New Regulators of Drug Sensitivity in the Family of Yeast Zinc Cluster Proteins*

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The Gal4p family of yeast zinc cluster proteins comprises over 50 members that are putative transcriptional regulators. For example, Pdr1p and Pdr3p activate multidrug resistance genes by binding to pleiotropic drug response elements (PDREs) found in promoters of target genes such as PDR5, encoding a drug efflux pump involved in resistance to cycloheximide. However, the role of many zinc cluster proteins is unknown. We tested a panel of strains carrying deletions of zinc cluster genes in the presence of various drugs. One deletion strain (Δdr1) was resistant to cycloheximide, whereas eight strains showed sensitivity to the antifungal ketoconazole or cycloheximide.Unnamed zinc cluster genes identified in our screen were called RDS for regulators of drug sensitivity. RNA levels of multidrug resistance genes such as PDR16, SNQ2, and PDR5 were decreased in many deletion strains. For example, cycloheximide sensitivity of a Δstb5 strain was correlated with decreased RNA levels and promoter activity of the PDR5 gene. We tested if activation of PDR5 is mediated via a PDRE by inserting this DNA element in front of a minimal promoter linked to the lacZ gene. Strikingly, activity of the reporter was decreased in a Δstb5 strain. The purified DNA binding domain of Stb5p bound to a PDRE in vitro. Mutations in the PDRE known to affect binding of Pdr1p/Pdr3p showed similar effects when assayed with Stb5p. These results strongly suggest that Stb5p is a transcriptional activator of multidrug resistance genes. Thus, we have identified new regulators of drug sensitivity in the family of zinc cluster proteins.

Multidrug or pleiotropic drug resistance (PDR)¹ is a phenomenon found in various organisms, ranging from prokaryotes to eukaryotes, such as yeast and humans. The ability of cells to become resistant to toxic compounds such as drugs is of major importance because the treatment of many diseases is hampered by the ability of either the body’s own malignant cells or of foreign pathogenic organisms to develop PDR and thereby become resistant to drugs. Saccharomyces cerevisiae has been widely used to study PDR, allowing us to gain insight into the mechanisms behind PDR in pathogenic fungi and in higher eukaryotes.

There are mainly three types of proteins involved in PDR: 1) ATP-binding cassette (ABC) proteins, 2) major facilitator superfamily (MFS) proteins, and 3) transcription factors. ABC proteins are found in organisms ranging from bacteria to humans and are involved in many important processes in the cell (1, 2). Most ABC proteins are ATP-powered membrane transporters, although some function as ion channels, channel regulators, receptors, proteases, and sensing proteins (3). ABC proteins are able to transport a wide variety of compounds including ions, heavy metals, anticancer drugs, steroids, mycotoxins, antibiotics, and whole proteins (1, 4–6). Two well-characterized ABC transporters, Pdr5p and Snq2p, confer PDR. They are functional homologues of mammalian P-glycoprotein (7, 8). In contrast to ABC proteins, MFS members do not use ATP. Instead, proton-motive force is used to transport substrates across the membrane. Atr1p is one member of the MFS shown to be involved in drug resistance (9).

Various transcription factors have been shown to regulate the expression of genes encoding ABC or MFS proteins (10). There are two major families of transcription factors involved in PDR: 1) the bZip protein family (Yap family), and 2) zinc cluster proteins. Yap1p is the best characterized member of the bZip family and is an important regulator in the stress response (11–13). Yap1p regulates the expression of the ABC transporter, Ycf1p (14). Another class of transcription factors involved in PDR is composed of zinc cluster or binuclear zinc cluster proteins. They form a family of transcription factors found exclusively in fungi. Zinc cluster proteins are characterized by a zinc finger, which contains the Zn(II)2Cys6 (or C6 zinc) binuclear cluster DNA-binding motif with the consensus sequence of Cys_{1-2}Cys_{X}\times Cys_{X-12}Cys_{X-5}Cys_{X-6-8}Cys. The cysteines mediate the binding of two zinc atoms, which are necessary for the zinc finger to bind DNA (15, 16). Many zinc cluster proteins bind DNA as homodimers to recognition sites that usually fall within three types: inverted, direct, and everted repeats (17). These proteins have been shown to be involved in various processes in the cell including regulation of primary and secondary metabolism, drug resistance, and meiotic development (18), e.g. Gal4p is involved in the activation of genes that encode enzymes for galactose metabolism (19), whereas Hap1p activates genes involved in respiration (20, 21). Two zinc cluster proteins, Pdr1p and Pdr3p, have been shown to positively control the expression of genes involved in multidrug resistance (10).

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‡ The abbreviations used are: PDR, pleiotropic drug resistance; MFS, major facilitator superfamily; ABC, ATP binding cassette; ORF, open reading frame; PDRE, pleiotropic drug resistance element; RDS, regulator of drug sensitivity; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; 4-NQO, 4-nitroquinoline N-oxide; DBD, DNA binding domain.
Target genes of Pdr1p and Pdr3p include PDR5, SNQ2, and YOR1 encoding ABC transporters, as well as HXT9 and HXT11 encoding hexose transporters which belong to the MFS family (22–25). Overexpression of the ABC transporters renders yeast resistant to drugs. However, the overexpression of the hexose transporters leads to drug sensitivity. Even though Pdr1p and Pdr3p recognize the same pleiotropic drug response element (PDRE), with Pdr3p binding an inverted repeat CCGGCG, they have different roles (26, 27). The reporters are low resistant to drugs. However, the overexpression of the hexose transporters leads to drug sensitivity. Even though Pdr1p and Pdr3p recognize the same pleiotropic drug response element (PDRE), with Pdr3p binding an inverted repeat CCGGCG, they have different roles (26, 27). The PDRE3 promoter contains two PDREs, allowing for autoregulation (26). Another zinc cluster protein, Yrr1p, is implicated in PDR, e.g., Yrr1p has been shown to regulate the expression of SNQ2 (28).

The yeast genome contains 55 genes encoding putative zinc cluster proteins (for a complete list, see Refs. 29 and 30). However, the function of many of these putative zinc cluster proteins is unknown. A phenotypic analysis was carried out on 33 genes encoding yeast zinc cluster proteins to better understand their role (29). For example, we have shown that deletion of eight different zinc cluster genes impairs growth on nonfermentable carbon sources. In this study, we have extended our previous analysis by assessing the growth of these deletion strains in the presence of various drugs. Our results show that none of these deletion strains are either resistant or sensitive to at least one drug.

EXPERIMENTAL PROCEDURES

Strains—The wild-type strain used was BY4742 (MATa his3Δ1 leu2Δ0 lys2Δ0 trp1Δ0) (Ref. 31). The deletion strains were obtained from Research Genetics (Huntsville, AL) (32). Deletions for a number of strains were verified by Southern blot analysis (see list below). Research Genetics strain 11677 does not carry a deletion of the ORF YOR380W (2). Deletion of the YOR380W ORF was performed using the PCR method of Baudin et al. (33) using oligonucleotides with homology to the target gene at its 5′ end and 3′ sequences complementary to the KanMX (G418) selection marker. Plasmid pAD4 (34) was used as a template for PCR with the oligonucleotides TAATCTAGCACTTTCCTACATTTAAAGTGGCCGACAGAACACATCA- CATTGCACGTGCGGAGAC and ATTATTCTCCGATCACAATAACATTAATATTCTTTATCTGCGCCAGCTATGACGTATCGAT-GAATTCAAGCTCG.

Media and Drug Assays—Media were prepared according to Adams et al. (35).YPD contained 1% yeast extract, 2% peptone, 2% glucose. SD contained 2% glucose, 0.67% yeast nitrogen base (without amino acids) and was supplemented with adenine and appropriate amino acids at a final concentration of 0.004%. Drugs were obtained from Sigma. Stock solutions were prepared as described below and stored at −20°C: cycloheximide, 2 mg/ml in 100% ethanol; ketoconazole, 5 mg/ml in H2O; chloramphenicol, 34 mg/ml in 100% ethanol; 4-nitroquinoline N-oxide (4-NQO), 10 mg/ml in dimethyl sulfoxide; rhodamine 6-G, 10 mg/ml in 100% ethanol; olomoucine, 5 mg/ml in 100% ethanol. Cycloheximide, ketoconazole, chloramphenicol, and 4-nitroquinoline N-oxide assays were performed with glucose as a carbon source, whereas rhodamine 6-G and olomoucine were tested with glycerol as a carbon source. Concentrations of drugs used for the assays are indicated in Table II.

β-Galactosidase Assays—The lacZ reporters PDR5-lacZ and SNQ2-lacZ have been described previously (36). Briefly, the reporters are low copy plasmids (ARS5CEN) containing a UR43 marker. The PDR5 and SNQ2 reporters contain 1000 and 700 bp of sequences upstream of the ATG, respectively. Reporters PDR5-lacZ, PDR3-lacZ, and PDR3B-lacZ are high copy (2-μm) URA3-marked plasmids containing a single Pdr1P/Pdr3p binding site inserted upstream of minimal CYC1 promoter driving lacZ transcription (36). β-Galactosidase assays were performed as described previously (36) with permeabilized cells. Results were obtained from at least two independent transformations performed at least with duplicate samples. Variation between assays was typically less than 20%.

Southern and Northern Blot Analysis—Northern blot analysis and probes have been described previously (36). Southern blot analysis was performed as described (37), and the probe was obtained by purifying a KanR fragment by digesting pFA6 (34) with ClaI. Strains YBR033W, YBR150C, YCR238C, YDR520C, YJR103C, YKR064W, YLR228C, YLR278C, YMR019W, and YPR196W were verified by Southern blot analysis; strains YBL066C, YDR213W, YDR421W, YHR117W, YJL089W, YLM076C, and YPR094W had been characterized previously (29). Research Genetics deletion strain 11677 (YOR380W) did not give a band of the expected size with a probe corresponding to the promoter region of the YOR380W gene (data not shown; see also “Strains”).

Electrophoretic Mobility Shift Assay (EMSA)—A DNA fragment encoding the DNA-binding domain of Stb5p (amino acids 1–163) was amplified by PCR using the oligonucleotides CGGAGATCCCATGGAATGTTCCCAATTTTGTCG and GAAATCTTGGTACAGCTTGTGGGCGC as a template. The PCR product was digested with BamHI and EcoRI and subcloned into plasmid pGEX-F (27) cut with the same enzymes to give pGST-STB5. The DNA-binding domains of Stb5 and Pdr3 fused to GST were expressed in Escherichia coli and purified as described (27). The GST moiety was removed by thrombin cleavage. EMSA was performed according to Ref. 27. The probes used in the EMSA correspond to site number 3 of the PDR5 promoter (39) and span sequences –372 to –337 bp relative to the ATG. Oligonucleotides were annealed and filled-in with Klenow and dGTP, dTTP, dATP, and [32P]dCTP.

Oligonucleotides for PDRE3 were TCGAAAGAAGAAGATCCTCGCCGAACAGCCGCGCGCG and its complement TCAGCCGCGTGACAAAGATCTCGCTGGCGAACCTCTTGTCCGGCGCCGACACCATTTTCTTCTTTT. Oligonucleotides for PDRE3B were TCGAAAGAAGAAGATCCTCGCCGAACAGCCGCGCGCG and its complement TCAGCGCCGATAGAAGGTCTCGCGCCGGAGGATCTTTT (mutations are in bold characters and underlined).

RESULTS

Our study focused on 32 members of the Gal4p family of yeast zinc cluster proteins (Table I). Many members are putative proteins of unknown function. We determined whether these zinc cluster genes play a role in multidrug resistance by testing the ability of strains carrying deletions of these genes to grow in the presence of six different drugs: cycloheximide, ketoconazole, chloramphenicol, 4-NQO, rhodamine 6-G, and olomoucine. The mode of action of these drugs is listed in Table II. Wild-type and deletion strains were serially diluted and spotted on plates containing the drugs and grown for the time indicated in Table II. As expected (28), deletion of YRR1 resulted in hypersensitivity to the mutagen 4-NQO (Table III). However, none of the 31 other strains showed altered sensitivity to 4-NQO, olomoucine, rhodamine 6-G, and chloramphenicol (data not shown).

When assayed with the antifungal ketoconazole or the translation inhibitor cycloheximide, nine strains demonstrated a clear phenotype with at least one drug (Table III). Three of the genes deleted were not named previously. Because they potentially encode transcriptional regulators and show altered drug sensitivity, we named them RDS1–RDS3 (for regulator of drug sensitivity; see Tables II and III). Two strains (Δnpk2 and Δrsd2) were hypersensitive to ketoconazole (Fig. 1). Deletion of RDS3 resulted in a slightly decreased resistance, as seen from the reduced number of colonies at low cell concentration. The Δrsd3 strain was also hypersensitive to cycloheximide (see below). Moreover, seven strains revealed a phenotype when grown in the presence of cycloheximide. One strain (Δdrd1) was resistant to that drug. The same phenotype was observed when RDR1 was deleted in the strain FY73 (36). A more detailed analysis of RDR1 will be presented elsewhere (36). Strains carrying deletions of YIL130W or YKL222C were slightly resistant to cycloheximide (data not shown). Because (38) of the substraphenotype observed with these two genes, they were not scored as regulators of drug sensitivity. Six other deletion strains showed sensitivity to cycloheximide (Fig. 2). For example, deletion of STB5 or RDS3 abolished growth on plates containing cycloheximide, whereas normal growth was observed in the absence of the drug when compared with the
Regulators of Drug Sensitivity

TABLE I
Genes tested in this study

| Systematic name | Gene | Function | Ref. |
|----------------|------|----------|------|
| YBL066C        | SEF1 | Suppressor of essential function | (55) |
| YBR033W        | Unknown | | |
| YBR150C        | Unknown | | |
| YBR210C        | Unknown | | |
| YCR240C        | THI2 (PHO6) | Activator of thiamin biosynthetic genes | (56) |
| YCR106W        | RDS1 | Regulator of drug sensitivity | This study |
| YDR213W        | UPC2 | Activator of sterol biosynthetic genes | This study |
| YDR421W        | ARO80 | Activator of the gene encoding aromatic aminotransferase | (58) |
| YDR520C        | Unknown | | |
| YER184C        | Unknown | | |
| YFL052W        | Unknown | | |
| YHR178W        | STB5 | Binds Sin3p in two-hybrid assay | This study |
| YIL130W        | Unknown | | |
| YJL089W        | SIP4 | Involved in Snt1p-regulated transcriptional activation | (59,60) |
| YIL103C        | Unknown | | |
| YJL206C        | Unknown | | |
| YKL229C        | Unknown | | |
| YKR064W        | Unknown | | |
| YLL054C        | Unknown | | |
| YLR228C        | ECM22 | Activator of sterol biosynthetic genes | This study |
| YLR266C        | Unknown | | |
| YLR278C        | Unknown | | |
| YML078C        | Unknown | | |
| YMR019W        | STB4 | Binds Sin3p in two-hybrid assay | (51) |
| YNR063W        | HAL9 | Involved in salt tolerance | This study |
| YOL089C        | Unknown | | |
| YOR162C        | YRR1 | Activator of multidrug resistance genes | This study |
| YOR172W        | Unknown | | |
| YOR360W        | RDR1 | Repressor of multidrug resistance genes | (36) |
| YPL133C        | RDS2 | Regulator of drug sensitivity | This study |
| YPR094W        | RDS3 | Regulator of drug sensitivity | This study |
| YPR196W        | MAL63 | Activator of maltose genes | (61) |

TABLE II
Conditions used for drug assays

| Drug                  | Target                | Drug concentration used | Growth time |
|-----------------------|-----------------------|-------------------------|-------------|
| Chloramphenicol       | Inhibits DNA synthesis | 3 mg/ml                 | 2           |
| Cycloheximide         | Inhibits protein translation | 1 µg/ml                | 9           |
| Ketoconazole          | Antifungal; inhibitor of the ERG11 gene | 4 µg/ml              | 2           |
| 4-NQO                 | DNA mutagen           | 0.35 µg/ml              | 2           |
| Oligomycin            | Inhibits oxidative phosphorylation | 1 µg/ml              | 4           |
| Rhodamine 6-G         | Inhibits oxidative phosphorylation | 5 µg/ml              | 4           |

wild-type strain. Two strains showed phenotypes on more than one drug: strain 

\[ \Delta rds1 \] was sensitive to 4-NQO and cycloheximide, whereas \[ \Delta rds3 \] was sensitive to both ketoconazole and cycloheximide. In summary, our study has assigned new drug sensitivity phenotypes for nine genes encoding zinc cluster proteins.

Deletion strains that showed a phenotype most probably lack a transcriptional regulator. Thus, we tested whether these strains had altered expression of selected genes involved in multidrug resistance. RNA was isolated from the wild-type strain and the deletion strains that showed altered drug sensitivity and probed for PDR5, SNQ2, and PDR16 mRNAs (Fig. 3). As stated above, SNQ2 and PDR5 encode multidrug transporters. For example, Pdr5p has been shown to be a major mediator of cycloheximide resistance (40–42). As expected (28), the level of SNQ2 mRNA was reduced in cells lacking YRR1 (Fig. 3, lane 8). Interestingly, SNQ2 RNA was also reduced in a \[ \Delta stb5 \] strain (Fig. 3, lane 11). However, actin level was also reduced with that strain. We doubled the amount of \[ \Delta stb5 \] RNA and repeated the Northern blot analysis (Fig. 3, lanes 12 and 13). Clearly, the levels of SNQ2 mRNA were reduced in a \[ \Delta stb5 \] strain, whereas signals with an actin probe were similar in wild-type and deletion strains. The levels of PDR16 mRNA were reduced in \[ \Delta ecn22, \Delta rds2, \Delta hal9, \Delta stb5 \] strains as compared with the wild-type strain (Fig. 3, compare lanes 4, 6, 7, and 11 with lane 1). PDR5 mRNA levels were reduced in many strains, but the decrease was not as severe as with PDR16 and SNQ2. Strains \[ \Delta ecn22 \] and \[ \Delta stb5 \] had the lowest amount of PDR5 mRNA when compared with a wild-type strain, whereas a decrease was also observed in \[ \Delta rds1, \Delta rds2, \]
Zinc cluster genes whose deletion results in altered drug sensitivity are listed. Phenotypes (resistance or sensitivity to ketoconazole, cycloheximide, and 4-NQO) are also indicated.

| Systematic name | Gene               | Ketocanazole | Cycloheximide | 4-NQO |
|-----------------|--------------------|--------------|---------------|-------|
| YCR106W         | RDS1               | –            | Sensitive     | –     |
| YDR213W         | UPC2               | Sensitive    | –             | –     |
| YHR178W         | STB5               | –            | Sensitive     | –     |
| YIL130W         | –                  | –            | –             | –     |
| YKL292C         | RDS2               | –            | Slightly resistant | – |
| YLR225C         | ECM22              | –            | Slightly resistant | – |
| YOL089C         | HAL9               | –            | Sensitive     | –     |
| YOR162C         | YRR1               | –            | Sensitive     | –     |
| YOR380W         | RDR1               | –            | Resistant     | –     |
| YPL133C         | RDS2               | –            | Slightly sensitive | – |
| YPR094W         | RDS3               | Sensitive    | Sensitive     | –     |

=, no phenotype.

FIG. 1. Deletion of the UPC2, RDS2, or RDS3 genes results in altered sensitivity to ketoconazole. Wild-type or deletion strains were grown overnight in YPD. Cells were spun down, resuspended in water, and serially diluted (left to right: ~1.25 × 10⁴, 2.5 × 10⁴, 5 × 10⁴, and 1 × 10⁵ cells). Cells were then spotted on YPD plates either with (lower panel) or without (upper panel) ketoconazole. Gene deletions are indicated on the right part of the figure. WT, wild-type strain.

FIG. 2. Deletion of various genes encoding zinc cluster proteins results in altered sensitivity to cycloheximide. Wild-type or deletion strains were grown overnight in YPD. Cells were spun down, resuspended in water, and serially diluted (left to right: ~1.25 × 10⁴, 2.5 × 10⁴, 5 × 10⁴, and 1 × 10⁵ cells). Cells were then spotted on YPD plates either with (lower panel) or without (upper panel) cycloheximide. Gene deletions are indicated on the right part of the figure. WT, wild-type strain.

Δhal9, Δupc2, and Δrds3 strains. No major changes in PDR5, PDR16, and SNQ2 mRNAs were observed with deletion of ORFs YKL22C and YIL130W, in agreement with their slight resistance to cycloheximide. All the drug-sensitive strains had lower mRNA levels for either one or more of the tested RNAs. Thus, the observed phenotypes correlate with the reduced amount of the tested mRNAs. Strikingly, a strain deleted of STB5 is sensitive to cycloheximide and has reduced mRNA levels for PDR5 (as well as SNQ2 and PDR16). Our data strongly suggest that Stb5p is an additional regulator of genes encoding ABC transporters.

To determine whether changes in PDR5 and SNQ2 mRNA levels are because of altered promoter activity, we transformed PDR5 and SNQ2 lacZ reporters into the wild-type and the deletion strains (Table IV). Only a slightly reduced activity of the SNQ2 reporter was observed with the Δyrr1 strain, even though SNQ2 mRNA levels were drastically reduced in the absence of Yrr1p. Similar results were obtained in another study (43). We do not know the reason for the discrepancy between the Northern blot analysis and the reporter assay. The activity of the PDR5 reporter in strain Δhal9 was decreased ~2-fold, whereas the activity of the SNQ2 reporter was slightly decreased (Table IV). Deletion of STB5 decreased activity of the PDR5 and SNQ2 reporters 2- and 7-fold, respectively. In addition, deletion of RDS3 decreased activity of the SNQ2 and

PDR5 promoters 2- and 3-fold, respectively.

Because both PDR5 and SNQ2 promoters contain PDREs, known to be important in regulating transcription by binding of the transcriptional regulators Pdr1p and Pdr9p, we wanted to determine whether the decrease in activity was mediated through this response element. A lacZ reporter was constructed with a PDRE (derived from the PDR5 promoter) inserted upstream of minimal CYC1 promoter driving lacZ transcription. Activity of that reporter was greatly increased (more than 50-fold) when compared with a similar construct lacking the PDRE (data not shown). No difference in activity of the PDRE-CYC1 reporter was observed between the wild-type and the strains deleted of HAL9 or RDS3 (Table V). Therefore, the decreased activity of the PDR5 reporter in Δhal9 and Δrds3 strains may be the result of an element other than the PDREs within the PDR5 and SNQ2 promoters (or indirect effects).
Experimental Procedures.

Twenty deletion strains were grown in rich medium and RNA isolated. About 50 μg of total RNA were loaded per lane (lanes 1–11) for Northern blot analysis (see “Experimental Procedures”). For the Δstb5 strain, RNA samples were adjusted, reloaded, and probed with SNQ2 and actin (lanes 12 and 13). Probes are indicated at the right of the autoradiograms and the strains on top.

TABLE IV
Activity of PDR5-lacZ or SNQ2-lacZ reporters is decreased in cells lacking Stb5p, Hal9p, or Rds3p

| Strain         | PDR5-lacZ | SNQ2-lacZ |
|---------------|-----------|-----------|
| WT            | 56        | 13        |
| Δrds1         | 62        | 13        |
| Δupe2         | 51        | 12        |
| Δstb5         | 23        | 1.7       |
| Δecm22        | 50        | 12        |
| Δhal9         | 33        | 10        |
| Δyr1          | 87        | 10        |
| Δrds2         | 80        | 13        |
| Δrds3         | 33        | 3.4       |

However, deletion of STB5 reduced activity of the PDRE-CYC1 reporter by a factor of 2.7 (Table V). These results suggest that activation of the PDR5 and SNQ2 genes by Stb5p is mediated by PDREs. This possibility is supported by mutational analysis of the PDRE. Indeed, we tested two PDREs containing mutations located in either of the CGG triplets that are crucial for binding of Pdr3p (27). As expected, activity of the two mutants was decreased in a wild-type strain. A mutation in the first CGG triplet (mutant PDRE3A, Table V) resulted in a modest decrease of activity in a Δstb5 strain as compared with the wild-type strain. However, mutating the second CGG triplet (mutant PDRE3B) reduced reporter activity 2.6-fold in cells lacking Stb5p. These results suggest that the first CGG triplet is important for maximal activation by Stb5p. In addition, our data suggest that Stb5p and Pdr1p/Pdr3p recognize highly related DNA elements.

Because our results suggest that Stb5p activates transcription through PDREs, we tested whether it can bind directly to that DNA element. The putative DNA-binding domain (DBD) of Stb5p was fused to GST, expressed in bacteria, purified, and the GST moiety removed by thrombin cleavage. The DBD of Stb5p was then assayed by EMSA using a Pdr1p/Pdr3 binding site (Fig. 4). In the presence of the DBD of Stb5p, two major retarded complexes were observed. It is possible that the two complexes correspond to monomeric and dimeric forms of Stb5p. Strikingly, mutations that prevent binding of the activator Pdr3p (Ref. 27 and data not shown) also greatly diminished binding of Stb5p (Fig. 4; mutants PDRE3A and PDRE3B). Thus, our results strongly suggest that Stb5p activates transcription of multidrug resistance genes by binding to PDREs that are also recognized by the well-characterized activators Pdr1p and Pdr3p.

DISCUSSION

The zinc cluster proteins Pdr1p, Pdr3p, and Yrr1p are well known transcriptional activators of multidrug resistance genes (4, 10, 28). However, the role of many other zinc cluster proteins is unknown. We have performed a systematic phenotypic analysis of strains with zinc cluster genes deleted to determine whether additional members of this family are involved in conferring multidrug resistance. Interestingly, we found that nine different strains lacking zinc cluster proteins showed a phenotype when assayed with the antifungal ketoconazole and the translation inhibitor cycloheximide (Table III). Eight strains were sensitive to a drug, whereas one (Δdrd1) was resistant to cycloheximide. In another study (36), we performed whole-genome analysis of gene expression and have shown that Rdr1p is a transcriptional repressor of five genes including PDR5. Thus, the effect of Rdr1p is highly specific, e.g. expression of SNQ2 is not affected by removal of Rdr1p, whereas expression of PDR5 is increased ~5-fold, in agreement with the increased cycloheximide resistance. Furthermore, we have shown that a PDRE derived from the PDR5 promoter mediates the repression effect.

With the exception of RDR1, all strains were sensitive to drugs (Table III). For example, Yrr1p was previously shown to confer 4-NQO resistance by controlling expression of SNQ2 (28). Our results show that removal of Yrr1p also results in cycloheximide sensitivity. Similarly, Hal9p confers salt resistance (44), and our study shows that this protein is also involved in conferring resistance to cycloheximide. Upc2p and Ecm22p are activators of the sterol biosynthetic genes (45). Deletion of UPC2 results only in ketoconazole sensitivity, whereas deletion of ECM22 yields a strain sensitive to cycloheximide but not ketoconazole (Table III, Figs. 1 and 2). Moreover, ECM22 but not UPC2 is sensitive to caffeine (29), an inhibitor of the mitogen-activated protein kinase pathway and cAMP phosphodiesterase (46). Thus, even though Upc2p and Ecm22p have been shown to have overlapping functions (45), our phenotypic analysis suggests that they also have specific targets.

Other genes identified in our screen were not named previously, and, because of their phenotype, they were called RDS for regulators of drug sensitivity. Two of these genes (RDS1, RDS3) are involved in conferring resistance to cycloheximide, whereas the third one (RDS2) mediates ketoconazole resistance. Thus, we have identified additional zinc cluster proteins responsible for drug resistance. The number of strains scored with a phenotype in our screen may seem to be high when considering the numerous studies on multidrug resistance in yeast. However, one must take into account that we have targeted the biggest family of transcriptional regulators in yeast.

Our phenotypic analysis raises the question of the mechanism of action of these zinc cluster proteins: do they play a direct role in regulating one or more genes involved in PDR, or do they have an indirect effect? To help distinguish between these two possibilities, we determined whether expression of some genes implicated in multidrug resistance is affected by removal of zinc cluster proteins. Northern blot analysis showed that deletion of STB5 greatly decreased RNA levels for SNQ2 and PDR16 (and to a lesser extent PDR5), whereas deletion of YRR1 reduced SNQ2 RNA (Fig. 3). Moreover, a strain deleted of RDS3 has lower PDR5 mRNA levels. However, we did not observe significant changes in PDR5, SNQ2, and PDR16 RNA levels for many other strains that showed drug sensitivity. Multidrug resistance genes not tested in our study may be responsible for the observed phenotype. Whole-genome analy-
was observed in deletion strains (Table IV). Reduced activity for these putative transcriptional regulators. The purified DNA binding domain of Stb5p was used in an EMSA. Core sequences of the probes are shown at the bottom with mutations underlined. Arrows correspond to CGG triplets known to be important for binding of Pdr3p. Top, −, no Stb5p.

**Fig. 4. Stb5p binds to a PDRE.** The purified DNA binding domain (amino acids 1–163) of Stb5p was used in an EMSA. Core sequences of the probes are shown at the bottom with mutations underlined. Arrows correspond to CGG triplets known to be important for binding of Pdr3p. Top, −, no Stb5p.

Activators Pdr1p and Pdr3p (23–25, 39, 47–50). Moreover, we have previously shown that another zinc cluster protein, Rdr1p, negatively regulates expression of PDR5 by acting on a PDRE (36). Thus, the regulation of multidrug resistance genes via PDREs is more complex than initially anticipated. Even though our work strongly suggests that Stb5p is a transcriptional activator, previous studies have shown that it interacts with Sin3p in a two-hybrid assay (51). Sin3p represses gene expression by interacting with the histone deacetylase Rpd3p (52). Therefore, Stb5p may be both a positive and a negative regulator of gene expression as observed with the zinc cluster proteins Ume6p and Rgt1p (53, 54).

Many questions remain to be answered. For example, does the binding affinity of Stb5p for different PDREs in the promoters of target genes differ? This would explain the differential effect of Stb5p on expression of the SNQ2, PDR16 and PDR5. What is the mechanism of action of the zinc cluster proteins (other than Stb5p) identified in our screen? Importantly, our studies have identified new players involved in multidrug resistance. Our work also shows the power of a systematic functional genomic approach.

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