A General Strategy to Uncover Transcription Factor Properties Identifies a New Regulator of Drug Resistance in Yeast*

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We demonstrate a genomewide approach to determine the physiological role of a putative transcription factor, Ylr266, identified through yeast genome sequencing program. We constructed activated forms of the zinc finger (Zn_{2}Cys_{6}) protein Ylr266, and we analyzed the corresponding transcriptomes with DNA microarrays to characterize the up-regulated genes. The direct target genes of Ylr266 were further identified by in vivo chromatin immunoprecipitation procedure. The functions of the genes directly controlled by YLR266c are in agreement with the observed drug-resistance phenotype of the cell expressing an activated form of Ylr266. These target genes code for ATP-binding cassette or major facilitator superfamily transporters such as PDR15, YOR1, or AZR1 or for other proteins such as SNG1, YJL216c, or YLL056c, which are already known to be involved in the yeast pleiotropic drug resistance (PDR) phenomenon. YLR266c could thus be named PDR8. Overlaps with the other PDR networks argue in favor of a new specific role for PDR8 in connection with the well known PDR regulators PDR1/PDR3 and YRR1. This strategy to identify the regulatory properties of an anonymous transcription factor is likely to be generalized to all the Zn_{2}Cys_{6} transcription factors from Saccharomyces cerevisiae and related yeasts.

With the advent of postgenomic approaches that provide a nearly complete analysis of the cell transcriptome, it has been disconcerting to discover the complexity of the cell genetic response to apparently simple physiological changes (1). This apparent complexity is likely to reflect the action of underlying regulatory networks that control gene-expression patterns characteristic of many different genetic changes (2). These transcriptional regulatory networks are under the combinatorial action of transcription factors, and dissection of the specific role of each transcription factor offers a good opportunity to decipher the complexity of genome expression (3).

One of the main challenges further the understanding of genome functions is to describe the set of genes that are directly regulated by the different specific transcription factors. DNA microarrays are very efficient tools to address such questions, but they have to be coupled with properly designed experiments if one wishes to distinguish direct and indirect effects of the activity of a transcription factor. Such data are already available for several transcription factors (4) that were previously characterized by classical biological approaches. However, it should be kept in mind that even in Saccharomyces cerevisiae, many direct target genes of identified or putative transcription factors are unknown. Any experimental approach to complete these data relies on the possibility to activate the relevant transcription factor. We have recently designed an approach for the artificial activation of yeast Zn_{2}Cys_{6} transcription factors. The Zn_{2}Cys_{6} family of transcription factors, exemplified by Gal4, represents more than 25% of the yeast transcription regulators. Our strategy to activate these factors relies on the postulate that the DNA-binding domain of transcription factors fused to a heterologous transcription activation domain isolated from Gal4 is sufficient to reveal the specific transcriptional response of the original protein. The target genes, whose expression is constitutively and strongly activated by the chimeric protein, are then identified by microarray analyses. We have validated this assumption and this strategy with Pdr1 and Yrr1 (5, 6), two regulators of the pleiotropic drug resistance (PDR) phenomenon.

The purpose of the present work is to use this approach to determine the network of genes regulated by a completely unknown transcription factor of the Zn_{2}Cys_{6} family and to deduce the biological functions of this factor from the properties of its target genes. The product of the unknown gene, YLR266c, was chosen because of its structural similarities with YRR1 (5). Yrr1 is a transcription factor (7, 8) that, when activated, confers resistance to reveromycin A, oligomycin, and 4-nitroquinoline-N-oxide. The strategy to activate the putative transcription factor Ylr266 was derived from our previous experiments with the transcription factors Pdr1 and Yrr1 (5, 6). Additionally, we have used the technique of chromatin immunoprecipitation to confirm the in vivo interactions of the transcription factor Ylr266 with the relevant promoters of the direct gene targets revealed by transcriptome analyses. These YLR266-regulated genes encode proteins like ATP-binding cassette or major facilitator superfamily transporters. In agreement with these modifications, we observed that the strain expressing an active form of Ylr266 is resistant to several unrelated drugs like ketoconazole and oligomycin; we thus

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† The abbreviations used are: PDR, pleiotropic drug resistance; ChIP, chromatin immunoprecipitation; ORF, open reading frame; HA, hemagglutinin.
propose to name this gene PDR8. Connections with other PDR regulatory networks (namely Pdr1 and Yrr1) show that Pdr8 is an element of a large regulatory program designed to allow the cell to adapt to diverse environmental conditions.

This strategy of artificial transcriptional activation, coupled to ChIP identification of direct targets in vivo, is the first step to a complete comprehensive description of the transcriptional regulatory networks in yeast and could be extended to other, more complex, organisms.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—The *S. cerevisiae* strain BY 4742 (MATa his3D 1 leu2D 0 lys2D 0) was used in this study. Cells were grown on YPD (1% yeast extract, 2% bactopeptone, 2% glucose) or minimal synthetic medium (0.67% yeast nitrogen base, 2% carbon source [glucose, galactose, or glycerol + ethanol]) supplemented with appropriate amino acids at 40 mg/ml. Osmotic or pH sensitivity and drug-resistance assays were performed by spot tests with serial dilutions (9). *Escherichia coli* TG1 (K-12 Δlac-pro) sup3 thi hsdS5 F’ traD36 proB “B” lacIq ZAM15) was used for plasmid manipulations.

Disruption of YLR266c—The BY 4742 ydr266c::LEU2 strain was prepared by one-step gene replacement. The LEU2 disruption marker was PCRamplified from plasmid pRS425 described previously (10) using oligonucleotides YLS66-up (5'-TCATCTTGGAGCTATGGGCG-3') and YLS66-down (5'-GAATTACAGAGAGATGATG-3') and amplified with Genepix 3.1 software (Axon).

The microarray experiments were performed with the pairwise comparison of the BY4742 wild-type strain and the three microarray experiments in which the Ylr266 overproduction was tested against the BY4742 wild-type strain.

**RNA Isolation and Northern Blot Analyses**—Total RNA was prepared using glass slides containing spots of open reading frames of *S. cerevisiae* control cells with Cy5-dUTP. The arrays after hybridization were washed and centrifuged, followed by the addition of 20 μl of salmon sperm DNA along with protein A-Sepharose beads. Immunoprecipitates were extensively washed and centrifuged to recover a protocol (17). Briefly, cells expressing HA-tagged Ylr266 under the control of the GAL1 promoter were collected at different times after galactose induction. Protein-nucleic acid complexes were fixed by formaldehyde treatment (15 min at 20 °C). Lysates were prepared by glass bead grinding followed by sonication to shear chromatin to an average length of 1–3 kb. A 20-μl aliquot of the lysate was saved as the input fraction. After incubation with mouse anti-HA monoclonal antibody (Babco), the samples were briefly centrifuged, followed by the addition of 20 μl of DNA with protein A-Sepharose beads. Immunoprecipitates were extensively washed and centrifuged to recover a protein pellet (bound) and supernatant (unbound). 20 μg of RaA was added to remove RNA. Protein was eluted from the Sepharose beads by treating with 1% SDS/0.1 M NaHCO3. Cross-links were reversed by adding 20 μl of 5 M NaCl to all reactions and heating at 65 °C for 5 h. The DNA was ethanol-precipitated, digested with proteinase K, phenol-extracted, and resuspended in TE (100 mM Tris, pH 7.5, 1 mM EDTA) prior to PCR analysis. Both input and bound DNA were dissolved in 20 μl of TE. 1 μl of DNA of each reaction was used for PCR. The different primer sets used for the 9 promoters are available on request. Linearity of PCR reactions was assayed by multiple template dilutions of input (IN) and immunoprecipitated (IP) DNA as indicated by Larscham and Winston (18). Gel images were captured by a fluorescent imager (Fuji) and quantified with Image Gauge (version 3.41) software. Calculation of the enrichment factor was made as follows: 1) the values at 10 h and 14 h were normalized to a reference product of YER184c promoter, amplified in the same reaction mixture. YER184c is a gene determined by microarray analyses to be transcriptionally stable in the conditions used here (data not shown). Moreover, we checked that in the described ChIP experiments its promoter behaves as the actin promoter ACT1 (data not shown). Mean values were used as the crude enrichment or occupancy value. 2) A general basal value was obtained when the transcription factor Ylr266 was not produced. A mean basal value was derived from the twelve independent experiments. The enrichment factor was obtained by dividing the normalized promoter-occupancy value by the mean basal value.

**RESULTS**

Design of Ylr266c*GAD, an Activated Form of Ylr266, and Overproduction of Ylr266—We recently developed a general genomewide strategy for the systematic analysis of regulatory networks under the control of Zn2Cys6 transcription factors (6). This approach is based on the conditional expression of a chimeric gene encoding the DNA-binding region of the transcription factor fused to GAL1 promoter used for the transcription-activating domain of Gal4. We used a similar strategy with YLR266c to create a chimera called Ylr266c*GAD (Fig. 1). The key point in this approach is that the chimera must contain a specific DNA-binding domain devoid of inhibitory activity known to be contained in the central flanking domain of this transcription factor family (5). Based on the assumption that the inhibitory region should be highly divergent among members of the Zn2Cys6 zinc finger family, we could define a DNA-binding domain from amino acids 1–149 that was included in the protein Ylr266c*GAD (Fig. 1). Moreover, we also overexpressed the complete form of Ylr266 under the control of the GAL1 promoter. This allowed us to analyze the transcriptional modifications induced by different forms of Ylr266 under different experimental conditions (Fig. 1).

**A Genomewide Search of Ylr266 Target Genes**—We investigated the YLR266c regulation network by microarray experiments to compare transcription factors generated along the time-course production of either the Ylr266c*GAD or the complete transcription factor. We performed three microarray analyses, testing the Ylr266 overproduction against the BY4742 wild-type strain and three microarray experiments in which the
strain producing Ylr266*GAD (pCB-PDR8*GAD) was tested against the control strain producing only the GAD domain. All of these data were clustered, and we then assumed that the minimum set of genes specifically activated in either conditions should represent the genes actually activated by Ylr266. The complete analysis criteria and data are available on request.

We identified by cluster analysis (Fig. 2A) one distinct group of genes that was similarly activated by the artificial chimera Ylr266*GAD and by the overproduction of the complete protein. In this group AZR1, CTT1, GTT2, YOR1, YJL216c, YLL056c, and YIL121w were strongly up-regulated in all conditions and clearly distinguish from the others by principal component analysis (Fig. 2B). In addition, eight genes are moderately activated (Fig. 2) and are likely to be indirectly activated by Ylr266 because no direct interactions were revealed by ChIP (see the case of GPH1, Fig. 3). Minor quantitative variations of gene activation levels by either forms of Pdr8 (Fig. 2A) are likely to reflect amount variations of each Pdr8 form rather than differences in specificity. This is supported by transcriptome analyses in which different amounts of factors were produced (data not shown).

Specific Promoter Occupancy by Ylr266 in Vivo—To discriminate among the putative Ylr266 target genes those which actually interact, in vivo, with the transcription factor, a
tagged version of the wild-type form of the Ylr266 protein was progressively expressed in vivo and covalently linked with formaldehyde to its target sites at different times. DNA fragments ranging from 1 to 3 kb long that were specifically cross-linked to Ylr266 were purified by immunoprecipitation. PCR analysis of immunoprecipitated DNA confirmed the specific association of Ylr266 with promoters of AZR1, CTT1, YOR1, YJL216c, and YLL056c (Fig. 3). The time-course analysis of the occupancy level of the different promoters is a good evidence of their direct in vivo interaction with Ylr266/Pdr8. The situation for the rest of the up-regulated group (Fig. 2A) is less clear-cut. Some genes are positive in Chromatin IP (SNG1, PDR15) whereas others are negative (GPH1, YGR052w). This further supports the necessity to associate ChIP and microarray analyses to identify direct target genes.

It is worth noting that most of the promoters that are positive in ChIP experiments contain sequences that are good candidates to be recognized by a zinc finger protein. Two more or less perfect CGG or CCG triplets repeated in dyads with a constant distance in between are a consensus signature of the binding site recognized by the Zn$_2$Cys$_6$ transcription factors studied so far. A sequence derived from TCCG(A/T/C)GGA is found in all but one of these promoters. The AZR1 promoter, which lacks this sequence, has nevertheless two closely related sequences TCCGCTGT and TCCGCGCT which are precisely localized in the promoter fragment which is positive in electrophoretic mobility-shift assays experiments (data not shown).

**A Function for Ylr266**—It is noteworthy that at least seven of the eight genes that are primary targets of Ylr266 are involved in stress response or multidrug resistance. This last function is carried out by ATP-binding cassette (YOR1, PDR15) or major facilitator superfamily (AZR1) membrane transporters. Other membrane proteins (SNG1) can also be involved. Such modifications of the plasma membrane induced by the activation of Ylr266 do suggest that the corresponding strain has altered drug sensitivity. We addressed this question using ketoconazole and oligomycin and testing serial strain dilutions (Fig. 4). Clearly, the production of Ylr266 confers resistance of the cell to both drugs. It is also very clear that the overproduction of Ylr266 leads to sensitivity to Na$^+$, Li$^+$, H$^+$, and to cationic drugs such as hygromycin B, whereas high concentrations of KCl have no significant effects (Fig. 4). All of these phenotypes are probably connected to membrane alterations induced by Ylr266. Because YIL121w, regulated by YLR266c, belongs to the H$^+$ antiporter DHA12 family, it is a good candidate to be involved in these phenotypes. On the other hand, resistance to ketoconazole or oligomycin are likely to be related to the overexpression of AZR1 and YOR1. These regulatory properties of Ylr266 are reminiscent of the phenotype controlled by PDR connected regulators (19), we then propose to name the ORF YLR266c as *PDR8*.

**CONCLUSION**

Studies of New Transcription Factors with Activated Chimeras and Microarrays—In this report we present the first evidence that the properties of a completely unknown yeast transcription factor can be inferred from its genomewide properties. Using global approaches, we could show that eight genes are directly regulated by Ylr266/Pdr8 which interact, in vivo, with their promoters. The drug-resistance phenotype observed when Ylr266/Pdr8 is either overproduced or activated is in agreement with the fact that at least seven of the eight regulated genes code for products involved in the properties of the plasma membrane. These functional properties of YLR266c substantiate the new name *PDR8*. We previously showed that known transcription factors of the zinc finger family like Pdr1 (6) or Yrr1 (5) can be engineered in a mini active form, keeping all of the DNA-binding specificity of the original protein. This is also the case for Pdr8, which presents further evidence that the short DNA-binding domain (in that case 173 amino acids long) of these zinc-finger transcription factors contain all of the specificity of the protein, the rest of the factor being probably involved in its regulation. This point is strongly supported by the chromatin immunoprecipitation experiments, which demonstrate that the wild-type form of Pdr8 can interact, in vivo, with the promoters of the genes activated by the engineered transcription factor, thus giving credence to our general experimental approach. Such a specificity of the DNA-binding do-

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**Fig. 3.** Promoter-occupancy of Ylr266 in vivo. Occupancy of a variety of promoters was measured in vivo by performing chromatin immunoprecipitation experiments with a α-HA antibody. Cells containing HA-tagged Ylr266 under control of GAL1-10 promoter were analyzed at different times after galactose induction and prior to immunoprecipitation (lane Start). Top lane, control corresponds to the same analysis of a strain carrying a vector devoid of the Ylr266c gene. The presence of promoter fragments listed at left was analyzed by the use of PCR and the appropriate primers (see “Experimental Procedures”). Each specific promoter analysis was conducted in parallel with the analysis of the promoter of YER184c, a promoter known to be independent of Ylr266. The results corresponding to this negative control are indicated by an asterisk. A quantitative value of the enrichment factor was obtained by taking into account the two experiments after 10 and 14 h, respectively, of protein production and the different negative controls (see “Experimental Procedures”).
main is all the more surprising because the suggested sequence features recognized by Pdr8 in the relevant promoters are very similar to those of the already characterized UAS_PDR8 for Pdr1 (20) or UAS_YRRE for Yrr1 (5). On the other hand, it is worth noting that no false-positive response arise from the numerous similar putative signals present in the genome. This again suggests that unknown elements of the local environment of these signals exert a discriminating role on the UAS recognition process. It is usually believed, as in the case of the well known zinc finger transcription factor Gal4, that chromatin obstructs most sites and leaves only a few relevant ones accessible (21, 22).

Plasma Membrane Modifications Induced by Pdr8—The Pdr8 target genes cluster in specific cellular functions mainly related to plasma membrane properties. Thus, all of the eight target genes, except CTT1, code for proteins that have at least one transmembrane segment. Some of these genes like AZR1 or YOR1 code for plasma membrane-localized proteins and most of them code for small molecule transporter like SNG1, or for active transporter involved in the multidrug resistance phenomenon like AZR1 (a member of the major facilitator superfamily) or like PDR15 and YOR1 (members of the ATP-binding cassette superfamily). In two cases, we have a better idea of the function of the transporter: YOR1 and AZR1 can confer resistance to oligomycin and azoles, respectively (23, 24). These features are in agreement with our observed phenotypes when Pdr8 is overproduced (Fig. 5). Interestingly, the set of genes regulated by PDR8 is distinct but overlapping with other PDR regulators.

Connections with the PDR Transcriptional Regulatory Network—Connections with other PDR regulatory networks show that PDR8 is an element of a large regulatory program designed to allow the cell to adapt to diverse environmental conditions. If the PDR8 network overlaps with the control of the regulation of CTT1 (the stress response program), it is, however, more involved in the drug response program. PDR1/ PDR3, YRR1, and PDR8 are four zinc-finger transcription factors for which the sets of direct target genes have been described (5, 6, 20, and this work). Important overlap exists between the three regulatory networks (Fig. 5). Strikingly, all of the promoters that are recognized by these four related transcription factors contain a signal sequence close to the UAS_PDR8 sequences that have been previously defined (6, 20). This is all the more surprising because each transcription factor activates a specific set of target genes. For instance, the PDR8 network is mostly included into the YRR1 network because five of eight genes are controlled by both Pdr8 and Yrr1. This may be connected to the close similarity between the different DNA-binding domains. The phenotypes conferred by the activation of either Yrr1 or Pdr8, however differ in agreement with the sets of genes that are overexpressed in these two conditions. Typically, genes like FMR1 or SNO2 which are activated by Yrr1 and not regulated by Pdr8, are involved in the resistance to benomyl (25, 26) or to 4-nitroquinoline-N-oxide (27). This is in agreement with the observed phenotypes of the strains activated either for Yrr1 or for Pdr8 and which are, respectively, resistant or sensitive to these two drugs. On the other hand, a common resistance to azole derivatives like...
ketoconazole could be observed in the two type of strains in connection, probably, with the fact that they both overexpress AZR1 (24). These are examples of the strong overlaps that characterize the regulatory networks controlled by Pdr1/Pdr3, Yrr1, and Pdr8. This however should not overlook the phenotype specificity controlled by each transcription factor. This family of binuclear cluster transcription factors appears to contain a battery of structurally related proteins that specifically control the responses of the yeast cell to its variable toxic environment. Recent systematic analyses of strains carrying deletions of zinc cluster genes have suggested that, directly or indirectly, one gene might be a repressor and seven new genes might be activators of the PDR phenomenon (28). It remains to determine how the regulatory networks of these twelve PDR-related transcription factors actually overlap and to decipher their own intricate regulation (8, 29).2 All of this information strongly suggests that the yeast S. cerevisiae has elaborated a highly sophisticated system to cope with the wide variety of biotic toxicants present in the environment. More generally, this study, which demonstrates the possibility to decipher the set of target genes directly connected to virtually any transcription factor, opens the way to a systematic analysis of the elementary networks that compose the yeast transcriptional network.

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