The Central Role of PDR1 in the Foundation of Yeast Drug Resistance*§

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The widespread pleiotropic drug resistance (PDR) phenomenon is well described as the long term selection of genetic variants expressing constitutively high levels of membrane transporters involved in drug efflux. However, the transcriptional cascades leading to the PDR phenotype in wild-type cells are largely unknown, and the first steps of this phenomenon are poorly understood. We investigated the transcriptional mechanisms underlying the establishment of an efficient PDR response in budding yeast. We show that within a few minutes of drug sensing yeast elicits an effective PDR response, involving tens of PDR genes. This early PDR response (ePDR) is highly dependent on the Pdr1p transcription factor, which is also one of the major genetic determinants of long term PDR acquisition. The activity of Pdr1p in early drug response is not drug-specific, as two chemically unrelated drugs, benomyl and fluphenazine, elicit identical, Pdr1p-dependent, ePDR patterns. Our data also demonstrate that Pdr1p is an original stress response factor, the DNA binding properties of which do not depend on the presence of drugs. Thus, Pdr1p is a promoter-resident regulator involved in both basal expression and rapid drug-dependent induction of PDR genes.

All living organisms have developed complex transcriptional responses for rapidly adapting genome expression to the presence of toxic compounds in the environment. These responses involve various types of cellular pathway. Genome-wide studies of drug responses in microorganisms have revealed that these responses comprise both specific effects depending on the precise chemical nature and cellular targets of the toxic compound and a general stress response (environmental stress response (ESR)) in the yeast Saccharomyces cerevisiae, reflecting cell adaptation to growth defects and cellular damages, regardless of the type of stress encountered by the cell (1). In between these very specific and very general responses, prokaryotic and eukaryotic cells have evolved multidrug resistance (MDR) pathways, which confer resistance to a broad spectrum of unrelated chemicals, but which are restricted to the stress responses associated with organic drugs. From bacteria to humans, MDR is essentially based on the overexpression of membrane transporters able to export a large number of chemically different compounds (2–4). MDR is a major concern for human health, as it leads to antibiotic resistance in pathogens and enables cancer cells to survive chemotherapy.

In the model yeast S. cerevisiae, MDR is referred to as PDR (pleiotropic drug response). The PDR network currently comprises 10 transcription factors regulating about 70 different target genes (Refs. 5–17; reviewed in Ref. 18). In this network, the Pdr1p transcription factor has the largest set of potential targets (about 50). Pdr1p and its functional homologue, Pdr3p, were identified in the early 1990s as regulators of the basal level of drug resistance in yeast cells (19, 20). Gain- or loss-of-function alleles of PDR1 and PDR3 confer resistance or sensitivity to a large panel of unrelated drugs, through constitutive modifications to the expression of ATP binding cassette transporters (e.g. Pdr5p, Snq2p, or Yor1p); major facilitator superfamily members (e.g. Flr1p, Tpo1p) or enzymes modifying the lipid composition of the plasma membrane (e.g. Pdr16p, Rsb1p, Lpt1p) (reviewed in Ref. 21). PDR1 and PDR3 display some functional redundancy, as the inactivation of both genes is required to have any major effect on the steady-state level of expression of their target genes, and Pdr1p and Pdr3p recognize the same DNA consensus motif (PDRE for pleiotropic drug response element) (16, 22). Pdr1p has been shown to modulate the in vivo expression and to bind in vitro to the promoters of genes encoding two other PDR regulators, namely PDR3 and YRR1, which are positively autoregulated (11, 16). Based on these findings, Pdr1p has been hypothesized to be at the top of a positive regulation loop leading to the overexpression of PDR effectors.

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5 The abbreviations used are: ESR, environmental stress response; MDR, multidrug resistance; PDR, pleiotropic drug resistance; ePDR, early pleiotropic drug response; PDRE, pleiotropic drug response element; STRE, stress response element; CORE, calcineurin-dependent response element; RT-qPCR, reverse transcription followed by quantitative polymerase chain reaction; ChIP-chip, chromatin immunoprecipitation followed by DNA chip analysis; IP, immunoprecipitation; WCE, whole cell extract; FDR, false discovery rate; ORF, open reading frame.

6 Recipients of Ph.D. grants from the MENRT.

This work was supported in part by grants from ARC (ARC3310) and CNRS (ACIIMPBIO 2004-45). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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upon drug treatment (16). However, although the role of Pdr1p in maintaining basal levels of yeast multidrug resistance has been clearly established, its role in the drug-mediated regulation of PDR genes remains unclear. Pdr1p is required for the PDR5 response to cycloheximide, a process which seems to require the auto-activation of PDR3 (16). The induction of PDR5 and TPO1 (encoding a MDR permease) expression in response to herbicides (methylene-chlorophenoxyacetic acid and dichlorophenoxyacetic acid) is much weaker in the absence of either Pdr1p or Pdr3p (10). However, Wehrschutz et al. (23) have shown that the deletion of both PDR1 and PDR3, despite strongly reducing PDR5 basal expression, does not abolish the induction of this gene upon cycloheximide or diazaborine treatment. Similar results have been reported for the ABC transporter Snq2p, the expression of which is slightly up-regulated by 4-nitroquinoline (4-NQO) independent of PDR1 and PDR3 (24).

Most of what we know about the PDR network has emerged from experiments carried out in the absence of drugs using gain-of-function or knock-out versions of the PDR transcription factors. Therefore, very little is known about the dynamic functionality of the PDR network in general, and the role of the Pdr1p transcription factor in PDR acquisition. The physiological relevance of the PDR network also remains to be investigated on a genome-wide scale. We carried out a genome-wide study of the role of Pdr1p in the early dynamic response of the yeast transcriptome to fluphenazine. We combined the data obtained with the results of a previous microarray analysis of the yeast response to benomyl, an inhibitor of microtubule assembly, carried out in our laboratory (25). Fluphenazine belongs to the phenotiazine family of antipsychotic drugs. Phenotiazines are calmodulin antagonists, as they bind to calmodulin in a Ca2+-dependent manner and alter its capacity to activate its cellular targets (26). We chose fluphenazine because: 1) It efficiently up-regulates the transcription of CDR1 and CDR2, the functional homologues of PDR5 and SNQ2 in the pathogenic yeast Candida albicans (27). 2) It differs from benomyl, in terms of both its chemical structure and its cellular targets. 3) Calcineurin, one of the main targets of calmodulin, modulates drug susceptibility in S. cerevisiae (28) and is important for PDR activation in C. albicans (29). We found that Pdr1p actually played a major role in the full fluphenazine induction of the expression of many PDR genes. The global fluphenazine and benomyl responses clearly differed, but the sets of Pdr1p-dependent genes induced by the two drugs were strikingly similar. The induction of this set of genes may thus represent the physiological early pleiotropic drug response (ePDR). Genome-wide chromatin immunoprecipitation experiments in the absence and presence of fluphenazine showed that Pdr1p was constitutively bound to its target DNA, even in the absence of drugs. Thus, Pdr1p is a promoter-resident protein involved in both the basal expression and response to drugs of PDR genes. This study provides a genome-wide view of dynamic Pdr1p functioning in the early drug response in yeast.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—All the strains used in this study were derived from the BY4742 (MATa; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0) background. The wild-type, pdr1Δ, pdr3Δ, pdr5Δ, yrr1Δ, ymr11Δ, elm1Δ, and ery1Δa strains were ordered from the Euroscarf collection. The pdr1Δpdr3Δ strain was constructed using the pdr3Δ strain, from which PDR1 was deleted with pAD-ΔPDR1 (30). Briefly, pAD-ΔPDR1 was digested with EcoRI and NotI. This generated a fragment including the URA marker flanked by sequences from the PDR1 promoter and terminator regions, respectively. This fragment was inserted in place of the wild-type PDR1 sequence, by homologous recombination, using a standard yeast transformation protocol (31). The deletion was controlled by PCR using forward primer (GCTATTCACTTCTCAGCCAAG) and reverse primer (AGGAGATCGCCCTAGAAAAACAG). Thirteen Myc tags were added to the part of the chromosomal PDR1 corresponding to the C terminus of Pdr1p as described in Ref. 32. The primers used were: GGAAGGAAGTTTTTGAGAATTTTATCTAT-TAAACACGTATACGTTACCAGCTCGTTTAAAC and GGACCTCTACAGTATCTCGTGATGCCGACGTTTTATCCAGATAGTCCGATCCCCCGGTTAATTAA. This PCR fragment was inserted into the yeast genome by homologous recombination, using a standard lithium acetate yeast transformation protocol (33). We checked that Myc-tagged proteins were correctly produced by Western blotting with the 9E10 antibody (anti-Myc mouse monoclonal antibody, Roche Applied Science).

**Growth Conditions and Drug Treatments**—For time course analysis of the fluphenazine response, strains were cultured in YPD (1%(w/v) bacto-yeast extract, 2% (w/v) bacto-peptone, 2% (w/v) glucose) up to an A600 of 0.5. The culture was split in two. Fluphenazine (Sigma) was dissolved in water to a concentration of 10 mg/ml (stock solution). Fluphenazine was added to one of the two cultures, to the concentration of 40 μg/ml. An equivalent volume of water was added to the other culture. Cells were incubating for various periods of time (2, 5, 10, 20, 40, 80 min) and were then harvested by pouring 15 ml of culture into 30 ml of ethanol, storing at −80 °C, and centrifuging.

**Transcriptome Analyses**—Microarrays containing oligonucleotides for probing most of the ORF of S. cerevisiae were produced by local microarray facilities. These microarrays contained 40-mer oligonucleotides from MWG deposited onto Corning ultragap slides, using an Omnimag II spotter from Biorobotics (Genomic Solutions), with each gene represented twice on the array. The protocol for these microarray experiments can be obtained from the authors. Each experiment was performed at least twice, with independent biological samples, using dye swap. The arrays were read with a Genepix 4000B scanner and analyzed with Genepix Pro 4.0 (Axon Instruments Inc.). Artificial, saturated or low-signal spots were excluded from the analysis. Print-tip lowess normalization was carried out using the Varan software with default parameters (34). The expression profiles of genes with more than 80% missing values were eliminated. The remaining missing values were replaced using the KNN-imputation method, available from GE-PAS (35). Gene expression profiles were clustered using Genesis (36).

**RT-qPCR Analyses**—RT-qPCR were performed as described in Ref. 37. The primers were: ICT1, ACCAGCTAACGTTTGAGG and ACCCTGATTGCTGTGGTGC;
YOR1, CGAAATGCACCTCCAGAGTC and GTG GACTT- ACCAGCACC TTGAC; SNQ2, CTGTGGTTA CAACTACGTTA C; ACT1, GGTGTCGCTT TGATTGATG and GACCCATACCA CCACTGATC.

Chromatin Immunoprecipitation on DNA Arrays (ChIP-chip)—Overnight cultures of tagged or wild-type yeasts were used to inoculate 100 ml of fresh YPD medium at 0.1 A600. Cells were grown to an A600 of 0.6–0.8. One 50-ml subculture was then treated with 40 μg/ml fluphenazine for 10 min, and the other 50 ml was incubated with an equivalent amount of water. Each culture was fixed by adding 1% formaldehyde for 15 min at room temperature. Fixation was stopped by adding 340 mM glycine and incubating for 5 min. Fixed cells were harvested by centrifugation and washed twice with TBS. ChIP extracts, IP, ligation-mediated PCR, labeling, slide preparation, and hybridization were carried out as described in Ref. 37. For qPCR analysis of specific promoters, we used 15% of each sample as described in Ref. 38. For ChIP-chip analysis, we used arrays previously described in Ref. 38, encompassing 14,178 PCR products for all yeast genomic elements. Immunoprecipitated DNA from tagged or wild-type cells was used for competitive hybridization against Whole Cell Extract (WCE) from the same culture. Each experiment was repeated at least four times, using dye swap. Arrays were scanned using a GenePix 4100A scanner and fluorescence ratios were determined with GenePix Pro 5.1. Artifactual spots were excluded and local background was subtracted from the intensity of the signal. R-software was used for further analyses. The signals were normalized using the print-tip median. Log2(IP/WCE) was calculated for each DNA sequence. The median log2 value for the four experiments was used to reflect the relative enrichment in the immunoprecipitate, for a given locus. Only loci with a significant signal in at least 3 of 4 experiments were considered. For each sequence, a Z-score, corresponding to the enrichment value after centering and scaling the global distribution of all log2 enrichment values (Z = (E-μ)/S, where E is the median log2 value of the enrichment of the sequence, μ is the median value, and S the standard deviation for all enrichments) was calculated. The IP was considered to be significantly enriched in DNA sequences with a Z-score of more than 3. Genes with a Z score above 3 in one of the conditions tested but an enrichment ratio in the IP lower than the enrichment ratio in the mock IP plus 2.5 standard deviations were considered to be nonspecifically bound by Pdr1p.

Microarray Data Download—All microarray data and supplemental materials are publicly available (see Supplementary Materials).

RESULTS

Time Course Analyses of the Fluphenazine Response in Yeast

The early fluphenazine response of yeast cells was analyzed, using a dose of fluphenazine with negligible impact on growth that nonetheless induced a clear transcriputional response. Preliminary studies indicated that the 40 μg/ml dose was the most appropriate for our purposes (data not shown). This dose is similar to that used in previous studies to induce MDR transporters in the pathogenic yeast C. albicans (27). As we were interested in studying the very early drug response rather than the long term adaptation of the cells, we analyzed the yeast transcriptome response after 2, 5, 10, 20, 40, and 80 min of exposure to fluphenazine, comparing this response with that of cells incubated with water for the same amount of time (mock treatment). We identified 160 genes with expression profiles clearly modified by fluphenazine addition. Hierarchical clustering analyses of these genes indicated that the response of yeast to 40 μg/ml of fluphenazine was transient, peaking at 10 min under our conditions. Repressed genes displayed similar, transient, and progressive changes in expression, whereas gene activation patterns were more heterogeneous, ranging from short, early pulses to late, progressive modifications (Fig. 1, left panel).

Fluphenazine Activates Msn2p/Msn4p-responsive Genes, a crz1p-dependent Calcium Response and Part of the PDR Network

Bioinformatic Analyses of the Fluphenazine Response—We used the T-profile software to investigate the functional significance of these expression profiles (39). We found that six different regulatory DNA motifs were predicted to play a role in the early fluphenazine response (Fig. 2). Three of these motifs, rRPE, rRNA, and PAC, were associated with transient repression. The rRPE and rRNA motifs have been identified in silico in the promoters of genes involved in rRNA processing (40, 41). The PAC motif is derived from the rRNA motif and has been identified in silico analyses of the promoters of ESR genes, the expression of which is repressed under stress conditions (1, 39). Genes containing the STRE motifs, corresponding to the DNA binding sequence of Msn2p/Msn4p, were induced very early and transiently (maximum t-value at 5 min) in our experiments. Msn2p/Msn4p are known to control the induction of stress-responsive genes involved in the ESR (1). Finally, the last two groups of motifs were associated with the induction of a second wave of genes (20–80 min). The first of these two DNA motifs has been identified in the promoter sequences of target genes of the Crz1p transcription factor in in silico analyses (39). Crz1p is known to bind to the CDRE (calcineurin-dependent response element) and is thought to be the major transcription factor target of the calmodulin-dependent phosphatase, calcineurin (42, 43). The final group of motifs are variations of the PDRE, which is recognized by the Pdr1p/Pdr3p transcription factors (5). This last result suggests that the pleiotropic drug response is an important component of the early response to fluphenazine.

Four Main Functional Groups Are Involved in the Fluphenazine Response—These bioinformatic predictions were fully confirmed investigating the functional annotation of the 160
Transcription Regulation of Yeast Early Drug Response

FIGURE 1. The early fluphenazine response of the yeast transcriptome. Left panel, hierarchical clustering of the genes with levels of expression changing more than 1.6-fold for at least two consecutive times. Right panel, genes annotated as belonging to the general stress response, the calcium response, the PDR network, or the rRNA biogenesis pathways have been extracted from the general cluster. The description of each gene is taken from the SGD. The presence in the promoters of these genes of a STRE (pink), a potential Crz1p regulation motif (AGCCWC; purple), a PDRE (yellow), or a rRPE (green) is indicated by dots. The log2 ratio values for each gene and each time point are available in supplemental Table S1.
Transcription Regulation of Yeast Early Drug Response

FIGURE 2. Six transcriptional regulatory motifs make a significant contribution to the early fluphenazine response. Results of the T-profiler search for DNA motifs significantly induced or repressed by fluphenazine treatment. Only motifs for which a significant change (E-value < 0.05 in T-profiler) was observed over at least two consecutive time points are represented. If several motifs were associated with the same transcription factor, the median t-value of all motifs was used. STRE is the binding motif for Msn2p/Msn4p, PDRE is the binding motif for Pdr1p/Pdr3p. The motif annotated as PDRE is the motif annotated as STRE in their promoters. Many of these genes encode proteins of the carbohydrate and energy reserve metabolism pathways, e.g., the glycogen phosphorylase (GPH1), the glycogen synthase (GSY2), the trehalose 6-P phosphate synthase subunits (TSI1, TPS2, and TPS3), the hexokinase (HXK1), the glucokinase (GLK1), and the glucose transporter (HXT6/7, the glyceral phosphatases (GPD1 and HXR2). Some genes encoding chaperones, including cytosolic (Hsp42p, Hsp104p) and mitochondrial (Hsp78p) stress proteins, were also induced by fluphenazine. Most of these genes were induced early and transiently (Fig. 1, right panel).

25% of genes induced by fluphenazine in Fig. 1 are known to be dependent on calcineurin and the major calcineurin-dependent transcription factor, Crz1p for their stress-mediated expression (43, 44). These genes encode proteins involved in vacuole morphogenesis and protein maturation (e.g., the peptidases [PEP4, CPS1, and YPS1; the mannosyl transferase [MNT4]), a calmodulin-dependent protein kinase (CMK2p) and Tis11p, involved in the regulation of mRNA stability. Most of these genes were induced after the Msn2/Msn4 response (10–40 min), with the notable exception of CMK2, which was induced as early as 5 min after fluphenazine treatment. Thus, in contrast to what had been observed in calcineurin mutants or in cells treated with the calcineurin inhibitor FK506 (43), the treatment of yeast with fluphenazine led to activation of the crz1p/calcineurin-dependent pathway of transcription regulation.

Finally, we found that 15 genes encoding proteins of the pleiotropic drug response pathway were induced by fluphenazine (Fig. 1). These genes encode multidrug transporters (PDR5, YOR1, SNQ2), proteins involved in lipid metabolism and membrane properties (RBP1, SNQ1, PLM1) or the stress response (ICT1, YLR346c, YGP1, GRE2, HXK1). Some of these genes (RBP1, YLR346c, HXK1) were already induced at 5 min and their level of expression subsequently decreased; some (ICT1, SNQ2, PDR5, YOR1, SNQ1, YGP1, GRE2, YAL061w) were induced from 10 min and were still significantly overexpressed at 80 min. Among the 15 PDR genes activated, 13 are potential targets of Pdr1p and/or Pdr3p (Fig. 1). The remaining two genes (PLB1 and SNQ1) are potential targets of Yrr1p, Pdr8p, and Yr1p (7, 8, 15). In addition, two genes encoding a ceramide synthase (IPT1) and a polyamine transporter (TP1), which were induced only at 10 min and are therefore not shown on Fig. 1, could also be included in this list of fluphenazine-dependent PDR-responsive genes (see complete microarray data, Supplementary Materials). A significant PDR response therefore occurred very rapidly after drug exposure.

PDR1 Contributes to the Early Fluphenazine Response

We investigated the contribution of Pdr1p to the early fluphenazine response, by analyzing changes in genome expression in pdr1Δ cells, 2, 5, 10, 20, 40, and 80 min after fluphenazine treatment. The impact of PDR1 deletion on the early fluphenazine response was assessed quantitatively, by comparing time course gene expression profiles in the wild-type and knock-out strains using a specific cluster method as described in Ref. 25. Briefly, this method resulted in the hierarchical clustering of gene groups, from the most affected genes to genes insensitive to PDR1 deletion. PDR1 deletion had few visible effects on gene expression in the absence of drugs (data not shown). However, 9 genes displayed markedly lower levels of activation in the presence of fluphenazine in the strain with the deletion than in the wild-type (Fig. 3A). Seven of these genes (YOR1, SNQ2, RBP1, YAL061w, YLR346c, ICT1, GRE2) are potential Pdr1p targets and contain a PDRE in their promoter (Fig. 3A). Only two genes (YPR027c and RTN2), have not previously been identified as PDR1-dependent, contain no PDRE in their promoter and encode proteins of unknown function. The contribution of Pdr1p to the fluphenazine response therefore seems to be limited to the regulation of PDR genes. Surprisingly, PDR5, one of the main Pdr1p targets, was found in a second group of genes, which exhibited few or no dependence on Pdr1p (supplemental Fig. S3). PDR5 expression induction was actually slightly reduced and delayed in the pdr1Δ strain. The significance of this slight defect in PDR5 induction was confirmed by quantitative RT-qPCR and by direct competitive microarray hybridization of wild-type and pdr1Δ RNAs from
cells exposed to fluphenazine for 5 and 10 min (data not shown).

Pdr1p is a major regulator of the fluphenazine-mediated PDR response. We investigated the requirement for other known PDR regulators in the fluphenazine response, by carrying out similar experiments with strains in which the PDR3, PDR8, YRM1, or YRR1 genes had been deleted. None of these strains differed significantly from the wild-type in terms of PDR gene induction by fluphenazine (data not shown). Thus, Pdr1p was the only PDR regulator tested that was absolutely required for a full fluphenazine response, over the time period considered. The induction of the PDR genes and of the calcium-responsive genes was simultaneous (Fig. 1, right panels). Little overlap between the known targets of Crz1p and Pdr1p has been identified (only ICT1 and YGP1 belong to these two groups). However, there may be cross-talk between PDR and calcium pathways in response to fluphenazine. Indeed, strains defective for calcineurin are particularly sensitive to antifungalazole derivatives, suggesting a possible role for Crz1p in drug resistance (28). The fluphenazine-mediated induction of PDR5 was only slightly dependent on Pdr1p (supplemental Fig. S3), and the product of this gene plays a role in calcium homeostasis (45). It is therefore tempting to speculate that PDR5 induction in pdr1A strain was, at least partly, sustained by Crz1p. We therefore carried out kinetic analyses of the fluphenazine response in strains deleted for CRZ1. We identified 6 groups of genes affected by CRZ1 deletion in the presence of fluphenazine (Fig. 3B). All but one of the calcium-responsive genes induced by fluphenazine (Fig. 1) were affected by CRZ1 deletion (Fig. 3B). Crz1p seemed to control a larger set of genes than Pdr1p in response to fluphenazine, but no Pdr1p-dependent gene was identified among the Crz1p-dependent genes. These results confirm that Crz1p is a major regulator of calcium-dependent transcriptional regulation pathways and that fluphenazine treatment stimulates the Crz1p pathway of transcriptional regulation, as predicted by T-profiler. However, Crz1p does not make a significant contribution to the induction of PDR genes, including PDR5, in response to fluphenazine. Similarly, Karababa et al. (46) demonstrated that the C. albicans Crz1p homologue is not involved in the regulation of drug resistance in this pathogenic yeast.

**Pdr1p Constitutively Binds to Its Target Promoters in Vivo**

We analyzed the Pdr1p DNA binding pattern in presence and absence of fluphenazine on a genome-wide scale, to identify genes directly regulated by Pdr1p in response to fluphenazine. We constructed a chromosomal C-terminal Myc-tagged version of Pdr1p and performed Pdr1p-Myc chromatin immunoprecipitation experiments coupled with DNA microarray identification of bound DNA fragments (ChIP-chip), 10 min after fluphenazine or mock treatment. We checked that Pdr1p-Myc was active and produced correctly by Western blotting, phenotypic analyses of cycloheximide resistance, and transcriptome analyses of the fluphenazine response of cells expressing Pdr1p-Myc (data not shown). Each ChIP-chip experiment, with or without fluphenazine, was reproduced four times, comparing immunoprecipitated DNA (IP) with input DNA (whole cell extract or WCE). The median log2 value of the

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**FIGURE 3.** *PDR1* is essential for the early PDR response. The genes were clustered according to differences in their fluphenazine expression profile in a strain in which *PDR1* (A) or *CRZ1* (B) was deleted and the wild-type strain. Only the groups of genes clearly affected by the deletions are shown. The functional annotation (yellow and purple dots) corresponds to the annotation used in Fig. 1. The complete clusters, with all genes, can be found in supplemental Fig. S3.
enrichment ratio (IP/WCE), was calculated for each DNA sequence present on the slide (Fig. 4A). We assessed the significance of these enrichments, by assigning a Z-score (i.e. the distance in standard deviations of each enrichment from the mean of the global distribution) to each DNA sequence. We controlled for nonspecifically enriched DNA sequences, by carrying out four mock IP experiments with an untagged strain and estimating the mock IP enrichment for all sequences. Supplemental Table S4 lists the DNA sequences with a Z-score of more than 3 in at least one of the conditions tested (i.e. with or without fluphenazine) and with a significantly higher enrichment ratio in the Pdr1p-Myc than in the mock IP experiments. Table 1 presents a shorter list restricted to the Pdr1p potential targets which were found to be significantly bound using these criteria.

We used SAM (47) to identify significant differences in Pdr1p-DNA binding pattern between fluphenazine-treated and mock-treated cells. SAM provides an estimation of the false positive discovery rate (FDR), i.e. the number of genes likely to be found by chance as being differentially bound by Pdr1p in treated cells compared with nontreated controls. No significant difference (FDR < 5%) in the Pdr1p DNA binding patterns was found between fluphenazine-treated and mock-treated cells by this method (data not shown). Thus, the genome-wide location of Pdr1p was independent of the presence of drugs, in the conditions tested.

Pdr1p binding was observed with the promoter of most of the PDR genes which were dependent of PDR1 for their fluphenazine induction (Table 1). These genes included PDR5, SNQ2, RSB1, GRE2, YLR346c, YOR1 and ICT1, RTN2, YAL061w, and YPR027c, which were clearly PDR1-dependent in our experiments (Fig. 3A), show no significant binding. The effect of Pdr1p on the transcription of these genes may be indirect. The promoter sequences of VHR1 and TPO1, for which fluphenazine induction did not require PDR1, were bound by Pdr1p, indicating that Pdr1p may regulate the expression of these genes, while being dispensable for their fluphenazine induction. Finally, many of the DNA sequences that bound to Pdr1p were promoters of genes which were not.
induced by fluphenazine. These sequences included the potential Pdr1p target genes YGR035c, PDR16, PDR10, PDR15, YCR61w, IPT1, and YMR102c (Table 1). They also included the promoters of genes encoding two transcriptional regulators: Rpn4p, which controls proteasome subunit expression, and Pdr3p (Table 1). However, some genes identified as Pdr1p targets in gain-of-function studies were not bound by the wild-type Pdr1p in the conditions tested. These genes are mostly related to general stress responses, (YLL056c, YGP1, YDL061c, YLR061c, YJL216c, HXT9, HXT11). Also, the gene encoding the PDR transcriptional regulator, YRR1, which had been shown to be bound by Pdr1p in vitro (11), was not enriched in our experiments. Our genome-wide studies of Pdr1p binding properties showed that most of the PDRE present in the yeast genome were not bound by Pdr1p under the conditions tested (Fig. 4B). However, promoters containing PDRE were clearly overrepresented among the sequences bound by Pdr1p (Fig. 4B), confirming the importance of this motif for Pdr1p binding, as demonstrated both in vitro (48) and in vivo (49). Four subtypes of PDRE: A, B, C, and D have been defined (5). In our experiments, only type A and B bound Pdr1p (Table 1).

**The Pdr1p-dependent Response Is Not Fluphenazine-specific**

We assessed the specificity of the Pdr1p-dependent response to fluphenazine by comparing the results described above with the results obtained in a previous genome expression study of the response of yeast to benomyl (25). Benomyl is a standard antifungal drug from the benzimidazol family, which inhibits mitotic spindle biosynthesis. This drug therefore differs from fluphenazine, in terms of both its molecular structure and cellular targets. Kinetic transcriptome experiments have been carried out with benomyl, using experimental procedures and a time scale very similar to that described here. This made possible to compare the genes induced by the two drugs, and also to take into account the timing of these induction events. This analysis was conducted by comparing, at each time point, the induction ratio of the genes induced in fluphenazine and/or in benomyl. Fig. 5A shows the results obtained for the comparison at 10 min, corresponding to the peak in gene induction for both drugs. The induction of many genes was drug-specific. For instance, fluphenazine activated calcium-responsive genes in a characteristic manner (Fig. 5A, triangles). By contrast, benomyl strongly induced oxidative stress response genes (Fig. 5A, diamonds). Strikingly, about half of the genes which were induced similarly by both drugs at 10 min belonged to the group of genes defined as regulated by Pdr1p (Fig. 3A) or to the group of genes with promoters directly bound by Pdr1p (Table 1). These genes included PDR5, SNQ2, YOR1, TPO1, YLR346c, ICT1, and VHR1 (Fig. 5B). Thus, the early PDR response described in this work is not specific to fluphenazine and may represent the standard physiological Pdr1p response to various, unrelated drugs.

**Early PDR Response Is Partially Dependent on Elm1p Kinase**

The Elm1p kinase was recently reported to be an important modulator of the activity of Pdr1p gain-of-function mutants (50). We therefore used RT-qPCR to assess the effect of ELM1 deletion on the expression of ICT1, SNQ2, and YOR1, three genes highly dependent on Pdr1p for fluphenazine induction. We observed a significant decrease in the activation of the ICT1 and YOR1, but not in the case of SNQ2 (Fig. 6). These effects were smaller than 2-fold. Thus, ELM1 deletion does not affect

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**TABLE 1**

| Regulated gene | DNA features | Z-score no drug | Z-score fluphenazine | PDRE | Fluphenazine induction |
|---------------|--------------|-----------------|---------------------|------|------------------------|
| SNQ2          | YDR010C      | 15.5            | 12.2                | A, B, C | Yes                    |
|               | iYDR010C     | 13.7            | 11.4                |       |                        |
|               | YDR011W      | 2.8             | 4.8                 | A, B, B | Yes                    |
|               | iYOR1252C-1  | 12.6            | 10.9                |       |                        |
| PDR5/YOR152c | YOR153W      | 5.2             | 5.8                 | A, B, B | Yes                    |
| YGR035c/YGR035w-a | YGR035C  | 10.3            | 9.8                 | B, B   | No                     |
| YPR4          | YDL020C      | 3.1             | 2.8                 | B, B   | No                     |
|               | iYDR049C     | 7.7             | 5.9                 |       |                        |
| RSB1          | YO050C       | 10.2            | 7.1                 | A      | Yes                    |
|               | iYOR050C     | 8.6             | 5.9                 |       |                        |
|               | iYDR049C     | 3.3             | 3.6                 |       |                        |
| VHR1          | iS(UGA)      | 8.1             | 6.4                 | A      | Yes                    |
|               | YNL231C      | 7.9             | 6.5                 | A      | No                     |
|               | YNL230C      | 3.1             | 3.0                 |       |                        |
| TPO1          | YL029W       | 7.4             | 6.1                 | B      | Yes                    |
| PDR3/YBL006c | YBL006C      | 5.6             | 3.5                 | A, A   | No                     |
| YMR102c       | YMR103C      | 4.8             | 3.9                 | A, D   | Yes                    |
| ICT1/YLR099w-a | YLR099C  | 3.5             | 3.0                 | B      | Yes                    |
| PDR10/SNC2   | YO327C       | 4.6             | 3.7                 | B, B   | No                     |
| PDR15         | iYDR405W     | 4.2             | 2.6                 | A, D   | No                     |
| YOR1/PRX1    | iYGR280C     | 3.7             | 2.5                 | B      | Yes                    |
| YLR346c      | iYLR346C     | 3.3             | 2.7                 | A      | Yes                    |
| YCR061w      | iYCR060W     | 3.6             | 2.0                 | B      | No                     |
| GRE2/DCP1    | YOL151W      | 1.6             | 3.6                 | B      | Yes                    |

The DNA binding pattern of Pdr1p in the presence and absence of fluphenazine was analyzed using ChIP-chip. Table 1 lists the potential targets of Pdr1p with DNA sequences significantly (Z-score >3) and specifically (IP enrichment > mock IP enrichment + 2.5 standard deviations in mock IP measurements) bound by Pdr1p. The complete list of sequences satisfying these criteria is available in supplemental Table S4. The ChIP-chip microarray data can be downloaded from Footnote 9. The Saccharomyces Genome Database was used to identify the regulated genes from DNA sequences as follows: when intergenic regions (feature name beginning with an “i”) were enriched, the regulated genes were presumed to be the downstream ORFs. In cases of divergent promoters, both ORFs are considered as intergenic regions (e.g.: YDR010k was assimilated as part of the SNQ2 promoter). The names of genes previously shown to be regulated by Pdr1p are indicated in bold typeface. The type of PDRE present in the sequences is indicated according to Ref. 5. The sensitivity of gene expression to fluphenazine is indicated in the last column. The results obtained for the promoters of RPN4, PDR5, SNQ2, and YGR035c were checked by qPCR (supplemental Fig. S5).
the pleiotropic drug response the same way than the \textit{PDR1} deletion. Hence, Elm1p may play a minor role in the Pdr1p-dependent induction of PDR genes following exposure to fluphenazine.

**DISCUSSION**

\textit{The Yeast Early Pleiotropic Drug Response}—The primary aim of this study was to determine which of the genes involved in constitutive pleiotropic drug resistance are actually involved in the dynamic response of the yeast transcriptome to various, unrelated drugs. Using fluphenazine as a model, we observed rapid activation of a subset of 15 PDR genes after the addition of fluphenazine. Transcriptome analysis of the benomyl response carried out in almost identical experimental conditions (25) revealed that the timing of expression and the nature of the PDR genes induced by these two drugs were almost identical (Fig. 5). As benomyl and fluphenazine have different chemical structures and cellular targets, the set of genes defined in this study probably correspond to the basis of a generic PDR response. This ePDR includes the up-regulation of \textit{PDR5}, \textit{SNQ2}, \textit{YOR1}, \textit{TPO1}, \textit{YLR346c}, \textit{VHR1}, and \textit{ICT1}. It could potentially be adapted to many different toxic conditions, as it involves four transporters for broad, complementary, but overlapping drug spectra (51, 52). This ePDR is induced as rapidly as
the fluphenazine- and benomyl-specific responses, *i.e.* Crz1p and Yap1p pathways, respectively (Fig. 1 and Ref. 25), indicating that the mechanisms triggering ePDR upon drug sensing are very efficient.

**A Dual Role for Pdr1p in Basal Gene Expression and ePDR**—
Pdr1p was one of the first transcriptional regulators of pleitropic drug resistance to be identified in eukaryotes (19). However, only two genes, encoding the ATPase Pdr5p and the polyamine transporter Tpo1p, have been shown to depend on Pdr1p for their induction in the presence of drugs (10, 16). The results obtained in this study (Fig. 3A) and in Ref. 25 clearly show that the ePDR response is altered in the absence of PDR1. PDR1 was the only one of the PDR regulators that were tested, for which gene deletion had a significant effect on ePDR. Thus Pdr1p plays two roles, one in the basal expression of PDR genes and the other in ePDR induction. These two activities differ as: 1) some genes that depend on Pdr1p for basal expression are not induced by ePDR and 2) the role of Pdr1p in basal expression can be almost fully complemented by Pdr3p, whereas a complete ePDR specifically requires Pdr1p. Basal PDR expression and ePDR may therefore involve different mechanisms.

**Pdr1p Is a Promoter-resident, Drug Response Factor**—The ePDR involves only a limited subset of the Pdr1p targets identified to date (Fig. 3A, and Refs. 5 and 6). We investigated whether Pdr1p displayed promoter selection with modulation of its DNA binding properties, as reported for other transcription factors in yeast (*e.g.* Ref. 53). Pdr1p has been shown to be a constitutive nuclear protein (54). However, its DNA binding properties in vivo are largely unknown. Pdr1p binds in vitro to the PDRE (\(T(C/G)CG(C/T)GG(A/G)\)). The physiological function of this motif for basal Pdr1p-dependent regulation has been demonstrated by mutational analyses in vivo (*e.g.* Refs. 9, 16, 20, 22, 48). We used ChIP-chip to analyze the genome-wide DNA-binding pattern of Pdr1p, in both the presence and in the absence of drug. Our data demonstrate that, as previously suggested (5, 6), the PDRE is a major determinant for Pdr1p regu-
Promoters of genes involved in ePDR (proteasome activity (55)). Similarly, Pdr1p binds in vivo to the promoters of genes involved in ePDR (e.g. PDR5, SNQ2, YOR1, TPO1), encoding the functional counterpart of Pdr1p, Pdr3p, and other potential targets of Pdr1p (e.g. PDR10, PDR15, etc.) (Table 1). Following the submission of this article, a similar Pdr1p DNA binding pattern was described in another study (56), providing further support to our observations. Strikingly, we found that the DNA binding pattern of Pdr1p was largely independent of the presence of drugs. This key result is consistent with previous observations that cycloheximide modifies neither Pdr1p binding nor mediator subunit recruitment to the PDR5 promoter (57). The functioning of Pdr1p therefore differs from that of the other transcription factors implicated in the early fluphenazine and benomyl responses (Fig. 2 and Ref. 25). Indeed, Msn2p, Crz1p, and Yap1p are all regulated at the level of nucleocytoplasmic translocation and bind to their target promoters only under stress conditions (58–60).

Pdr1p DNA Binding Properties Do Not Dictate the Specific Activation of ePDR Genes—The constitutive binding of Pdr1p to its target promoters, independently of the presence of drugs, indicates that Pdr1p DNA binding properties alone cannot account for the selection of Pdr1p targets involved in ePDR. This situation is similar to that observed when a subset of Yap1p target genes is activated following the addition of benomyl (25). Benomyl triggers the translocation of Yap1p into the nucleus, but the promoter binding properties of Yap1p were not limited to the set of activated genes. Promoter binding therefore seems to be necessary, but not sufficient for the activation of gene expression. This suggests that Pdr1p, as a resident protein involved in the basal expression of ePDR genes, may play a particular role in facilitating the activation of these genes by drugs, through other as yet unidentified factors conveying a specific signal related to the chemical nature of the drug and, probably, the chromatin structure of the relevant promoters. Different sources of oxidative stress are known to trigger different modifications to Yap1p, potentially resulting in different transactivation and/or protein-protein interaction properties (61). Pdr1p has been shown to be phosphorylated in vivo and to contain several inhibitory motifs (49, 62). We have shown that the Eml1p kinase, which is known to affect Pdr1p gain-of-function activity (50), plays only a limited role in ePDR. Transduction pathways involved in Pdr1p activation in the presence of drugs remain unknown.

The Early Drug Response Results from a Combination of Transcriptional Inputs—The ePDR response combines with other early transcriptional responses to delineate a transcriptional drug-specific response. Indeed, PDR1 deletion strongly reduces the induction of PDR5 by benomyl (25), whereas it has a rather limited effect on the activation of this gene by fluphenazine, diazaborine, and cycloheximide (Fig. 3A and Refs. 16 and 23). The opposite is true for SNQ2, which is more strongly affected by PDR1 deletion in the presence of fluphenazine than in the presence of benomyl (Fig. 3A and Ref. 25). The robust induction of PDR5 in presence of a calmodulin inhibitor may be connected to the role played by Pdr5p in calcium homeostasis (45). By contrast, the SNQ2 expression is less sensitive to PDR1 deletion in the presence of benomyl, because of Yap1p (25). Fluphenazine does not trigger the relocation of Yap1p to its target promoters, confirming that different transcriptional regulators are required for ePDR to fluphenazine and benomyl. All these observations suggest that a drug is sensed as a combination of chemical properties (e.g. redox properties, hydrophobicity, etc.) and cellular targets (e.g. calcium-binding proteins in the case of fluphenazine), converted into transcriptional inputs (e.g. Yap1p for oxidation). These different transcriptional inputs combine, at the promoter of target genes, to delineate a drug-specific response from a rather limited set of general transcriptional pathways (Fig. 7). This suggests that fluphenazine, benomyl, and other drugs stimulating ePDR have certain properties in common, which are recognized by unknown factors and lead to activation of the same set of Pdr1p-dependent genes. These common properties and the upstream signals triggering ePDR remain completely unknown. The identification of a putative common signal triggering ePDR in the presence of various, apparently unrelated drugs, would be extremely useful to control MDR in pathogenic yeasts.

Acknowledgments—We thank M. Werner, M. Kabani, O. Haris-mendy, and V. Borde for technical assistance with ChIP-chip, X. Gidrol, F. Amyot, and the SGE/CEA staff who made the intergenic yeast arrays, L. Amirneni and M. Longtime for Myc tag plasmids, and D. Sanglard for communicating data before publication. Plate-forme transcriptioniste IFR36 is funded by the RGN.

REFERENCES
1. Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000) Mol. Biol. Cell 11, 4241–4257
2. Borst, P., and Elferink, R. O. (2002) Annu. Rev. Biochem. 71, 537–592
3. Li, X. Z., and Nikaido, H. (2004) Drugs 64, 159–204
4. Prasad, R., and Kapoor, K. (2005) Int. Rev. Cytol. 242, 215–248
5. DeRisi, J., van den Hazel, B., Marc, P., Balzi, E., Brown, P., Jacq, C., and Goffeau, A. (2000) FEBS Lett. 470, 156–160
6. Devaux, F., Marc, P., Bouchoux, C., Delaveau, T., Hikkel, I., Potier, M. C., and Jacq, C. (2001) EMBO Rep. 2, 493–498
7. Hikkel, I., Lucau-Danila, A., Delaveau, T., Marc, P., Devaux, F., and Jacq, C. (2003) J. Biol. Chem. 278, 11427–11432
8. Le Crom, S., Devaux, F., Marc, P., Zhang, X., Moye-Rowley, W. S., and Jacq, C. (2002) Mol. Cell. Biol. 22, 2642–2649
9. Kolaczkowska, M., Kolaczkowska, A., Gaigg, B., Schneider, R., and Moye-Rowley, W. S. (2004) Eukaryot. Cell 3, 880–892
10. Teixeira, M. C., and Sa-Correia, I. (2002) Biochem. Biophys. Res. Commun. 292, 530–537
11. Zhang, X., Cui, Z., Miyakawa, T., and Moye-Rowley, W. S. (2001) J. Biol. Chem. 276, 8812–8819
12. Akache, B., and Turcotte, B. (2002) J. Biol. Chem. 277, 21254–21260
13. Hellauer, K., Akache, B., MacPherson, S., Sirard, E., and Turcotte, B. (2002) J. Biol. Chem. 277, 17671–17676
14. Devaux, F., Carvajal, E., Moye-Rowley, S., and Jacq, C. (2002) FEBS Lett. 515, 25–28
15. Lucau-Danila, A., Delaveau, T., Lelandais, G., Devaux, F., and Jacq, C.

6 V. Farudeau, unpublished results.
