ml). Strain A, *pdr5*::Tn*5* (JG436); strain B, wild type (RW2802); strain C, *PDR1-3*, *pdr5*::Tn*5* (JG365-5C); strain D, *pdr1Δ1::URA3* (D1-3); strain E, wild type (IL125-2B); strain F, *PDR1-3* (US50-18-C); strain G, *pdr1Δ1::URA3* (D1-3); strain H, D1-3-16B = mutant 16; strain I, D1-3-4A = mutant 4; strain J, D1-3-8D = mutant 8; strain K, *pdr4-1* (BJ5590); strain L, *pdr3-1* (BJ6673); strain M, *pdr3-2* (BJ6676).

rized in Table 2. The D1-3-4A (strain I), D1-3-8D (strain J), D1-3-16B (strain H), *pdr4-1* (BJ5690-strain K), *pdr3-1* (BJ6673-strain L) and *pdr3-2* (BJ6676-strain M) strains are resistant to cycloheximide and sulfometuron methyl. In addition, the *pdr3-1*, *pdr3-2*, and the D1-3-8D mutant bearing strains were also oligomycin resistant, although as we discuss later, this phenotype segregates independently of the multiple drug resistance one.

Preliminary genetic analysis: To determine whether the putative mutants were dominant or recessive, each was crossed to two wild type strains: JG200 and RW2802. Diploids, selected on omission medium were compared to haploid mutants and isogenic controls with regard to their relative cycloheximide resistance. The results are shown in Figure 3. When heterozygotes were made using JG200, all of the mutants exhibited a semidominant resistant phenotype as heterozygotes. They grew as strongly as homozygous mutant controls on plates containing 1.0 μg/ml cyclohexmide. At higher doses, homozygous mutants grew better (data not shown). Interestingly, the D1-3-8D and D1-3-16B mutants behaved differently when crossed to a second wild-type strain RW2802. Though the D1-3-4A as well as the *pdr3* and *pdr4* heterozygotes continued to exhibit a resistant phenotype, the D1-3-8D and D1-3-16B mutants were drug sensitive and thus appears recessive. To determine which of these phenotypes is the general rule, mutants were crossed to seven other sensitive (wild type) strains (JG225, JG282, JG283, STX84-5A, STX145-13D, X4119-19C and X4126-6D). The resulting heterozygotes were tested on cycloheximide media as above. Crosses between the mutants and JG225, JG282, JG283 and STX84-5A were drug sensitive. Crosses with X4119-19C and X4126-6D

TABLE 2

Drug phenotypes of various strains

Strain	Pertinent genotype[a]	At 24 hr[b] Cyh 0.5	Cyh 1.0	Oli 0.8	SM 5
RW2802		±	−	−	−
ILI25-2B		+	−	−	−
JG436	*pdr5*::Tn*5*	−	−	−	−
JG365-5C	*PDR1-3*, *pdr5*::Tn*5*	−	−	+	−
US50-18C	*PDR1-3*	+	+	+	+
D1-3	*pdr1Δ1::URA3*	−	−	−	−
D1-3-16B	*pdr1Δ1::URA3*, mutant 16B	+	+	−	+
D1-3-4A	*pdr1Δ1::URA3*, mutant 4A	+	+	−	+
D1-3-8D	*pdr1Δ1::URA3*, mutant 8D	+	+	+	+
BJ5690	*pdr4-1*	+	+	−	+
BJ6673	*pdr3-1*	+	+	+	+
BJ6676	*pdr3-2*	+	+	+	+

[a] A given strain is wild type for *PDR* genes except as indicated by allelic designation. US50-18C and IL125-2B are isogenic strains as are RW2802 and JG436. The BJ5690, 6673 and 6676 strains are not isogenic to the others, though their resistance is striking when compared to standard wild-type strains.

[b] Drug doses are given in micrograms/ml. Cyh, cycloheximide; Oli, oligomycin; SM, sulfometuron methyl.

exhibited the same semidominant phenotype encountered with JG200.

The *pdr* × JG200 heterozygotes were sporulated and the resulting tetrads dissected. The crosses involving mutants D1-3-4A, D1-3-8D, and D1-3-16B showed high viability (>92%). Not surprisingly, all three exhibited a standard (2:2) Mendelian segregation of the resistance phenotype (Table 3), even though the *pdr1Δ1::URA3* mutation was also segregating. This result means that the newly isolated mutations do not require the *PDR1* gene product for their hyperresistant phenotype. It is critical to note that all of the tetrads (typically 85–90 spores) were screened on both cycloheximide and sulfometuron methyl medium. In every instance analyzed, the resistance phenotypes cosegregated. Similar results were also obtained for heterozygotes made with RW2802 (data not shown).

All of the mutants map to the centromere region of chromosome *II*: Since the crosses described above were also heterozygous for the centromere-linked gene *LEU2*, centromere linkage of each mutant was tested. All of the mutants exhibited centromere linkage (Table 4) as do several previously characterized loci that influence multiple drug resistance. Therefore, it was necessary to determine the possible allelic relationship between these mutants and the centromere-linked *YAP1*, *PDR3* and *PDR4* loci. Linkage to chromosome *II* (and therefore possible allelism with *PDR3* and/or *PDR4*) was tested by crossing each mutant to a strain containing a *gal1* mutation (STX 445-2A). The resulting diploids were sporulated and subjected to tetrad analysis. The results, recorded in Table 5, indicated that each mutant was linked to

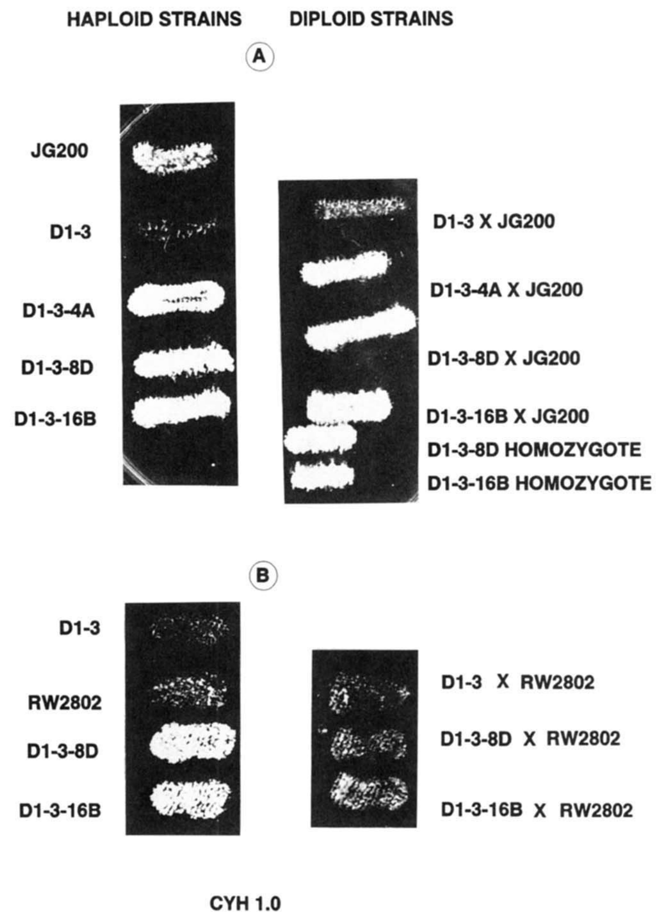

FIGURE 3.—Phenotypes of diploid strains bearing *pdr* mutations. Diploids heterozygous for various mutations as well as control strains were constructed and tested on three doses of cycloheximide (CYH, 0.5, 1.0 and 1.5 μg/ml). The results are shown for a 24-hr incubation on plates containing 1.0 μg/ml cycloheximide at 30°. Heterozygotes were made using two different wild-type strains: JG200 and RW2802. Panel A shows that diploids remain drug resistant when the mutants are crossed to JG200. In contrast (panel B), diploids constructed from a cross between RW2802 and either D1-3-16B or D1-3-8D are drug sensitive. The D1-3-8D homozygote is a cross between D1-3-8D and JG396-12A. The D1-3-16B homozygote was made by crossing D1-3-16B and JG422-4D.

TABLE 3

Monofactorial inheritance of the 4A, 8D and 16B mutants in four-spored tetrads

Cross	No. of resistant spores in four-spored tetrads 4	3	2	1	0
D1-3-4A × JG200	1	2	37	0	0
D1-3-8D × JG200	1	2	19	0	0
D1-3-16B × JG200	1	1	14	2	0

GAL1. At issue, however, is whether the mutants are allelic to each other and to *PDR3* or *PDR4*. During this study, we learned that RUTTKAY-NEDECKY *et al.* (1992) identified an additional cycloheximide resistance mutation in the original *pdr3-1* strain. We verified that this was caused by backcrossing BJ6673 (*pdr3-1*, *cyh^r*) to a wild-type strain (RW2802). The

TABLE 4

Centromere linkage of hyperresistant mutants and the *GAL1* locus

Cross	CEN[a] marker	FDS[b]	SDS[c]	Distance (cM)
a. CYH:CEN segregation[d]				
D1-3-4A × JG200	*LEU2*	34	5	3.0
JG396-12A (D1-3-8D) × STX445-2A	*URA3*	51	22	9.1
D1-3-16B × JG200	*LEU2*	20	10	16.2
D1-3-16B × JG284	*MET3*	21	14	17.0
JG424-1C (*pdr3-1*) × STX445-2A	*LEU2*	35	6	2.9
b. *GAL1*:CEN segregation				
JG424-1C × STX445-2A (*gal1*)	*LEU2*	33	8	5.3
JG396-12A × STX445-2A	*URA3*	28	15	9.4

[a] CEN, centromere.
[b] FDS, first division segregation.
[c] SDS, second division segregation.
[d] In calculating centromere linkage the *LEU2*-centromere and *MET3*-centromere distances are 4.4 and 3 cM, respectively. *URA3* is 8 cM from the centromere. CHY, cycloheximide.

multiple drug resistance phenotype, scored as the cosegregation of resistance to cycloheximide and sulfometuron methyl, segregated as a single gene. Other spores segregated the other cycloheximide resistance factor, but remained sensitive to sulfometuron methyl. A *pdr3-1* segregant (JG424-1C) which did not contain the additional *cyh^r^* gene was used in the subsequent mapping experiments.

To determine the allelic relationships among mutants, pairwise crosses were made between the newly isolated mutants as well as the previously identified genes. The resulting tetrads were subjected to analysis as follows. An ascus containing four resistant spores is assumed to be a parental ditype, while those yielding a single sensitive segregant are tetratypes. Asci with two sensitive spores are nonparental ditypes. These results are also found in Table 5. Several conclusions can be reached. First, D1-3-4A and *PDR4* appear to be allelic to one another since only one recombinant was found in 86 tetrads. Therefore, D1-3-4A is now called *pdr4-2*. In contrast, pairwise crosses between D1-3-4A and either D1-3-8D or D1-3-16B yield frequencies of recombinant asci that are higher than expected for two alleles of the same locus. Furthermore, their locations relative to the centromere are different. Crosses between strains containing *pdr3-1* and D1-3-8D showed significant genetic distance between them (about 20 cM). D1-3-8D was also separable from D1-3-16B. The data found in Tables 4 and 5 indicate that these mutants define new *PDR* genes. D1-3-16B is now known as *PDR7*, while D1-3-8D is *PDR9*. Figure 4 comprises the mapping data. Our results do not allow us to position *PDR4* and *PDR3* with respect to the centromere, other than to conclude that they are tightly linked. PRESTON *et al.* (1991) made a reasonable argument for placing *PDR4* on the

TABLE 5

Mapping of drug-resistant mutants with respect to *PDR3* and *PDR4*

Cross	PD[a]	NPD[b]	TT[c]	Distance (cM)[d]
a. Linkage to *GAL1*				
JG396-12A (mutant D1-3-8D) × STX445-2A (*gal1*)	46	1	19	18.9
JG391-2B (mutant D1-3-4A) × STX445-2A	13	0	5	13.9
JG422-4D (mutant D1-3-16B) × STX445-2A	33	1	20	24.1
JG424-1C (*pdr3-1*) × STX445-2A	37	0	5	6.0
b. Mapping of D1-3-4A, D1-3-8D, D1-3-16B				
D1-3-4A × JG396-12A (D1-3-8D)	31	0	10	12.2
391-2B (*pdr4-2*) × BJ5690 (*pdr4-1*)	85	0	1	0.6
JG396-12A × BJ5691 (*pdr4-1*)	59	1	3	7.1
D1-3-8D × JG424-1C (*pdr3-1*)	16	0	6	
D1-3-8D × JG424-1B (*pdr3-1*)	12	1	5	
	28	1	11	21.3
JG422-4D (mutant D1-3-16B) × BJ5691	46	2	11	19.5
JG410-38A (mutant D1-3-16B) × JG396-12A	54	1	10	12.3

[a] Parental ditype.
[b] Nonparental ditype.
[c] Tetratype.
[d] Map distance (m.d.) computed using the formula m.d. = 6(NPD) + TT/2 × no. of tetrads (PERKINS 1949).

opposite side of the centromere from *GAL1*. They also concluded that *PDR3* and *PDR4* are most likely non-allelic (they were about 5.5 cM apart). The location of the *PDR3* gene with respect to the centromere is unclear. SUBIK *et al.* (1986) placed it *ca.* 5 cM from the centromere on the side opposite *GAL1*. This was based in part on the relatively tight linkage (11.6 cM) between *PDR3* and *PET9* (*PET9* is *ca.* 20 cM from the centromere). Our data, however, seem more compatible with the placement of *pdr3-1* on the same side of the centromere as *GAL1*. Otherwise, *PDR3* and *PDR9* should be tightly linked (about 6 cM apart). This was not observed. The locations of *PDR7* and *PDR9* seem unambiguous. The *PDR7/PDR9/PDR4/CEN2/GAL1* intervals are reasonably additive given the sample sizes and small errors inherent in mapping. There are some exceptions, however, that bear mention. *PDR9/GAL1* gives a slightly shorter distance than *PDR9/PDR3*, although other data strongly suggest that the order is *PDR9/CEN/PDR3/GAL1*. In fact, the *PDR9/CEN/GAL1* as well as the *CEN/PDR3/GAL1* intervals are additive, whereas the *PDR9/PDR3* distance is larger than expected. Similarly, *PDR7/PDR4* gives a slightly larger distance than *PDR7/CEN*.

The positions of *PDR7* and *PDR9* suggest that they could be allelic to either *CYH1*, *CYH10* or *AMY2*. We have not been able to obtain a *CYH1* or a *CYH10*

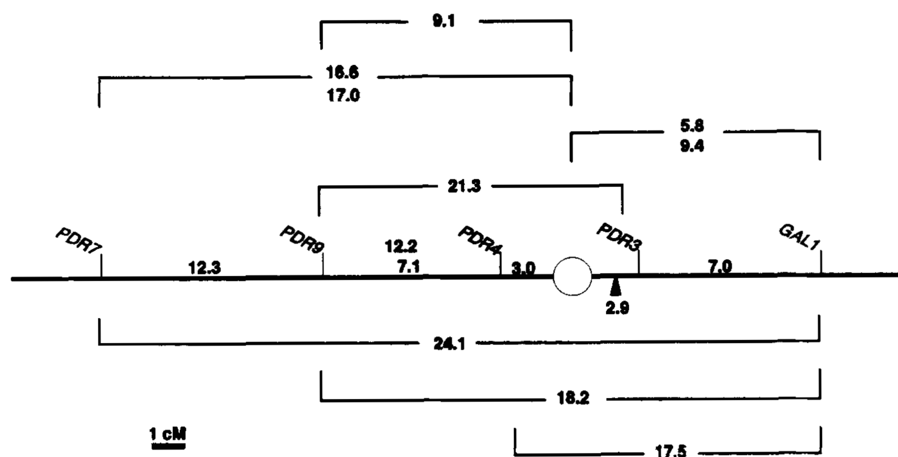

FIGURE 4.—Genetic map of various *PDR* genes. The map was constructed from the data in Tables 4 and 5 and shows the location of four *PDR* genes found on chromosome *II*. Some intervals are represented by more than one cross, thus the map distance for each one is indicated.

strain. We tested our mutants with two concentrations of antimycin (0.01 and 0.04 μg/ml) to which the *AMY2* mutants are known to be resistant. The *pdr7-1* and *pdr9-1* alleles were drug sensitive.

Dependence of *pdr3-1*, *pdr4-1*, *pdr4-2*, *pdr7-1* and *pdr9-1* cycloheximide drug resistance on a functional *PDR5* locus: Each of the mutants exhibited a marked increase in the steady state levels of *PDR5* when Northern hybridization was performed. At issue is whether *PDR5* is the major target for these genes, at least with respect to cycloheximide drug resistance. To determine whether this was the case, each mutant was crossed to one or more strains containing the *pdr5*::Tn*5* disruption previously described (see LEPPERT *et al.* 1990). This mutant results in little or no detectable *PDR5* transcript (see Figure 1). If a hyperresistant phenotype depends upon *PDR5*, two-gene segregation would be observed. Thus, many four-spored tetrads would yield only one resistant segregant and some would yield none. In contrast, if the *PDR5* gene product is not required for hyperresistance, the regular 2 resistant:2 nonresistant pattern would result. The results of this analysis are found in Table 6. The *pdr3*, *pdr4* and *pdr7* hyperresistance alleles all exhibit two-gene segregation on plates containing 1.0 μg/ml cycloheximide, suggesting that the double mutant (*pdrx*, *pdr5*::Tn*5*) is hypersensitive. That this was the case was tested with respect to the *pdr7-1* mutation. From the *pdr7-1* × *pdr5*::Tn*5* heterozygote, we identified tetrads in which two members were wild type (sensitive) and two segregants were hypersensitive. Two hypersensitive segregants (JG406-3C and JG406-13B) from two such tetrads were assumed to be *pdr7-1*, *pdr5*::Tn*5*. These were backcrossed to a standard wild-type strain (JG200) and the resulting diploids were sporulated and dissected. The meiotic segregants were screened for the re-appearance of the resistant *pdr7-1* allele. Table 7 indicates that resistant segregants were recovered in ratios close to the expected value for two interacting genes. Thus, there was a prevalence of tetrads with one resistant spore.

TABLE 6

Dependency of drug resistance on a functional *PDR5* locus

Cross	Segregation in four-spored tetrads[a]				
	4^r:0^s	3^r:1^s	2^r:2^s	1^r:3^s	0^r:4^s
D1-3-4A (*pdr4-2*) × JG415-5B (*pdr5*::Tn*5*)	0	0	8	16	7
D1-3-4A × JG385-19A (*pdr5*::Tn*5*)	0	0	5	7	4
BJ5690 (*pdr4-1*) × JG436 (*pdr5*::Tn*5*)	0	0	7	15	5
D1-3-8D (*pdr9-1*) × JG385-19A (*pdr5*::Tn*5*)	0	0	37	7	3
JG396-12A (*pdr9-1*) × JG349-9B (*pdr5*::Tn*5*)	0	0	16	35	10
JG424-1C (*pdr3-1*) × JG349-9B (*pdr5*::Tn*5*)	0	0	2	18	4
JG410-38A (*pdr7-1*) × JG415-5B	0	0	10	25	5

[a] r means resistant, s means either sensitive or hypersensitive.

TABLE 7

Segregation of drug resistance in crosses involving putative *pdr7-1*, *pdr5*::Tn*5* double mutants

Cross	No. of resistant spores in four-spored tetrads		
	2	1	0
JG406-3C (*pdr7-1*, *pdr5*::Tn*5*) × JG200	3	18	7
JG406-13B (*pdr7-1*, *pdr5*::Tn*5*) × JG200	3	13	8

Our results with the *pdr9-1* allele are more complicated. In a cross to one strain (JG385-19A) that contained a *pdr5*::Tn*5* mutation, *pdr9-1* (D1-3-8D) mediated resistance continued to exhibit a 2:2 segregation in a majority (37/47) of the tetrads analyzed. Furthermore, among these asci, the resistance showed the tight centromere linkage expected of *PDR9* (23 FDS, 11 SDS). In the cross between JG396-12A and a second *pdr5*::Tn*5* bearing strain (JG349-9B), we noted two-gene segregation. The basis for this difference is under investigation. Possible explanations will be outlined in the DISCUSSION.

TABLE 8

***ADE2* genotype of resistant spores**

Cross	No. of resistant spores that are *ADE2*	*ade2*
JG410-38A (*pdr7-1, ADE2*) × JG415-5B (*pdr5*::Tn*5*, *ade2*)	42 (0.77)	13
D1-3-4A (*pdr4-2*) × JG415-5B	32 (0.78)	9
JG424-1C (*pdr3-1*) × JG349-9B (*pdr5*::Tn*5*, *ade2*)	41 (0.85)	8
JG396-12A (*pdr9-1*) × JG349-9B	20 (0.77)	6

The data from this analysis also indicate that the aberrant segregation observed with all the *pdr* hyperresistance mutants is likely due to *pdr5*::Tn*5* and not to some other factor fortuitously segregating in our strains. This is determined as follows. In a previous communication, we showed linkage of *PDR5* with *ADE2* (LEPPERT *et al.* 1990). The genes are about 25 cM apart. Since many of the diploids were also heterozygous at the *ADE2* locus, we determined whether the resistant spores were predominantly (*ca.* 75%) of one *ADE2* genotype. Because *pdr5*::Tn*5* was linked to *ade2*, while the hyperresistant spores were in a *PDR5*, *ADE2* background, we anticipated that most of the resistant spores should be *ADE2*. The data in Table 8 show this to be the case for each mutation analyzed.

Segregation of oligomycin resistance: In our initial phenotypic characterization of mutant strains (Figure 2), we observed that D1-3-8D (mutant 8 = *pdr9-1*), BJ6673 (*pdr3-1*, *cyh*^{r}) and BJ6676 (*pdr3-2*) were oligomycin-resistant relative to the other strains. It is known that the *PDR5* gene does not mediate oligomycin resistance [see LEPPERT *et al.* (1990) and MEYERS *et al.* (1991)]. Nevertheless, we observed that *pdr3-1* and *pdr9-1* are dependent upon *PDR5*. It therefore becomes critical to determine whether this resistance cosegregated with the *pdr* phenotype of these strains (*i.e.*, coresistance to sulfometuron methyl and cycloheximide). We therefore looked at the segregation of resistance to these inhibitors by analyzing tetrads from the JG200 × D1-3-8D (heterozygous for *pdr9-1*) and RW2802 + pKV2 × BJ6673 (heterozygous for *pdr3-1*) crosses. In both cases, the oligomycin resistance segregated independently of the *pdr* phenotype and was therefore not a property of either *pdr3-1* or *pdr9-1*. Interestingly, the original *pdr3-1* strain (BJ6673) contained no fewer than three unlinked resistance factors: *PDR3* (*pdr3-1*), *cyh*^{r} and *oli*^{r}. As was previously observed, all sulfometuron methyl-resistant ascosporal clones spores showed concomitant resistance to cycloheximide. These were classified as *pdr3-1*. In a majority of the cases (10/11), four-spored tetrads contained two such segregants.

Effect of *pdr3*, *pdr4*, *pdr7* and *pdr9* hyperresistant mutants on a fusion (*pKV2*) between β-galactosidase and the *PDR5* promoter: Preliminary analysis indicated that all of the hyperresistant mutants described above result in increased steady-state levels of *PDR5* transcript. To determine more conclusively whether these mutants act through the *PDR5* promoter, we tested them to see if they increased the level of β-galactosidase activity in the pKV2 fusion described in MATERIALS AND METHODS. In addition, we also tested the levels in diploids that were heterozygous for each mutation. The pKV2 plasmid was used to transform a standard sensitive yeast strain RW2802. This strain, in turn, was mated to *pdr3-1* (BJ6673), *pdr4-1* (BJ5691), *pdr7-1* (JG410-38A), and *pdr9-1* (JG426-8D)-bearing stains and the diploids selected on appropriate double omission medium. The results, found in Table 9, clearly indicate increased levels of activity for strains that are heterozygous for the *pdr3-1* and *pdr4-1* mutations when compared to controls. The *pdr7-1* and possibly the *pdr9-1* mutation, however, appear nearly recessive since the levels are at or near those found in wild-type strains. This is consistent with their behavior on cycloheximide media (see text and Figure 3). The heterozygous diploids were also sporulated and subjected to tetrad analysis. For each cross, resistant and wild-type haploid segregants that were Ura$^+$ and therefore contained pKV2 were identified and tested for β-galactosidase activity. With regard to *pdr3-1*, the analysis was carried out as follows. Segregants from the BJ6673 × RW2802 + pKV2 diploid which is known to contain three resistance factors (*pdr3*, *cyh*^{r}, *oli*^{r}) were first scored to determine whether they were *pdr3* (resistant to cycloheximide and sulfometuron methyl), or only *cyh*^{r} or *oli*^{r}. Following this, the β-galactosidase activity was assayed. The results found in Table 9 indicate that, to overproduce β-galactosidase, a *pdr3-1* mutation is necessary. The presence or absence of the other resistance factors does not matter. Thus, for example, JG424-15B is *cyh*^{r}, *oli*^{r}, but *PDR3*. It exhibits wild-type β-galactosidase activity. In contrast, JG424-17A contains only *pdr3-1*, but shows very high levels of enzyme activity.

Lack of interdependency between *PDR5* and *YAP1*: The *YAP1* locus (MOYE-ROWLEY *et al.* 1989), formerly called *PDR4* in one study (LEPPERT *et al.* 1990), causes multiple drug resistance when it is amplified. Interestingly, both the *YAP1* and *PDR5* genes were identified in the same screen of a 2μ plasmid library as loci that lead to multiple drug resistance when amplified (LEPPERT *et al.* 1990). *YAP1* encodes a transcriptional regulatory protein containing a leucine zipper motif (MOYE-ROWLEY *et al.* 1989). Nucleotide sequencing of *PDR5* has identified a possible binding site for such a regulator (E. BALZI and A. GOFFEAU, unpublished results). To test the hypothesis that *YAP1* might confer drug resistance through the regulation of *PDR5* mRNA levels, the role of this

TABLE 9

Effect of *pdr* mutants on *PDR5* promoter activity as measured with the pKV-2 fusion

Strain designation	Allele	Average specific activity (β-galactosidase)	±SD
RW2802-pKV-2	Wild type	24.0	1.60
RW2802 + pKV-2	Wild type	161	16.9
RW2802 + pKV-2 × SEY6210	Wild type	75.4	4.57
RW2802 + pKV-2 × JG410-38A	*pdr7-1/PDR7*	160	1.92
RW2802 + pKV-2 × BJ6673	*pdr3-1/PDR3*	410	8.79
JG426-8D × RW2802 + pKV-2	*pdr9-1/PDR9*	231	12.0
RW2802 + pKV-2 × BJ5691	*pdr4-1/PDR4*	617	14.1
JG423-2A	*PDR7*	171	1.00
JG423-2D	*pdr7-1*	484	1.40
JG423-3C	*pdr7-1*	667	4.70
JG423-5C	*PDR7*	99.3	12.7
JG423-9A	*PDR7*	76.5	4.71
JG423-21A	*PDR7*	54.7	1.35
JG423-21C	*PDR7*	50.0	1.30
JG423-28B	*pdr7-1*	440	21.6
JG423-22C	*pdr7-1*	749	16.3
JG423-24B	*PDR7*	64.0	0.57
JG423-24C	*PDR7*	89.3	2.40
JG424-1C[a]	*pdr3-1 oli^r*	2420	6.00
JG424-2A	*PDR3*	112	3.13
JG424-5C	*PDR3*	84.7	0.00
JG424-11A	*PDR3*	161	2.93
JG424-11D	*PDR3*	34.7	4.65
JG424-15A[b]	*pdr3-1*	1090	18.6
JG424-15B	*PDR3 cyh^r oli^r*	75.0	5.00
JG424-15C[b]	*pdr3-1 oli^r*	1210	9.09
JG424-16A	*PDR3 cyh^r*	63.5	3.47
JG424-16C[b]	*pdr3-1*	1760	15.0
JG424-17A[a]	*pdr3-1*	3828	38.7
JG424-20A	*pdr3-1 oli^r*	466	24.7
JG424-20B	*PDR3 oli^r*	133	2.5
JG414-20C	*PDR3*	183	2.4
JG425-3D	*pdr4-1*	2380	27.2
JG425-4B	*PDR4*	194	10.3
JG425-4C	*PDR4*	226	11.4
JG425-5A	*pdr4-1*	1770	4.77
JG425-21A	*PDR4*	77.0	4.00
JG425-21C	*PDR4*	96.3	1.25
JG425-24A	*pdr4-1*	907	10.5
JG425-24B	*PDR4*	90.6	0.29
JG425-24C	*pdr4-1*	1040	3.47
JG425-24D	*PDR4*	82.4	25.2
JG425-28A	*pdr4-1*	925	21.8
JG425-28B	*pdr4-1*	536	37.0
JG429-2C	*pdr9-1*	955	18.7
JG429-15C	*pdr9-1*	2220	20.0
JG429-4A	*PDR9*	86.0	5.00
JG429-4B	*PDR9*	129	13.9
JG429-25A	*pdr9-1*	887	152
JG429-25B	*PDR9*	113	47.6
JG429-25C	*PDR9*	67.4	24.6
JG429-25D	*pdr9-1*	695	180
JG429-35A	*PDR9*	64.9	3.15
JG429-35B	*pdr9-1*	885	57.7
JG429-35C	*pdr9-1*	1130	79.4
JG429-35D	*PDR9*	40.4	0.40

[a] This strain does not contain *cyh^r*.
[b] This strain may contain *cyh^r*. The backcross to determine whether this is the case was not performed.

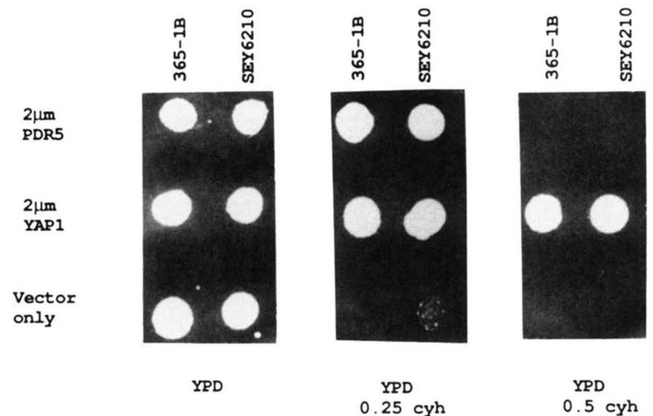

FIGURE 5.—YAP1 high copy suppression of *pdr5*::Tn*5* hypersensitivity. Spot tests were performed on two concentrations of cycloheximide (YPD + 0.25 μg/ml cyh, YPD + 0.50 μg/ml cyh) medium as described in MATERIALS AND METHODS. Introduction of *YAP1* into *pdr5*::Tn*5* results in significant drug resistance when compared to transformants that receive only the vector pSEY18. SEY6210 is a standard wild-type strain (*YAP11*, *PDR1*, *PDR5*). Plates were photographed after 48 hr of incubation.

gene in *PDR5* mediated pleiotropic drug resistance was evaluated.

A *YAP1* allele (*yap1Δ1::HIS3*) in which the DNA encoding the DNA binding domain of the factor was deleted and replaced with *HIS3* was used to determine whether this locus affects *PDR5*-mediated drug resistance. When a strain (SM10) carrying the deletion was compared to an isogenic *YAP1* strain, no increase in cycloheximide hypersensitivity was seen. Nevertheless, it could be argued that *YAP1* serves a supplementary role which would be seen in a *pdr1* deletion. To explore this possibility, we intercrossed marked deletions in both genes (*pdr1Δ1::URA3* JG393-18C × *yap1Δ1::HIS3* SM10). The phenotypes of *URA3,his3* (deletion of *PDR1*), *ura3,HIS3* (deletion of *YAP1*), and *URA,HIS3* (double deletion) segregants were compared on several doses of cycloheximide (0.3, 0.5 and 1.0 μg/ml) and sulfometuron methyl (1, 3 and 8 μg/ml). The lowest dose would allow wild-type cells to exhibit growth by 48 hr, but would kill a hypersensitive strain such as *pdr5*::Tn*5*. The double mutants did not exhibit greater hypersensitivity to cycloheximide or sulfometuron methyl than did the single *pdr1Δ1::URA3* deletion (data not shown).

The result described above argues that even in the absence of *PDR1*, *YAP1* does not substitute as an activator of *PDR5*. Other genes besides *PDR1* might encode a function similar to *YAP1*. Alternatively, *YAP1* might stimulate other gene products that could partly substitute for the *PDR5* encoded protein. To test the latter idea, a strain (JG365-1B) bearing a *pdr5*::Tn*5* mutation was subjected to transformation with a high copy plasmid containing a functional *YAP1* gene (pSEY 18-R2.5). The relative cycloheximide resistance of *URA$^+$* transformants and untransformed controls was compared. As shown in Figure 5, *YAP1*

high copy transformants exhibit significantly greater cycloheximide resistance when compared to a control in which transformants received only a vector. The latter remained hypersensitive. In fact, transformants bearing *YAP1* in high copy number are even more resistant than those containing the *PDR5* gene (pDR3.3) in a high copy vector. We also performed Northern analysis to look for enhanced *PDR5* transcript in multicopy *YAP1* transformants but failed to find elevated *PDR5* mRNA levels (data not shown). Thus, it is most likely that *YAP1*-mediated cycloheximide resistance is conferred by a *PDR5*-independent pathway.

DISCUSSION

In this report, we describe four nuclear mutations that cause overproduction of *PDR5* and enhanced multiple drug resistance, even when the *PDR1* gene product is not functional. One of these maps in the previously identified *PDR4* locus (PRESTON *et al.* 1991); another is the *pdr3-1* mutation (SUBIK *et al.* 1986). Two new genes, *PDR7* and *PDR9*, were also identified. In a previous communication, we presented evidence that the *PDR1* gene is a positive regulator of the *PDR5* gene (MEYERS *et al.* 1992). In contrast, the *YAP1* gene product does not appear to act on *PDR5* even when the *PDR1* gene product is absent. Thus, *yap1Δ1::HIS3*, *pdr1Δ1::URA3* double mutants do not cause added cycloheximide hypersensitivity. Furthermore, overproduction of *YAP1* does not increase the level of *PDR5* transcript. Finally, amplification of *YAP1* in a *pdr5*::Tn*5* strain increases cycloheximide resistance. Thus, there seem to be regulatory loci that are relatively *PDR5* specific (*PDR3*, *PDR4* and *PDR7* and probably *PDR9*–see below), while *YAP1* mediated cycloheximide resistance is *PDR5* independent. The situation with *PDR1* may be a bit more complex (MEYERS *et al.* 1992). *PDR1-3* and *PDR1-7* hyperresistant alleles require a functional *PDR5* for cycloheximide resistance, but not for resistance to a host of other drugs (MEYERS *et al.* 1992). Whether this will be the case for the other *pdr* mutants remains to be determined. Given the extreme hypersensitivity of the *pdr5*::Tn*5* mutation, the *PDR5* independent resistance observed in the *YAP1* amplified strains is probably due to the overproduction of gene products that do not necessarily mediate cycloheximide resistance when they are present at *wild-type* levels.

The semidominance of the *PDR3* and *PDR4* mutant alleles and their function in the absence of a *PDR1* gene argues for (but does not prove) some positive regulatory function. Whether these are related in structure and/or function to *PDR1* remains to be determined. In contrast it is hard to predict the function of the *PDR7* and *PDR9* genes because the *pdr7-1* and *pdr9-1* mutants appear recessive in some genetic

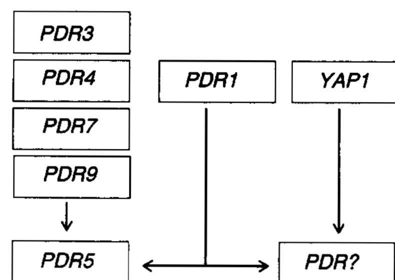

FIGURE 6.—The interrelationships of various *PDR* genes. The diagram illustrates a possible set of interrelationships between the genes analyzed in this study. As drawn, the putative regulatory genes are assumed to be independent of one another although this has not been proven. It is known from this study that *PDR4*, *PDR7* and *PDR9* hyperresistant alleles do not require *PDR1* since they were isolated in a *pdr1Δ1::URA3* background.

backgrounds, but not in others. Figure 6 summarizes the relationship of the *PDR* genes with respect to the *PDR5* locus.

Differential behavior of *pdr9-1* in two *pdr5*::Tn*5* strains: To test the dependency of *pdr9-1* resistance on a functional *PDR5* gene, two crosses were analyzed: D1-3-8D (*pdr9-1*) × JG385-19A (*pdr5*::Tn*5*) and JG396-12A (*pdr9-1*) × *JG349-9B* (*pdr5::Tn5*). In the latter, two gene segregation was observed. In the former, however, the *pdr9-1* mutation showed a segregation pattern that was much closer to that expected for a single gene. At least two explanations can be given that might account for this discrepancy. The original cross could contain a modifier present in JG385-19A which permits resistance in a *pdr9-1*, *pdr5*::Tn*5* background, but by itself does not affect resistance. If this were true, there would be an increase in the number of tetrads showing a 2:2 segregation. Theoretically, for a modifier that is unlinked to *PDR9*, but brings about resistance in a *pdr9-1*, *pdr5*::Tn*5* double mutant, the ratio of 2:2 *vs.* 1:3 would be almost 1:1 rather than 1:4. The number of 0:4 segregants should be quite low (1/36). Our observations are not in complete agreement with such a model because the proportion of tetrads showing a 2:2 segregation is significantly higher than expected (about 5:1). An alternate explanation which fits the data better posits that the original D1-3-8D strain actually contained two linked *pdr* mutations: one *PDR5* dependent and one *PDR5* independent. In the construction of JG396-12A, the former remained, while the latter recombined out. We would therefore define *pdr9-1* as the mutation present in JG396-12A which confers *PDR5* dependent resistance resulting from overexpression of *PDR5* transcript.

Clustering of *pdr* genes: The relatively tight linkage of four genes on chromosome *II* suggests that these sequences might have arisen from a single gene via duplication. It will be interesting to examine the DNA and protein sequences of these genes once they are cloned. Finally, it is important to bear in mind that the normal function of these putative *PDR* regu-

latory genes in a yeast cell is unknown. It may be that the wild type equivalents of these genes are normally regulators of other cellular functions. The drug resistance provides a useful platform from which to manipulate and identify these genes, but it may not be the physiological role of this group of proteins. Nevertheless, from the standpoint of a clinician, mutations in any of these genes could lead to resistance and failure of chemotherapeutic regimes.

Strains with more than one drug resistance factor: In the course of this study, we observed that several strains segregated up to three different drug resistance genes. BJ6673 yielded no less than three different unlinked determinants: *pdr3-1*, *cyh*^{r} and *oli*^{r}. Similarly, the original *pdr9-1* strain (D1-3-8D) has at least two (*pdr9-1* and *oli*^{r}) and perhaps a third (tightly linked to *pdr9-1*–see above). This second example is particularly intriguing because it is known that the other isogenic strains (D1-3, D1-3-4A and D1-3-16B) do not contain these factors. Several explanations could account for these observations. Since the frequency of second site mutation seems high, we considered the possibility that because of enhanced efflux of substances involved in detoxification, *pdr* strains have a mutator phenotype. Our preliminary results indicate no differences in the rate of mutation to either canavanine or oligomycin resistance. Alternatively, for other reasons that are not clear, mutation to resistance in one gene may cause or select for resistance mutations in other loci over a long period.

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