Multiple or pleiotropic drug resistance often arises in the yeast Saccharomyces cerevisiae due to genetic alterations of the functional state of the Cys6-Zn(II)2 transcription factors Pdr1p and Pdr3p. Single amino acid substitutions give rise to hyperactive forms of these regulatory proteins, which in turn cause overproduction of downstream target genes that directly mediate multidrug resistance. Previous work has identified a novel Cys6-Zn(II)2 transcription factor designated Yrr1p as mutant forms of this protein confer high level resistance to the cell cycle inhibitor reveromycin A and DNA damaging agent 4-nitroquinoline-N-oxide. In the present study, we demonstrate that Yrr1p also mediates oligomycin resistance through activation of the ATP-binding cassette transporter-encoding gene YOR1. Additionally, insertion of triplicated copies of the hemagglutinin epitope in the C-terminal region of Yrr1p causes the protein to behave as a hyperactive regulator of transcription. We have found that YRR1 expression is both controlled in a Pdr1p/Pdr3p-dependent manner and autoregulated. Chromatin immunoprecipitation experiments also show that Yrr1p associates with target promoters in vivo. Together these data argue that the signal generated by activation of Pdr1p and/or Pdr3p can be amplified through the action of these transcriptional regulatory proteins on downstream target genes, like YRR1, that also encode transcription factors.

A common feature underlying acquisition of multiple or pleiotropic drug resistance is the overexpression of genes encoding ATP-binding cassette (ABC) transporters. Multidrug resistant tumor cells often exhibit amplification of the gene encoding the Mdr1p, a prototypical drug efflux pump (1). Gene amplification is typically not seen in Saccharomyces cerevisiae, which contains two PDR1 homologues (PDR1 and PDR3). Hyperactive alleles of PDR1 lead to marked overproduction of target genes, which in turn allow cells to tolerate otherwise toxic levels of drugs (12). Mutant forms of a PDR1 homologue (PDR3) produce a similar effect on both drug resistance and ABC transporter expression (13, 14). Biochemical experiments indicated that Pdr1p and Pdr3p bind to the same DNA element that was designated the Pdr1p/Pdr3p response element (PDER) (15). Interestingly, the PDR3 structural gene contains two PDERs and is regulated by Pdr1p and autoregulated at the transcriptional level (16). The presence of at least one PDER has been found associated with every gene regulated by Pdr1p or Pdr3p.

Recently, another transcription factor has been identified that shows a partially overlapping regulatory network with Pdr1p and Pdr3p. YYR1 was cloned as a locus that could be genetically altered to give rise to high level resistance to the cell cycle inhibitor reveromycin A (17). A mutant form of this gene (YYR1-1) contained a duplicated segment of the protein in the C-terminal region of the factor and strongly activated expression of the ABC transporter-encoding genes SNQ2 and YOR1. Construction of a SNQ2-lacZ fusion gene demonstrated that Yrr1p was able to regulate the promoter of this gene (18).

In this work, we show that Yrr1p acts to regulate YOR1 transcription at the level of its promoter by a Pdr1p/Pdr3p-independent mechanism. Analysis of expression of the YYR1 gene indicates that this locus is both responsive to Pdr1p and Pdr3p and autoregulated. Two different insertions of a 3× hemagglutinin (HA) epitope tag into the C terminus of Yrr1p convert this protein into a hyperactive regulator of gene expression. Chromatin immunoprecipitation experiments using these 3×-HA-tagged forms of Yrr1p demonstrate that this protein associates with both the YYR1 and YOR1 promoters in vivo.

**MATERIALS AND METHODS**

Yeast Strains and Media—The S. cerevisiae strains used in this study were all derived from SEY6210. The genotypes of the strains are listed in Table I. PB4 is a pdr1Δ and pdr3Δ mutant yeast strain as previously described (19). ZYX1 and ZYX2 were made by transforming a yrr1-1::TRP1 gene disruption plasmid (17) digested with BamHI and PstI into SEY6210 and PB4, respectively. Trp+ transformants were selected and analyzed by Southern blotting analysis to confirm introduction of the disruption allele. ZYX12 to ZYX17 were generated by using mTn-3X HA/lacZ transposon clones from the Yale Genome Analysis Center and used as described (20). ZYX12, ZYX13, and ZYX16 were generated with one-step transformation of plasmids of clone V41A2 (YRR1::lacZ-URA3-3X HA-695), V54F11 (YRR1::lacZ-URA3-3X HA-570), and Biophysics, 5-612 Bowen Science Bldg., University of Iowa, Iowa City, Iowa 52242 and the Department of Microbiology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima, 739-8526 Japan

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HA-730), and V123G2 (YOR172w::lacZ-URA3-3X HA-774) for NoI digestion, respectively. These three strains were then processed for lysis excision by inducing the expression of cre recombinase from the pB227/GAL-cre plasmid with galactose. The resulting 3X-β-galactosidase was evaluated using a luminometer. All enzyme assays were performed at least twice on independent transformants, and the values reported are expressed ± S.D.

**DNase I Footprint Assay**—The vector pPTS-No12 expressing a Myc-tagged Pdr1p N-terminal 248 amino acids or vector only was transformed into the Escherichia coli strain AR68. Protein extracts were then made from heat-shocked cells as previously described (19). An Asp718/SacI fragment of pXTZ118 was labeled with 32P at the 3′-end and used as a footprint probe. The binding reaction was carried out at room temperature for 5 min followed by a 30-s DNase I digestion on ice. The reaction mix was resolved by 6% denaturing polyacrylamide gel electrophoresis after phenol extraction and ethanol precipitation.

**RESULTS**

**Yrr1 Confers Oligomycin Resistance in S. cerevisiae**—Yrr1 was originally cloned by its ability to confer reveromycin A resistance (17). Our data previously implicated YOR1 in resistance to both reveromycin A (7) and the mitochondrial ATPase inhibitor oligomycin (6). Additionally, previous work showed that the mRNA expression level of YOR1 was increased in presence of YRR1 (17). To further examine the effect of the loss of YRR1 on oligomycin resistance of cells, yrr1Δ deletion alleles were made in both wild-type or pdr1Δ, pdr3Δ strains by homologous recombination. All four yeast strains were grown in YPD medium to an A600 of 1, and 1000 cells of each were spotted on a YPE plate containing a gradient of oligomycin.

**Transcription Factor Crosstalk in Yeast**

**Table 1**

| Strain | Genotype | Reference |
|--------|----------|-----------|
| SEY6210 | MATa leu2-3 – 112 ura3-52 his3 Δ300 trpl-199 lys2-801 suc2-39 Mel- | Scott Emr (19) |
| PB4 | MATa leu2-3 – 112 ura3-52 his3 Δ300 trpl-199 lys2-801 suc2-39 Mel- pdr1 Δ2::hisG, pdr3 Δ1::hisG | This work |
| XZY1 | MATa leu2-3 – 112 ura3-52 his3 Δ300 trpl-199 lys2-801 suc2-39 Mel- yrr1::TRP1 | This work |
| XZY2 | MATa leu2-3 – 112 ura3-52 his3 Δ300 trpl-199 lys2-801 suc2-39 Mel- pdr1 Δ2::hisG, pdr3 Δ1::hisG yrr1 Δ1::TRP1 | This work |
| XZY12 | MATa leu2-3 – 112 ura3-52 his3 Δ300 trpl-199 lys2-801 suc2-39 Mel- YRR1::lacZ-URA3-3X HA-695 | This work |
| XZY13 | MATa leu2-3 – 112 ura3-52 his3 Δ300 trpl-199 lys2-801 suc2-39 Mel- YRR1::lacZ-URA3-3X HA-730 | This work |
| XZY14 | MATa leu2-3 – 112 ura3-52 his3 Δ300 trpl-199 lys2-801 suc2-39 Mel- YOR172w::lacZ-URA3-3X HA-774 | This work |
| XZY15 | MATa leu2-3 – 112 ura3-52 his3 Δ300 trpl-199 lys2-801 suc2-39 Mel- YOR172w::lacZ-URA3-3X HA-774 | This work |
| XZY16 | MATa leu2-3 – 112 ura3-52 his3 Δ300 trpl-199 lys2-801 suc2-39 Mel- YOR172w::lacZ-URA3-3X HA-774 | This work |
| DPTY11 | MATa leu2-3 Δ2::hisG trpl-199 lys2-801 ade2-101 thy1 Δ1::TRP1 pRS153::X HATrplp | Tony Weil |
introduction of the yrr1Δ deletion mutation into the pdr1Δ, pdr3Δ reduced the ability of the resulting triple mutant to tolerate oligomycin. These data indicate that, although YRR1 does contribute to oligomycin resistance in S. cerevisiae, its contribution is masked in the presence of Pdr1p and Pdr3p.

YRR1 Activates YOR1 Gene Expression Independent of the PDRE and Its Binding Proteins—We have previously demonstrated that Pdr1p and Pdr3p regulate YOR1 gene expression via directly binding to the single PDRE (Pdr1p/Pdr3p response element) located in the YOR1 promoter (28). To examine if the PDRE is also required for Yrr1p function, two different YOR1-lacZ fusion plasmids were used that either contained (YOR1-PDRE) or lacked (mpDRE-YOR1-lacZ) the PDRE in the YOR1 promoter. This mutant form of the PDRE contains a two-nucleotide substitution, which blocks Pdr1p/Pdr3p binding (28). To examine if the Yrr1p activation of YOR1-lacZ expression depends on a DNA element other than the PDRE and its binding proteins, we were interested in examining expression of the YRR1 gene itself. Inspection of the YRR1 promoter showed strong resistance to oligomycin in comparison to either wild-type or pdr1Δ, pdr3Δ cells transformed with YRR1. It was also found that the resistance of cell to oligomycin by YRR1 requires the presence of YOR1 structure gene (data not shown). These data are consistent with the idea that YRR1 confers oligomycin resistance by regulating gene expression of YOR1 in a Pdr1p/Pdr3p- and PDRE-independent manner.

Deletion Mapping of YOR1 Promoter—The above analysis suggested that Yrr1p did not act through the intact PDRE. To localize the Yrr1p binding site in the YOR1 promoter, a series of deletion derivatives of the YOR1-lacZ reporter gene was utilized. Low copy number plasmids containing the indicated YOR1-lacZ fusion with varying amounts of 5'-promoter sequences were transformed into a wild-type yeast strain along with a second low copy number plasmid carrying either wild-type or hyperactive Yrr1p were tested for oligomycin resistance using a gradient plate as described above.

A 5' deletion to −222 bp upstream of the YOR1 transcription start site was as responsive to the presence of the YRR1-1 allele as the fusion gene containing 1065 bp of upstream DNA. Further deletion to −129 bp upstream eliminated the response to Yrr1p. An internal deletion lacking the −190 to −299 region of the YOR1 promoter was still induced in the presence of Yrr1p, whereas the −299 to −50 deletion derivative lacked any response to the YRR1-1 allele. The elevated expression of the −299 to −50 internal deletion construct has been observed before (28). These data suggest that Yrr1p acts through the −222 to −190 bp region upstream of the YOR1 transcription start site. Note that the PDRE corresponds to position −218 to −209 but is not required to mediate the response of YOR1 to Yrr1p (see above).

Transcriptional Control of the YRR1 Promoter—Along with localizing the Yrr1p response element in the YOR1 promoter, we were interested in examining expression of the YRR1 gene itself. Inspection of the YRR1 promoter suggested that this gene might be transcriptionally regulated by Pdr1p and/or Pdr3p. A 9 of 10 match with the consensus PDRE was detected in the YRR1 promoter (Fig. 4). To facilitate analysis of YRR1 gene expression, a YRR1-lacZ fusion gene was prepared. This fusion gene was introduced into wild-type cells along with low copy number plasmids carrying wild-type or the PDR1–3 allele of YRR1. PDR1–3 encodes a hyperactive form of Pdr1p that
leads to overexpression of Pdr1p target genes and drug hyperresistance (8). Transformants were assayed for YRR1-dependent β-galactosidase using a chemiluminescence method owing to the low level expression of YRR1.

The presence of the PDR1-3 allele increased expression of the YRR1-lacZ fusion gene by a factor of 4 compared with introduction of a second copy of wild-type PDR1. This analysis provided strong support for the view that YRR1 represents a new target gene for Pdr1p (and likely Pdr3p).

To directly demonstrate that Pdr1p was capable of binding to the YRR1 PDRE, a DNase I protection assay was performed using a probe containing this element from YRR1 promoter (Fig. 4). Incubation of this probe with protein extracts from bacteria expressing the DNA binding domain of Pdr1p but not with extracts from cells carrying the empty expression vector led to a region of DNase I protection corresponding to the YRR1 PDRE. Together, these data indicate that expression of YRR1 is regulated by Pdr1p.

During this analysis of YRR1 expression, we tested if Yrr1p might influence transcription of its own structural gene. To evaluate this possibility, low copy number plasmids expressing either wild-type or hyperactive alleles of Yrr1p were transformed into wild-type cells along with the YRR1-lacZ fusion gene. A series of 5′-truncated versions of the YRR1-lacZ reporter construct was prepared to localize important regulatory sequences. Transformants were processed for β-galactosidase activity measurements as described above (Fig. 5).

The presence of the YRR1-1 allele increased expression of the YRR1-lacZ fusion by nearly 600%. This same strong increase in expression was seen with reporter constructs containing 326 or 271 of 5′-noncoding DNA from YRR1. However, a fusion gene extending 220 bp upstream of the YRR1 ATG was no longer significantly responsive to YRR1-1. As we found for the YOR1 promoter, the YRR1 PDRE was localized from −269 to −260 and mapped to this Yrr1p-responsive region.

These data provide two important additional findings about control of YRR1 expression. First, YRR1 is autoregulated. Second, the element responsive to Yrr1p can be mapped to positions −271 to −220 upstream of the YRR1 ATG. This region of the YRR1 promoter was selected for further analysis to gain insight into the sequence elements required for response to Yrr1p.

**Fig. 3. Localization of the Yrr1p-responsive region in the YOR1 promoter.** A series of YOR1-lacZ fusion genes lacking different segments of the YOR1 promoter was introduced into wild-type cells along with plasmids expressing either wild-type or hyperactive forms of Yrr1p. All plasmids were maintained at low copy number. Transformants were grown to mid-log phase and assayed for β-galactosidase activity. The solid box indicates the position of the PDRE, whereas the arrow denotes the start site for YOR1 gene transcription. The numbers on the left refer to the extent of YOR1 5′-noncoding DNA remaining for the 5′-truncation mutants, whereas the DNA that has been deleted is shown for the internal deletion mutants.

**Fig. 4. Identification of a PDRE in the YRR1 promoter.** A, the DNA sequence of an element in the YRR1 5′-regulatory region matching the consensus PDRE is shown. The numbers refer to the position of this PDRE relative to the YRR1 ATG. B, a YRR1-lacZ fusion gene was transformed into wild-type cells along with plasmids carrying the wild-type (PDR1) or hyperactive form of Pdr1p (PDR1-3). Transformants were assayed for β-galactosidase activity in crude protein extracts using a chemiluminescent reagent (CLONTECH) as recommended by the manufacturer. C, a 5′-32P-end-labeled probe containing the YRR1 PDRE was prepared as described under “Materials and Methods.” This probe was incubated with either 10 or 20 μl of crude extract from bacterial cells expressing the DNA binding domain of Pdr1p (Pdr1p, volume indicated by bar of increasing width), 20 μl of crude extract from cells carrying the empty expression vector (Vector), or with buffer alone (No Protein). Samples were then digested with DNase I for 30 s, deproteinized, and electrophoresed through a denaturing polyacrylamide gel. Location of the PDRE was established by comparison with Maxam-Gilbert sequencing reactions on the same fragment (data not shown).
clustered mutations located near the PDRE (−259 and −255 in pXTZ112, 114, 115, 117) eliminated the ability of the corresponding oligonucleotide to be regulated by Yrr1p in every case. Importantly, these mutant oligonucleotides did maintain their ability to respond to Pdr1p with the exception of pXTZ117, which contains a 1-bp deletion in the PDRE. These data are consistent with the view that the Yrr1p response element and the PDRE are linked but separable in the YRR1 promoter.

The C Termini of YRR1 and Its Homologue YOR172W Regulate Factor Function—An invaluable resource in the analysis of S. cerevisiae genes is the availability of a collection of transposon insertions into a large fraction of the genome (20). This series of insertion mutations was found to contain two different in-frame insertions of E. coli lacZ into YRR1, one after codon 695 and one after codon 730. These insertions are in a region of the protein in which a gain-of-function mutation was previously isolated (17). The transposon used to make these mutations also contains loxP sequences that can be cleaved by the cre protein expressed in yeast to evict the lacZ cassette and associated URA3 marker, leaving behind a 3X hemagglutinin (3X HA) epitope tag inserted into the coding sequence. These two different transposon insertions were integrated in place of the wild-type YRR1 locus in an otherwise wild-type strain, the lacZ/URA3 sequences removed by expressing cre in appropriate integrants and the resulting mutant YRR1 loci designated YRR1::3X HA-695 (3X HA insertion after codon 695) and YRR1::3X HA-730 (3X HA insertion after codon 730).

Strains expressing wild-type or the two different 3X HA insertion variants of Yrr1p were assayed for their ability to confer resistance to oligomycin and 4-NQO using a gradient plate of each drug (Fig. 7). Both the Yrr1p::3X HA-695 and Yrr1p::3X HA-730 gave rise to an increased ability to grow in the presence of both oligomycin and 4-NQO. To determine whether this increased oligomycin resistance correlated with an elevation in YOR1 expression, a YOR1-lacZ fusion plasmid was used to vary the activity of the Yrr1p factor. Low copy number plasmids were used to introduce a single extra copy of PDR1 or PDR1–3. Transformants were grown to mid-log phase and assayed as described under “Materials and Methods.”
was introduced into these backgrounds. The Yrr1p::3X HA-695 and Yrr1p::3X HA-730 led to an increase in \( YOR1-lacZ \) expression to 25 and 31 units/optical density, respectively, whereas the presence of wild-type Yrr1p produced 11 units/optical density. These data indicate that insertion of foreign sequences into the C-terminal region of Yrr1p activate the function of this transcriptional regulatory protein.

Analysis of the \( S.\ cer\ ae\ ) genome detected the presence of a protein encoded by the \( YOR172w \) locus that shared strong sequence identity with Yrr1p (41%) identity (17). Search of the \( S.\ cer\ ae\ ) genome detected the presence of a large number of genes that increase in expression in response to activated forms of Pdr1p or Pdr3p (29). Although many of these genes contain at least one PDRE, requisites an intact C terminus for normal regulation.

\( YRR1 \) interacts with both the \( YRR1 \) and \( YOR1 \) promoters

A simple model that could explain the effect of Yrr1p on expression of \( YOR1 \) and \( YRR1 \) would be provided by the ability of this factor to directly bind to and activate these promoters. We tried a variety of bacterial expression systems but were not able to reproducibly detect Yrr1p DNA binding activity. We turned to the technique of chromatin immunoprecipitation (ChIP) (27) to determine if Yrr1p was capable of associating with these putative target promoters in vivo.

Three different strains were used to assess in vivo association of Yrr1p with the \( YOR1 \) and \( YRR1 \) promoters: a wild-type strain expressing no HA-tagged proteins (SEY6210), a strain expressing an HA-tagged Yrr1p (XZY15), and a strain expressing an HA-tagged form of TATA-binding protein (Tbp1p) as its sole source of this essential protein (DPY11). Three different primer pairs were used to evaluate the specificity of the ChIP assay. Primers corresponding to the promoter regions of \( YRR1 \) and \( YOR1 \) as well as a primer set that would amplify the promoter of the \( ATR1 \) gene (29), a locus not under Yrr1p or Pdr1p/Pdr3p control (data not shown). ChIP was performed using anti-HA antibody essentially as described (27) and analyzed by PCR amplification of total DNA prior to immunoprecipitation (input) or specifically immunoprecipitated DNA (anti-HA IP, Fig. 8).

Both the \( YRR1 \) and \( YOR1 \) promoters are detected in DNA recovered from ChIP reactions performed on chromatin lysates from the cells expressing the HA-tagged proteins but not on control lysates from wild-type cells. Importantly, the \( ATR1 \) promoter was identified in the immunoprecipitates from the HA-Tbp1p expressing strain but not from the HA-Yrr1p-expressing cells. These data provide support for the view that Yrr1p associates in vivo with target promoters and through this association leads to an increase in gene expression.

DISCUSSION

These data illustrate important new connections in the pleiotropic drug resistance pathway in \( S.\ cer\ ae\ ). The finding that Pdr1p (and likely Pdr3p) regulate expression of \( YRR1 \) suggest a potential new complexity in the analysis of Pdr1p/Pdr3p target genes. Genome microarray experiments have suggested the presence of a large number of genes that increase in expression in response to activated forms of Pdr1p or Pdr3p (30). Although many of these genes contain at least one PDRE,
not all do. These data provide a possible explanation for this observation, because activation of Yrr1p expression by Pdr1p or Pdr3p could lead to an increase in transcription of downstream target genes via Yrr1p binding to an element other than a PDRE.

With this possibility in mind, it is interesting to note that in the two genes identified here are Yrr1p targets, both contain PDREs. Additionally, previous work (17) has provided evidence that Yrr1p activates expression of the ABC transporter-encoding locus, SNQ2, which in turn leads to the increase in 4-NQO resistance seen in cells containing activated forms of Yrr1p. SNQ2, like YRR1 and YOR1, also contains a PDRE. Examination of these three genes cannot be viewed as representative of all Yrr1p target genes, because all three loci are involved in drug resistance. Less directed approaches such as microarray analyses must be undertaken to give a more accurate picture of the necessary linkage of Yrr1p response with a PDRE.

A second striking feature of the observed linkage of the PDRE with the Yrr1p response element is the finding that these two recognition sequences appear to be tightly physically linked. We have identified two base pairs, critical for Yrr1p activation, that are located immediately adjacent to the YRR1 PDRE. A 33-bp region containing the YOR1 PDRE appears to be necessary for the Yrr1p responsiveness of this gene. A similar tight linkage has been found for SNQ2 (18). At least for these three Yrr1p/Pdr1p/Pdr3p-coregulated genes, the possibility exists that these regulatory proteins may directly communicate during gene regulation.

Even though the actions of Pdr1p and Yrr1p occur through sites that are physically close on target promoters, we provide evidence that their actions are not through the same element. First, a mutant YOR1 promoter lacking a functional PDRE can still be activated by Yrr1p. Second, the presence of the YRR1-1 allele completely bypasses the requirement for Pdr1p or Pdr3p in terms of oligomycin resistance and YOR1-lacZ expression. Finally, mutant forms of the YRR1 promoter can be generated that fail to respond to Yrr1p but are normally activated by Pdr1p.

Although the ChiP analysis demonstrates that Yrr1p interacts with target promoters, a consensus binding site for Yrr1p has not yet been identified. Efforts to produce forms of Yrr1p in bacterial expression were not successful even though similar constructs were used to produce active forms of either Pdr1p or Pdr3p (15, 19). Inspection of the three known target promoters has not revealed any striking candidates for shared recognition elements. The identification of the precise binding site for Yrr1p is a high priority.

The finding that insertions of random sequences into the C termini of either Yrr1p or Yor172wp lead to an increase in function of these factors suggests that these proteins may normally be negatively regulated. The insertion of this extraneous sequence leads to a loss of the ability to respond to this negative signal and results in a constitutively active protein. Previously, a hyperactive form of YRR1 (YRR1-1) was identified that contained a duplication of amino acids 695–706 (17). Our observation that insertion of the 3X HA sequence after either position 695 or 730 indicates that there is not likely to be a special significance to the previously reported duplication but rather that this region of Yrr1p must be structurally intact for normal regulation of function. Other Zn(II)$_2$Cy$_6$ transcription factors like Gal4p (31), Pdr1p (32), Leu3p (33), or Hap1p (34) also possess central regulatory domains that can be mutationally altered to change the function of the resulting factor. We have previously reported that the activity of Pdr3p is tightly linked to the status of mitochondria (35) and find that Yrr1p also appears to be involved in this regulatory circuit. Together, these data illustrate the close communication between Pdr1p, Pdr3p, and Yrr1p activity (Fig. 9). Coordination of the activity of these transcription factors is a critical factor for normal drug resistance as shown by the interlocking systems of transcriptional control of the Pdr3p and Yrr1p structural genes. Identification of the precise regulatory signals controlling these factors will provide important new insight into the physiological basis underlying these multidrug resistance regulatory proteins.

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