

Competitive Promoter Occupancy by Two Yeast Paralogous Transcription Factors Controlling the Multidrug Resistance Phenomenon^{*§}

Received for publication, August 28, 2003, and in revised form, September 24, 2003
Published, JBC Papers in Press, September 25, 2003, DOI 10.1074/jbc.M309580200

Ancuta Lucau-Danila, Thierry Delaveau, Gaëlle Lelandais, Frédéric Devaux, and Claude Jacq[‡]

From the Laboratoire de Génétique Moléculaire, Ecole Normale Supérieure, 46 rue d'Ulm 75230 Paris cedex 05, France

Highly flexible gene expression programs are required to allow cell growth in the presence of a wide variety of chemicals. We used genome-wide expression analyses coupled with chromatin immunoprecipitation experiments to study the regulatory relationships between two very similar yeast transcription factors involved in the control of the multidrug resistance phenomenon. Yrm1 (Yor172w) is a new zinc finger transcription factor, the overproduction of which decreases the level of transcription of the target genes of Yrr1, a zinc finger transcription factor controlling the expression of several membrane transporter-encoding genes. Surprisingly, the absence of *YRR1* releases the transcriptional activity of Yrm1, which then up-regulates 23 genes, 14 of which are also direct target genes of Yrr1. Chromatin immunoprecipitation experiments confirmed that Yrm1 binds to the promoters of the up-regulated genes only in yeast strains from which *YRR1* has been deleted. This sophisticated regulatory program can be associated with drug resistance phenotypes of the cell. The program-specific distribution of paired transcription factors throughout the genome may be a general mechanism by which similar transcription factors regulate overlapping gene expression programs in response to chemical stress.

Promoter occupancy by transcription factors is a critical step in establishment of the developmental program. In several cases, the transcription factor binds all its potential target genes, and signaling events at specific promoters are responsible for determining which genes are expressed. Alternatively, the regulation of target gene selection could occur at the level of DNA binding. New tools have opened up new opportunities for studying the program-specific distribution of transcription factors (1), revealing a high level of sophistication in the DNA binding program. Paralogous transcription factors often seem to interact with similar promoters, although little is known about the specific mechanism involved. Gene duplication, by conferring new evolutionary possibilities, provides an important source of diversity in the regulatory processes controlling gene expression. This is particularly true of the genes encoding transcription factors. The genome of the yeast *Saccharomyces*

cerevisiae contains a number of genes encoding transcriptional activators that exist as protein pairs (2). A few representative cases deserve special mention because their interesting properties have stimulated several studies. Pdr1 and Pdr3 have highly similar Zn finger domains and activate similar sets of target genes (3, 4) but these two factors are regulated differently as Pdr3 displays autoregulation whereas Pdr1 does not (5). Moreover, Pdr3 has been shown to be activated by mitochondrial signals (6, 7) whereas Pdr1 is under the regulatory control of the Hsp70 protein Pdr13 (8), which is now known as Ssz1 (9). Swi5 and Ace1 recognize the same DNA promoter sequences *in vitro* but transactivate different genes due to context effects and negative regulators (10). Aft2, like its paralog Aft1, is a transcriptional activator that responds to iron. However, Aft1 and Aft2 regulate the iron regulon to different extents (11–13). Cat8 and Sip4, two very similar transcription factors, are two CSRE-binding proteins that contribute unequally to the activation of genes containing the carbon source-responsive element (CSRE) in their promoters (14). Interestingly, Cat8 is required for the derepression of SIP4 under nonfermentative growth conditions (15). One of the most salient features of differential regulation of two similar transcription factors have been recently described for Msn2/Msn4 (16). The cAMP-PKA pathway was shown to control the sensitivity of Msn2/Msn4 oscillatory shuttling and, in the absence of PKA, Msn4 continues to oscillate whereas Msn2 remains in the nucleus. All these examples suggest that the evolutionary duplication of genes encoding transcription factors has resulted in factors controlling similar sets of target genes that are themselves differently regulated. However, this is not a hard-and-fast rule because Haa1, a protein homologous to the copper-regulated transcription factor Ace1, acts independently of the copper status of the cell and does not regulate the three genes activated by Ace1 (17).

In this study, we focused on a new pair of transcription factors, Yrr1/Yrm1, with alternative DNA binding properties. Yrr1 was recently identified as a Zn₂Cys₆ transcription factor involved in control of the pleiotropic drug resistance (PDR)¹ phenomenon (18). Gain-of-function mutations in its activation domain confer high level resistance to the cell cycle inhibitor reveromycin A, to the DNA-damaging agent 4-nitroquinoline-N-oxide and to oligomycin (19, 20). Yrr1 affects oligomycin resistance by activating *YOR1* expression via a region in the *YOR1* promoter that is very similar, but not identical, to the PDRE elements recognized by Pdr1/Pdr3 (19). An intricate interplay of cross-regulation links *YRR1*, *PDR1*, and *PDR3*.

^{*} This work was supported in part by CNRS Grant Puces à ADN 2003 and a PRFMMIP grant from the Ministère de la Recherche et de la Technologie. 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.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains Supplementary Materials.

[‡] To whom correspondence should be addressed. Tel.: 33-1-44323546; E-mail: jacq@biologie.ens.fr; Web address: www.biologie.ens.fr/lmgml.

¹ The abbreviations used are: PDR, pleiotropic drug resistance; PKA, cAMP-dependent kinase; HA, hemagglutinin; MHR, middle homology region; GAD, Gal4 activation domain; YRM, yeast reveromycin resistance modulator; ChIP, chromatin immunoprecipitation assay.

TABLE I
Saccharomyces cerevisiae strains

Strains	Genotype	Ref.
BY4742	MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0	EUROSCARF
Δ YRM1	MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0 YOR172W::kanMX4	EUROSCARF
W303-1B	MAT α ura3-52 ade2-1 leu2-3,12 his3-11,15 trp1-289	(20)
Δ YRR1	MAT α ura3-52 ade2-1 leu2-3,12 his3-11,15 trp1-289 YRR1::His6-URA3-His6	(20)
SEY6210	MAT α leu2-3, 112 ura3-52 his3- Δ 200 trp1- Δ 190 lys2-801 suc2- Δ 9 Mel ⁻	(19)
YRR1-gof	MAT α leu2-3, 112 ura3-52 his3- Δ 200 trp1- Δ 190 lys2-801 suc2- Δ 9 Mel ⁻ YRR1::3X HA-774	(19)

PDR1 regulates *PDR3* and *YRR1*, both of which regulate their own expression (5, 19). In addition, different post-translational processes, including nuclear targeting or heterodimer formation (21), may be involved in different regulatory pathways. Genome-wide analyses have established that the sets of target genes directly regulated by these three transcription factors display considerable overlap (3, 4, 20). Most of these co-regulated genes encode for proteins involved in the structural organization of the plasma membrane. These proteins include ABC and MFS transporters, together with proteins involved in the control of specific steps in lipid biosynthesis. Accordingly, the drug resistance phenotypes conferred by the gain-of-function mutations for the three transcription factors reflect changes in plasma membrane composition. Thus discrete changes in plasma membrane properties can be accomplished through sophisticated cross-regulations between several transcription factors, not all of which have been identified (22, 23).

YOR172W, referred to here as *YRM1* (for yeast reveromycin resistance modulator) is homologous (41% identities) to *YRR1*. The degree of similarity is higher in the zinc finger domain, but the two proteins display considerable similarity throughout the entire length of their sequences. The sequences in the Cys regions are quasi-identical suggesting that the two factors recognize similar DNA binding motifs. Microarray experiments were carried out to determine whether Yrm1 was a transcription factor and to identify the corresponding direct target genes. The situation turned out to be more complex than expected: Yrm1 acts as a transcription factor and interacts with the promoter of the target genes only in the absence of Yrr1. The sets of target genes directly regulated by *YRR1* or *YRM1* are similar, but not identical. Alternative promoter occupancy thus plays a key role in this cross-regulation adding a new degree of complexity to the cell drug response.

EXPERIMENTAL PROCEDURES

Strains and Media—*Saccharomyces cerevisiae* strains are described in Table I. Cells were grown in minimal synthetic medium SC (0.67% yeast nitrogen base, 2% carbon source [glucose, galactose, or glycerol+ethanol] supplemented with appropriate amino acids). Drug resistance assays were performed by spot tests with serial dilutions. *Escherichia coli* TG1 (K-12*(lac-pro) supE thi hsdD5/F' traD36 proA⁺B⁺lacI^q lacZ^{*}M15) was used for plasmid constructions.

Plasmid Construction—pYES2-*YRM1* was obtained by homologous recombination in yeast of NotI-digested pYES2 (URA3, AmpR, 2 μ , *GAL1* promoter, three HA epitopes, and the CYC1 terminator) with PCR-amplified *YOR172W* ORF. For PCR amplification of *YOR172W* we used the genomic DNA from the BY 4742 strain and the following primers: 5'-TCCTATCATATGACGTTCCAGATTACGCTGCTCAGG-TGAGTAAGCGGGGTAGTTACAG and 3'-AACTAATTACATGATCGG-GCCCTCTAGATGCTGCTCTACTGCGTATCAAATAAATA. The inserts from six independent clones were checked by digestion and restriction mapping. Also their tested phenotype was the same. The expression of the cloned gene from two independent clones was checked by Western blot analysis (24), using mouse anti-HA IgG as the primary antibody (Babco) and anti-mouse IgG horseradish peroxidase-conjugated as the secondary antibody (Promega). The signal was visualized using the ECL kit (Amersham Biosciences).

The chimeric construct *YRM1**GAD was generated by inserting into pCB-GAD (4) the first 381 nucleotides of the *YOR172W* open reading frame. This sequence was amplified by PCR and inserted into the NotI site of pCB-GAD by homologous recombination in the Δ YRM1 strain.

The nucleotides used for PCR amplification and homologous recombination were: 5'-GACGTCCCGGACTATGCAAGGCCTGTTCCATCACACG-TGAGTAAGCGGGGTAGTTACAG and 3'-CTTTTGGAGGCTC-GGGAATTAATTCCGCTGCATGTCCCGAGCCTTTACATTGTAATAA. The insert was sequenced and production of the chimeric protein checked by Western blotting, as shown for pYES2-*YRM1*.

Microarray Experiments—The activity of the entire or chimeric Yrm1 protein, produced under control of the *GAL1* promoter, was induced on galactose medium by growing cells in glucose minimal medium to an OD₆₀₀ 0.5 and transferring them to galactose minimal medium for various times of induction. We carried out progressive galactose induction of pYES2-*YRM1* in the Δ YOR172W, Δ YRR1, and *YRR1*-gof strains and of the *YRM1**GAD in the Δ YOR172W and in Δ YRR1 strains for periods of 30' to 14 h. Cells were harvested by centrifugation, flash-frozen in liquid nitrogen, and stored at -80 °C. Total RNA was isolated and used to synthesize and to label cDNA as described on our web site (www.biologie.ens.fr/microarrays.html).

We performed a total of 12 independent microarray experiments, each of which was repeated at least once. Microarrays containing all the open reading frames of *S. cerevisiae* were produced in our laboratory with an Omnigrid II Biorobotics robot (www.transcriptome.ens.fr/sgdb). They were based on the principal of 40-mer oligonucleotides from MWG (www.mwg-biotech.com) covalent deposited onto Corning glass slides coated with pure gamma amino propyl silane (www.corning.com/life-sciences). Repeat experiments were carried out with microarrays obtained from Hitachi Software and Eurogentec S.A. The microarray protocol used is described on our web site (www.transcriptome.ens.fr/sgdb). A total of 20 μ g of total purified RNA was used for each experiment. In each experiment, the cDNA corresponding to cells expressing pYES2-*YRM1* or *YRM1**GAD was labeled with CY5-dUTP and cDNA from control cells was labeled with CY3-dUTP. The arrays were read using a Genepix 4000A scanner (Axon) and analyzed with Genepix 3.0 software. Artifactual, saturated or low-signal spots were eliminated from the analysis. Fluorochrome-channel normalization was carried out with Arrayplot (25). Up-regulated genes were selected with a custom-made data base, and the analysis was completed with the PCA module of the J-express program (26). Clusters were generated by Treeview (27).

Northern Blot Analyses—Cells were grown on minimal synthetic medium with glucose as carbon source supplemented with corresponded amino acids, to an OD of 0.5, harvested, and transferred to minimal synthetic medium with galactose as carbon source for various lengths of time, from 0 to 14 h. Total RNA, prepared by hot acidic phenol extraction, was used for Northern blot analysis. *AZR1*, *SNQ2*, *YOR1*, *YRR1*, *SNG1*, *FLR1*, *YLL056C*, *YGR035C*, *YPL088W*, *YLR046C*, *YMR102C*, *PLB1*, *APD1*, *YLR179C*, *SFK1*, *YPL127W*, *YJL216C*, *PDR16*, *YLR346C*, *SCS7*, *YOR084W*, *TPO4*, *YDR061W*, *ADH7*, and *YBR161W* were used as probes, PCR-amplified using gene-specific oligonucleotides and genomic DNA isolated from strain BY4742 and randomly labeled with ³²P. Blots were performed using standard procedures (28).

Chromatin Immunoprecipitation Assay—Two forms of Yrm1p were analyzed by ChIP assay: the entire protein produced from the pYES2-*YRM1* construct and the chimeric protein produced from *YRM1**GAD, both of which producing Yrm1p under the control of the *GAL1* promoter. Cells were grown on minimal synthetic medium, with glucose as carbon source supplemented with corresponded amino acids for maintaining of pYES2 or pCB*GAD plasmid to an OD of 0.5, harvested, and transferred to minimal synthetic medium with galactose as carbon source for 6 h. DNA-binding proteins were cross-linked to DNA with formaldehyde *in vivo* (treatment for 15 min at room temperature). The chromatin was isolated by grinding with glass beads followed by sonication to shear DNA along with bound proteins into small fragments (1–3 kb). A 20- μ l aliquot of the lysate was saved as the input fraction. To isolate the DNA-Yrm1 complex, the samples were incubated with mouse anti-HA monoclonal antibody (Babco), and with UltraLink

TABLE II
Primer sets used for the PCR amplification of promoter genes in the ChIP assay

Promoter gene	Upstream primer	Downstream primer
AZR1	TGCACTGTTACCATAGGATCTTACC	TTAATGTAGTGGTTCTGCTTGACAC
YLL056C	TAGAGAGGACGGACATAAGTCTTC	TCTTCAGGAAAGCCAAGAATAATG
YOR1	ACACATCTGTCAGAGGTAGCTAGC	GTATGGAAATGCGTTCTTCTCTGTT
YGR035C	AGACCCCTTTCCACGGATAATCAG	ATTGCCCTCAAACTGTATCTCATC
SNG1	TCACCACAATAGGTGGATCTC	GAAGACGCTTATGACTACGC
YPL088W	GTGGAAGTATTTACCCACGTATG	AGTTATCTCCGTTCCCTAACCTAC
YLR046C	TGGTGATAGTTGCGAAAAATGACAG	GAGGCAGTTGAGTTCTTTTATCGAC
YMR102C	CCTTTCTTAAGCTTCGTAAGCGAG	CCTCCTCTGTTTAAACAAGTTGGAG
PLB1	CCTCATACAGTAACATGTCCGCTT	CGTTTCCGTTGGTTAAATGTGGTG
YLR179C	GTGCCAAGCGTGCTCATTATTGGA	TTACTAAGTACTCGGATGGGTACC
APD1	CAAGTGCAAGGAGAGGGAGAGTTT	CCTCCTCCGAATATAGCCAAGTAC
SFK1	CTCTAAACATCTTCCACGGCTGTTT	CTAGATGACAAATCTTCCAGGCTG
YLR346C	CACCTGCTGAATGCTCAACGTTTG	TAGTCCTCTGTGAATGAGCATCAC
SNQ2	ACCTTCACGCCAGACTATGTATGAC	GGATTTAAACGGAATGGGCGGAC
FLR1	CTTAGGTAAAGGAGCAATAACAGTGC	GCAATTCTCGTCTTCTTCTCTGTT
YJL216C	TCTGGAATTTCTCTCCTTCTC	GGATTGTGACTATTTCAATGG
YPR127W	AGGAAGGGATCTGCGTTTTTGTTC	CTATTCTACGGTTCAATAGACACTC
PDR16	GAGTGCAAGCATGCGATATATCTC	CAGACAATGAGCACAATATTGTGCA
SCS7	GACAACAGGGAAAGTTGGACAAAC	CTGCGAAAAGATGTGCACACGTCAG
YOR084W	TGTCGAAACACTGATGCTATTGTC	CTCGTACTACCACATTAAGACGTC
TPO4	TGCTTACATGCTAATGTCTCTGTC	ACTAGAAAATCGATGTGTGTCAGGTAC
YDR061W	CCTTGAAGCGAAAGACGCATATCA	TCGTCTCAGCATGTGTCAGCAATCTC
ADH7	AGAGTTGAAGGGCGAACATTTGC	GGTGCTCAATTAGTGACATCTGC
YBR161W	ACTTGTCACCGTTGAATAACCGAG	CTACTTTAGCACTGTTGTAAGGTG
YRR1	GCGAATGTAGATTTCTGCCAGTATC	GTCTATTACATACGCTAGTGATGTGG
YOR172W	CGGTCAAGATAGCCCTAAGTT	CCAGGACCATTCAATGTGCTT
Control-cz V	GGCTGTCTAGAATATGGGGCCGTAGTA	CACCCCGAAGCTGCTTTTACAATAC

Immobilized Protein G (Pierce). Immunoprecipitates were extensively washed and centrifuged to recover a pellet (bound) and supernatant (unbound). Protein was eluted from the Sepharose beads by heating at 95 °C for 20 min. Cross-links were reversed by heating at 65 °C for 6 h. The DNA was purified with PCR QiaQuick columns prior to PCR analysis. Both input and bound DNA were dissolved in 40 µl of TE. 1 µl of DNA preparation from each reaction was used for PCR. The Qiagen QuantiTect SYBR Green PCR kit was used for quantitative real-time PCR. We used LightCycler3 and repeated experiments at least twice. The various primer sets used for amplification of the 27 promoters are presented in Table II. The enrichment factor was calculated as follows: 1) the values of immunoprecipitated amplified DNA were divided by those for the negative control (a non-coding fragment of chromosome V) included in each analysis, and their mean values retained as the crude promoter occupancy value, 2) the ratio of amplified unbound DNA to negative control constituted the background value. The enrichment factor was obtained by dividing the crude promoter occupancy value by the background value.

RESULTS

Yrm1 Can Act as a Specific Inhibitor of Yrr1—We recently described the genome-wide regulatory properties of YRR1, a gene encoding a Zn₂Cys₆ zinc finger transcription factor (20). We showed that 15 genes, mostly encoding plasma membrane proteins, are directly up-regulated by various mutated forms of Yrr1. One of these forms consisted of a chimeric protein containing the zinc finger domain of Yrr1 linked to the Gal4 activation domain (GAD). This short chimerical protein named Yrr1*GAD contains the specific DNA binding domain (positions 1–178), devoid of inhibitory activity known to be contained in the central flanking domain, fused to GAD as described (7, 20). This hybrid transcriptional activator has the physiological activity and the DNA binding domain of the natural activator.

As YRM1 encodes for an unknown protein with a very similar zinc finger domain, the two proteins might be expected to display similar DNA binding functions. We investigated this issue by first characterizing the properties of the intact form of Yrm1. To this end we constructed a GAL1/YRM1 fusion gene to enable galactose-inducible expression of YRM1. The episomal fusion gene containing the entire YRM1 open reading frame was transformed into a yrm1-deleted strain. Cells pregrown in glucose to mid-log phase were induced with galactose and har-

vested at various times after the addition of galactose. YRM1 expression was followed by Western blot analyses, using the HA epitope inserted at the C-terminal part of the protein (data not shown). Cells transformed with a control vector devoid of the YRM1 open reading frame were used as reference for transcriptome analyses with microarrays containing oligonucleotide probes for most of the *S. cerevisiae* genes. Four independent microarray analyses were conducted. Principal results are shown in Fig. 1A. To our surprise, all 14 genes known to be up-regulated by YRR1 (20) turned out to be repressed when YRM1 was overexpressed. Moreover the level of YRM1-dependent repression seemed to parallel YRR1 activation. Northern blot analyses were carried out to validate the microarrays data (data not shown). The observed phenotype (Fig. 1B) of the strain overexpressing YRM1 was consistent with low levels of expression for several genes. Many studies have pointed out the correlation between the level of expression of genes encoding membrane proteins and the drug-specific resistance phenotype (29). In our case the low levels of SNQ2 expression may be connected to the weak but significant decrease in growth on 4-nitroquinoline-*N*-oxide because SNQ2 is known to confer resistance to this drug (30). A similar situation has been reported for YOR1, which is required for oligomycin resistance (31).

Yrm1 Also Decreases the Transcriptional Activity of a Gain-of-Function Mutant of Yrr1—Consistent with the observed effects on the transcriptional activity of YRR1, YRM1 can reduce the gain-of-function phenotype of YRR1. A gain-of-function allele of YRR1 (YRR1::3XHA-730) resulting from insertion of three HA tags in the C-terminal region of Yrr1, has been described (19) and the corresponding transcriptome characterized by microarray analyses (20). We overexpressed YRM1 in a strain carrying the genomic version of YRR1::3XHA-730; the corresponding transcriptome displayed a clear reduction in expression of the 15 genes previously found to be up-regulated by YRR1 (Fig. 2A). Accordingly, this strain presented a phenotype more responsive to relevant drugs, such as 4-NQO (Fig. 2B). This is consistent with the large decrease (Fig. 2B) in SNQ2 mRNA levels following the expression of YRM1, whereas YOR1 mRNA level was only slightly affected, consistent with the slight modification in oligomycin resistance.

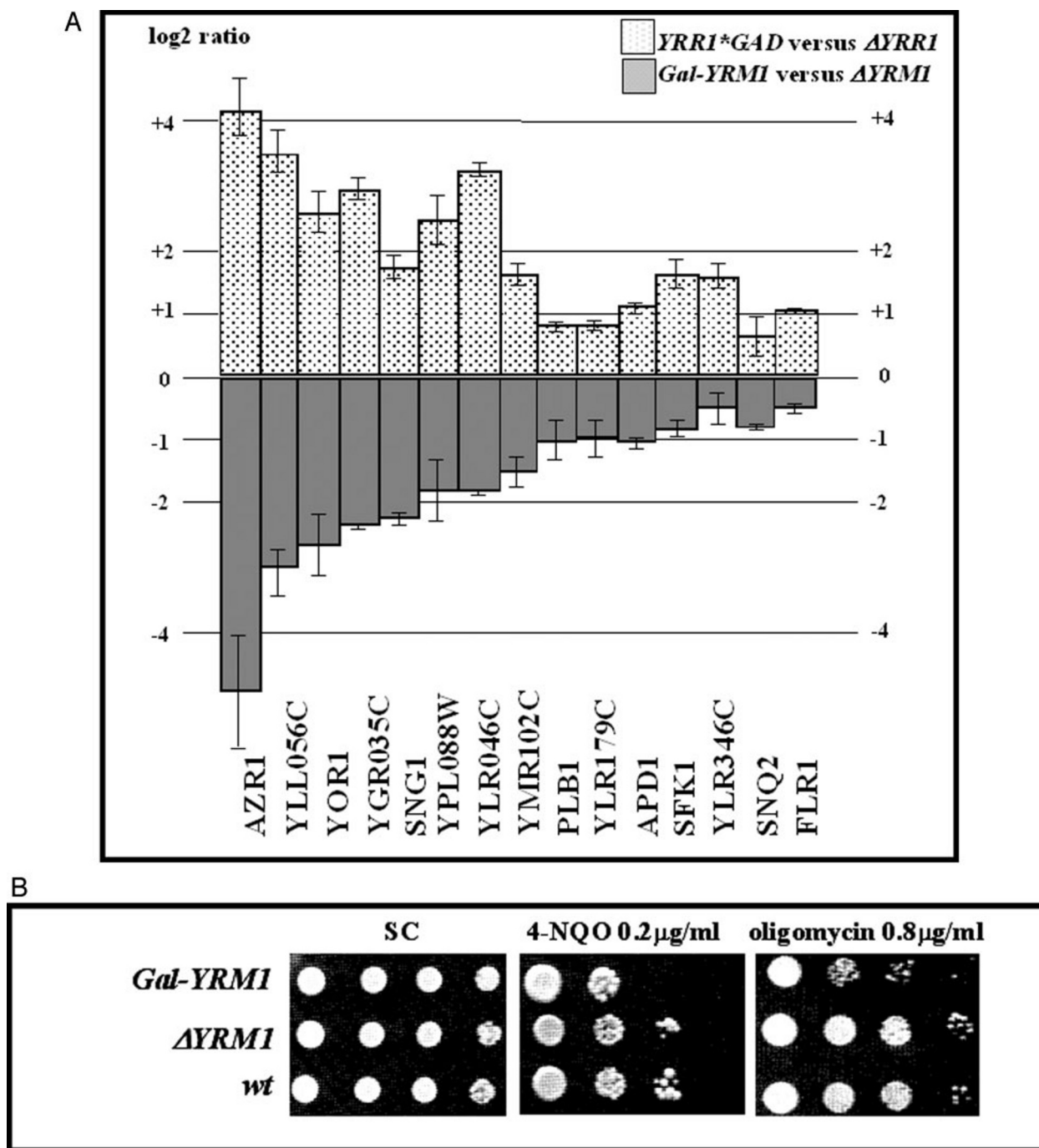


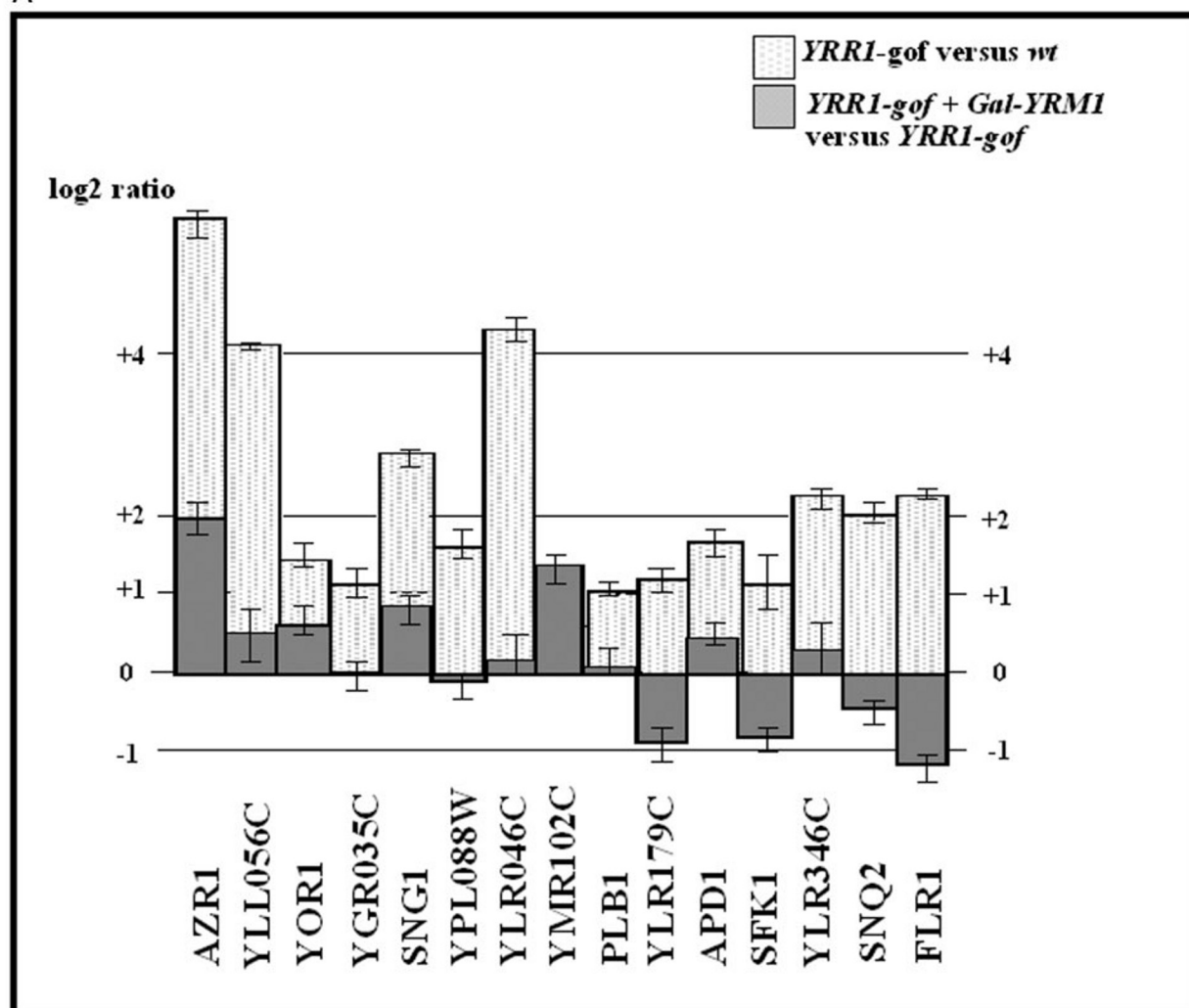
FIG. 1. *YRM1* overexpression results in the repression of genes that are activated by *YRR1*. A, schematic results of microarray experiments are presented. Overproduction of the Yrm1 protein in the wild-type cells reduces the expression of all genes directly up-regulated by the chimerical protein Yrr1*GAD, as previously reported (20). B, for phenotypic analyses, cells were grown on minimal synthetic medium with galactose as carbon source supplemented with amino acids for plasmid maintenance, to an optical density at 600 nm of 0.6. Four serial 1:10 dilutions were prepared and plated on media with the indicated drug concentrations. For oligomycin resistance analysis, cells were plated on minimal synthetic medium with glycerol and ethanol as carbon source. The overproduction of Yrm1 slightly reduced the growth rate on 4-NQO or oligomycin (B), consistent with the decrease in mRNA level for *SNQ2* and *YOR1* (A, left).

In the Absence of Yrr1, Yrm1 Activates the Transcription of Most of the Genes Regulated by Yrr1—Genome-wide expression analysis of a strain deleted for *YRR1* and expressing *YRM1* under the control of the *GAL1* promoter revealed (Fig. 3A, second column) that 23 genes are significantly up-regulated; 14 of them are known to be direct target genes of Yrr1, and 8 genes are specifically regulated by Yrm1. A very different situation was observed in the presence of *YRR1* (Fig. 3A, first column) in which all these genes were repressed. These two situations, in which *YRM1* displayed such different properties, are isogenic,

differing only in terms of the presence (left) or absence (right) of *YRR1*. Taken at face value, this result suggests that Yrm1 and Yrr1 reciprocally inhibit each other. However, the reciprocal effect is not equivalent. The key findings may be summarized as follows: 1) *YRM1* requires the total absence of *YRR1* to function as an activator (Fig. 3A, **). 2) *YRR1* can activate its cognate target genes even in the presence of *YRM1* (Fig. 1A). 3) The overexpression of *YRM1* reduces the activity of *YRR1* (Figs. 2A and 3A*).

The phenotype of the strains carrying the two genetic con-

A



B

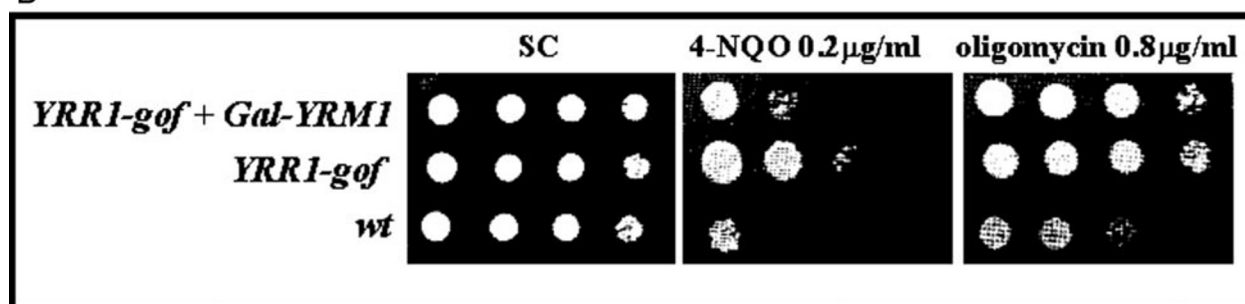


FIG. 2. *Yrr1* activity up-regulating its target genes is decreased if *YRM1* is overexpressed. A, compared with the wild-type strain, the genes directly up-regulated by a gain-of-function allele of *YRR1* are represented by gray bars (according to Ref. 20). The same microarray analysis conducted in the presence of Yrm1 overproduction leads to the results represented by dark bars. B, the phenotypes of the two strains, the transcriptome variations of which are shown above, are represented. Analyses were conducted as described in Fig. 1. The drug resistance phenotype conferred by the gain-of-function mutant (*YRR1-gof*) is modified if the Yrm1 protein is produced in the mutant strain (B, upper line). This modification is more visible with 4-NQO, consistent with the strong effect of Yrm1 on the expression of *SNQ2* (A).

texts is consistent with these molecular data. For instance, if Yrm1 is produced in the absence of *YRR1*, *YOR1* is up-regulated (Fig. 3A), and the strain can grow in the presence of oligomycin (Fig. 3B).

Specific Promoter Occupancy by Yrm1 in Vivo—To address the mechanism by which *YRM1* activates its target genes, chromatin immunoprecipitation experiments were carried out to assess the *in vivo* DNA binding properties of Yrm1 in the two different genetic contexts: presence and absence of *YRR1*. A

tagged version of Yrm1, which was first demonstrated to be transcriptionally active, was translated for 6 h from the episomal version of the entire gene. Control experiments were conducted to ensure that the results presented in Fig. 3A were reliable (see “Experimental Procedures”). Enrichment factor analyses (left and right columns of Fig. 3A) clearly indicated a difference between the DNA binding properties of Yrm1 in the presence and absence of *YRR1*. In the absence of *YRR1*, Yrm1 binds to most of the promoters of genes that are also Yrr1

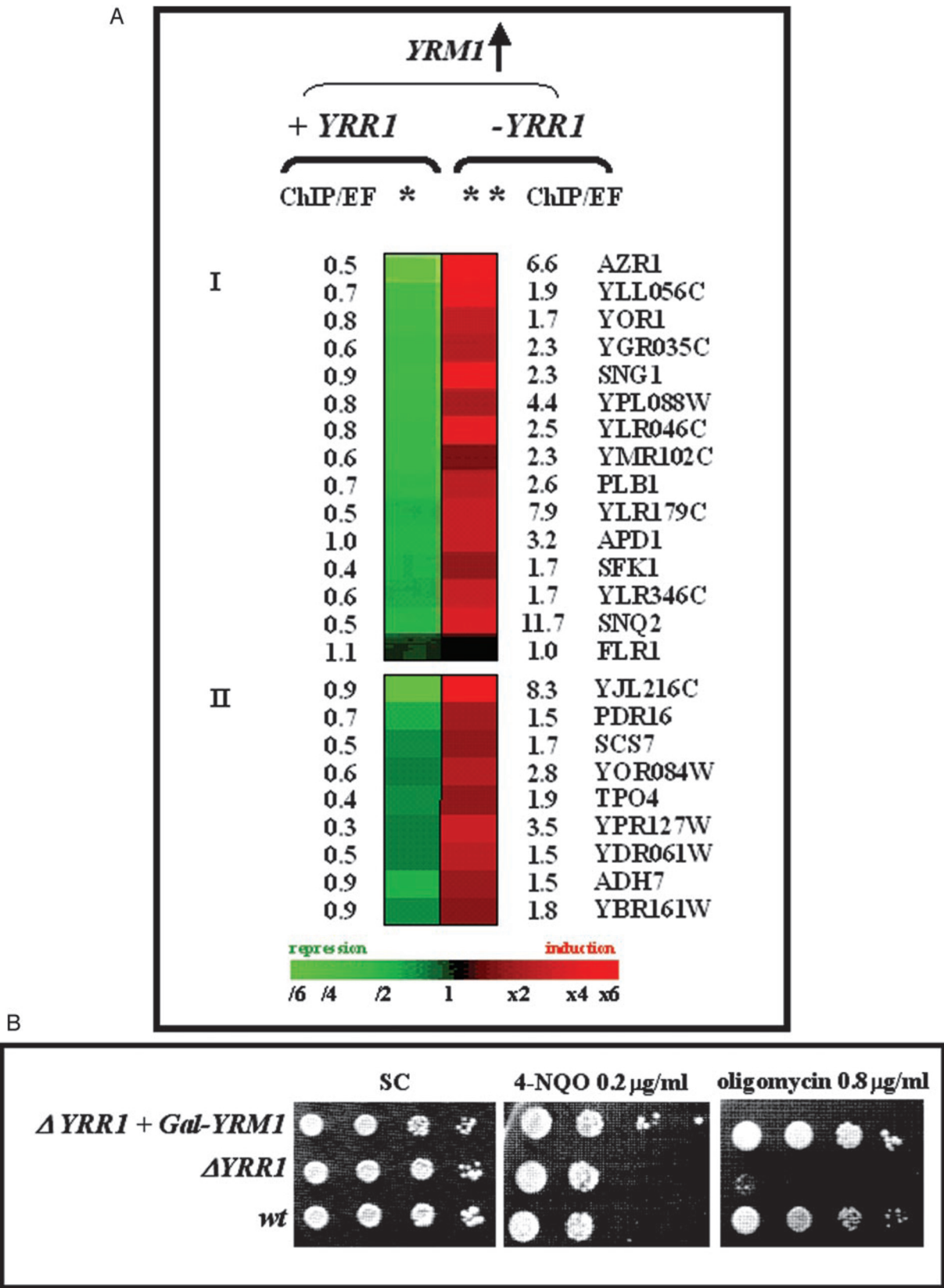


FIG. 3. In the absence of Yrr1, Yrm1 acts as a transcription activator that partially mimics the properties of Yrr1. A, microarray analyses of the effects of Yrm1 production, in the presence (*) or absence (**) of Yrr1, on the genes known to be regulated by Yrr1 (I) or on the genes specifically regulated by Yrm1 (II). Repression (green) or activation (red) effects are recorded by Treeview (27). Chromatin immunoprecipitation analysis results presented in the left and right columns (ChIP/EF) were carried out to assess the *in vivo* DNA binding properties of Yrm1 on the various promoters in the presence (*) or absence of Yrr1 (**). The mean enrichment factors have been calculated from two independent experiments (see "Experimental Procedures"). *, CY5-ΔYRR1 strain overexpressing pYES2-YRM1 (YRM1 ↑) after 6 h of galactose induction versus the CY3-ΔYRR1 strain containing the empty pYES2 plasmid. **, CY5-ΔYRR1 strain overexpressing pYES2-YRM1 (YRM1 ↑) after 6 h of galactose induction versus the CY3-ΔYRR1 strain containing the empty pYES2 plasmid. B, overexpression of YRM1 restores the drug resistance phenotype in a strain deleted for YRR1 (phenotype analyses as in Fig. 1).

target genes (20). *FLR1* is the only Yrr1 target gene for which the promoter is not recognized by Yrm1. This finding is consistent with the lack of activation of *FLR1* by Yrm1 in this genetic context.

The DNA Binding Domain Properties of Yrm1 Are Insensitive to the Presence of Yrr1—Yrm1 seems to act as a *bona fide* transcription factor, except in the presence of Yrr1. This raises the question as to whether specific regions of Yrm1 are involved in the inhibition by Yrr1 of the binding of this molecule to DNA. We have found that several Zn₂Cys₆ zinc finger transcription factors have a DNA binding domain that controls the specificity of the whole protein. Construction of a chimeric protein composed of the DNA binding domain fused to a heterologous activation domain, such as that of Gal4, results in a constitutively activated transcription factor that up-regulates the same genes regulated by the complete protein (4, 32). We created a similar construct, encoding Yrm1 containing the 127 amino acids of the DNA binding domain and the 103 amino acids of the Gal4 activation domain. The corresponding gene, *YRM1*GAD* was expressed under the control of the *GAL1* promoter, and time course analyses of transcriptome variations were carried out by microarray analyses. The results of duplicated experiments carried out seven different times for *YRM1*GAD* expression are presented in Fig. 4. Principal component analysis (Fig. 4A) clearly identified the genes up-regulated by Yrm1. The cluster of the most strongly up-regulated genes in the wild-type strain is presented in Fig. 4B and compared with that for the strain deleted for *YRR1*. In contrast to what was observed with the complete form of Yrm1, the presence or absence of *YRR1* had no significant effect. We also checked that the chimeric protein Yrm1*GAD interacted with the corresponding promoters of the activated genes. The results of chromatin immunoprecipitation experiments (Fig. 4B, right) were very similar to those obtained with the complete form of Yrm1 in the absence of *YRR1* (Fig. 3A). The ChIP enrichment factor was low (between 1 and 1.5) for some promoters even when the corresponding genes are up-regulated, possibly reflecting an indirect activation or weak *in vivo* binding affinity. Both time course expression analyses and chromatin immunoprecipitation studies showed that at least 23 genes were actually direct target genes of Yrm1.

The physiological properties of the yeast producing the chimeric Yrm1*GAD protein are consistent with the above observations. Clearly (Fig. 4C), even in the presence of *YRR1*, Yrm1*GAD can activate a gene expression program that confers resistance to both 4NQO and oligomycin. The specificity of Yrm1, in the absence of Yrr1, was fully conserved in its DNA binding domain. This was true for the nine genes, *YJL216C*, *PDR16*, *SCS7*, *YOR084W*, *TPO4*, *YPR127W*, *YDR061W*, *ADH7*, and *YBR161W*, activated by Yrm1 only and for the *FLR1* gene, which is activated only by Yrr1. Northern blot analyses were performed to confirm the regulation properties of the chimeric protein Yrm1*GAD, and the results were fully consistent with the microarray data (Fig. 4D).

DISCUSSION

Yeast cells, which have to cope with a large variety of chemical environments, have had to develop a wide panel of regulatory processes. Gene duplications undoubtedly served as a rich source for the creation of regulatory processes mimicking the original function but with new alternative outcomes. We describe here the case of two very similar transcription factors, *YRR1* and *YRM1*, which share the transcriptional activity of their putative common precursor but which have evolved to cross-regulate their own activities. As a result, the total absence of *YRR1* releases the transcriptional activities of *YRM1*, a new yeast transcription activator that could not have been

detected in a wild-type context. Alternatively, in the presence of either a wild-type or a gain-of-function allele of *YRR1*, *YRM1* decreases the level of expression of all the *YRR1* target genes. Many regulators are themselves transcriptionally regulated, but in this case, the deletion of *YRM1* does not stimulate the expression of *YRR1* (data not shown). Engineered forms of Yrm1 that had lost the central regulatory region displayed indifference to the presence of Yrr1, strongly suggesting that a post-transcriptional process is involved in the negative regulation of Yrm1.

Properties of the DNA Binding Region of YRM1—The genome-wide analyses presented here demonstrate that the DNA binding region contains all the information required to guide *in vivo* discriminating recognition of the correct promoter sequences. This finding is consistent with those of several previous analyses with the DNA binding regions of other members of the C6 zinc cluster family (4); (32); (20). Time-course analyses of the genes regulated by Yrm1 and chromatin immunoprecipitation analyses have revealed the genes directly activated by Yrm1. Interestingly, the vast majority of these genes are also up-regulated by activated forms of Yrr1 (20). This is consistent with the strong similarity between the two DNA binding regions and strongly suggests that the central regions of both proteins are involved in their global regulation. As a matter of fact the *in vivo* analyses of the complete form of Yrm1 have shown that the presence of the central region abolishes the DNA binding properties of the zinc cluster module in the presence of Yrr1. This inhibition may be mediated by a direct interaction between Yrm1 and Yrr1. Several conserved hydrophobic peptides in the central middle homology region (MHR) region are currently being tested to determine their putative role in this interaction (data not shown).

Several studies, for which Gal4 structure-function studies are the prototype, have shown that the C6 zinc cluster family of yeast transcriptional regulators contains an MHR between the C6 zinc cluster and the activation domain (33). It has been suggested that this central region assists the C6 zinc cluster in DNA target discrimination. We show here a more spectacular effect for Yrm1 as the central region, probably through interactions with Yrr1, completely abolishes the DNA binding properties of Yrm1.

Parallel Evolution of YRR1 and YRM1—Our observations suggest a simple evolutionary scenario in which *YRM1* and *YRR1* have conserved the DNA binding properties of their common ancestor while diverging in their central regulatory region. Three-dimensional models of the DNA binding domains of Yrm1 and Yrr1 have shown these domains to be very similar (data not shown). This is clearly connected to their recognition of the same set of 14 promoters. Moreover, the ability of Yrm1 to recognize nine additional promoters is also controlled by the DNA binding domain, which is the only part of the protein present in the engineered chimeric form (Fig. 4B). Thus, the central part of Yrm1 is involved exclusively in negative cross regulation with Yrr1. Interestingly, recent comparative genomic analyses conducted on three *Saccharomyces* species (*S. paradoxus*, *S. mikatae*, and *S. bayanus*) (34) revealed that proteins similar to Yrm1 and Yrr1 have conserved these structural features. This strongly suggests that what could be considered a marginal regulation process has in fact been conserved over 5–20 million years of evolution.

A New Level of Regulation for the Yeast Multidrug Resistance Process—The alternative binding of Yrm1 to cognate promoters and inhibition of the activity of Yrr1 by Yrm1 reflect the high level of complexity of the regulatory processes controlling plasma membrane properties and related drug-resistance phenotypes. This switching between Yrm1 and Yrr1 for DNA bind-

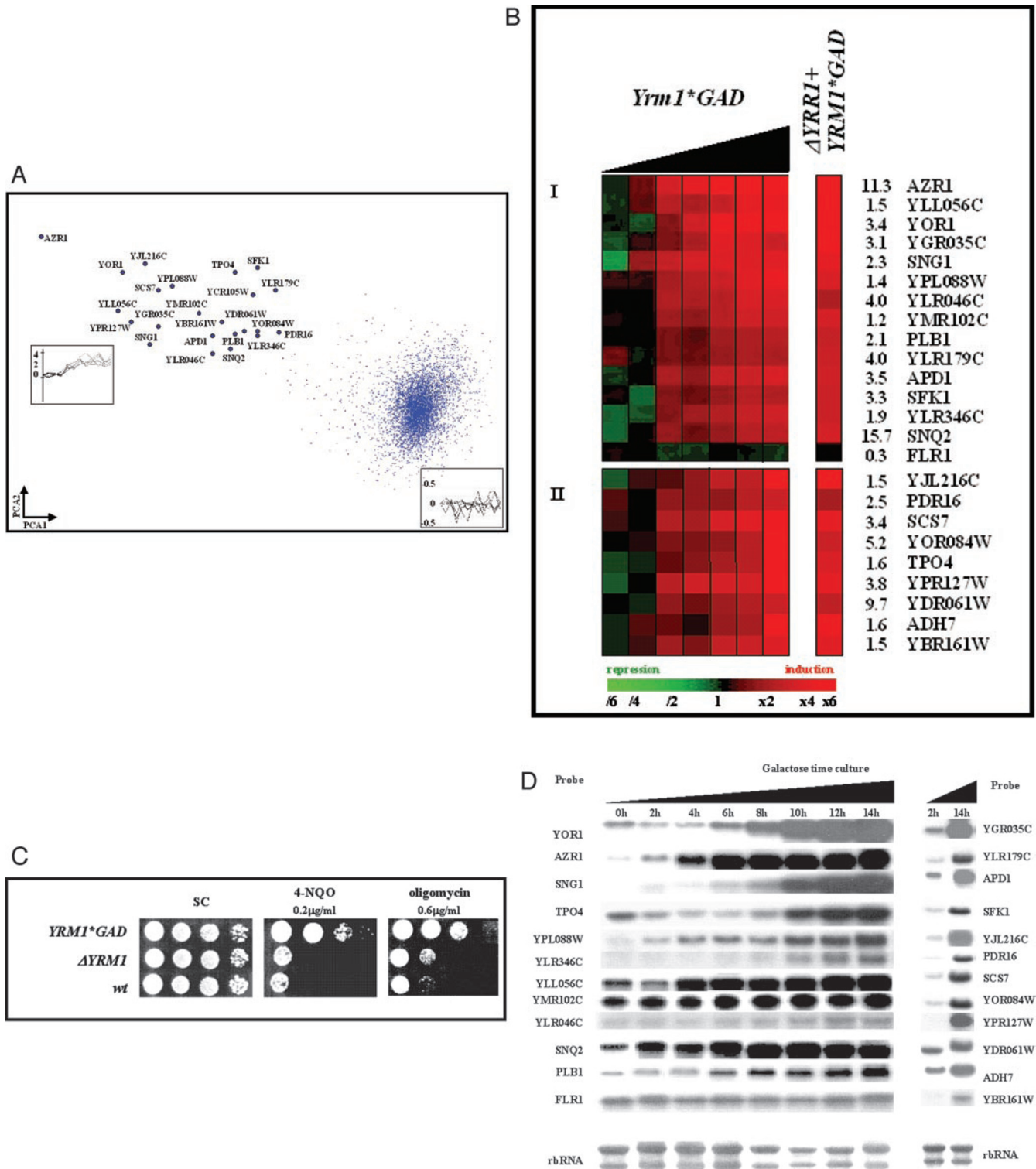


FIG. 4. The artificial chimeric protein Yrm1*GAD acts as a Yrr1-like transcription activator, even in the presence of Yrr1. A, the microarray results of time-course production of chimeric protein were analyzed by PCA, using J-express software (26) using 34.64% variance for the principal component nr.1 (PC1) and 14.69% variance for the principal component nr.2 (PC2). This analysis of 14 independent experiments clearly distinguishes the group of activated genes (left) from the bulk of the genes (right). The up-regulated genes are distributed along a gradient following the kinetic response (left square). Contrary, the rest of the genes do not respond to the time course expression of the chimera (right square). B, the chimeric gene YRM1*GAD encodes for the Yrm1 zinc finger DNA binding domain fused to GAD. It is expressed under the control of the GAL1 promoter. Time course production of this chimeric protein corresponding to 30 min, 4 h, 6 h, 8 h, 10 h, 12 h, and 14 h of galactose induction, and cluster analyses of the first activated genes are shown representing the results of 14 independent experiments. The last column shows the results of transcriptome analysis when YRM1*GAD was expressed for 10 h in a strain deleted for YRR1. The repression (green) or activation (red) effects were recorded with Treeview (27). The right-hand part of the figure presents the results of chromatin immunoprecipitation experiments conducted with the chimeric Yrm1*GAD protein and the promoters of the up-regulated genes. The chromatin immunoprecipitation enrichment factors (ChIP/EF) represent the mean values of at least two independent experiments and were calculated as indicated under "Experimental Procedures." C, phenotype analyses of strains producing the chimeric protein Yrm1*GAD. The drug resistance phenotypes of strain producing the chimeric protein in the presence of Yrr1 are presented and compared with the phenotype of the strain deleted for YRM1. D, Northern blot analysis in strain containing YRM1*GAD encoding the Yrm1 zinc finger DNA binding domain fused to GAD. The experiments were carried out as reported under "Experimental Procedures."

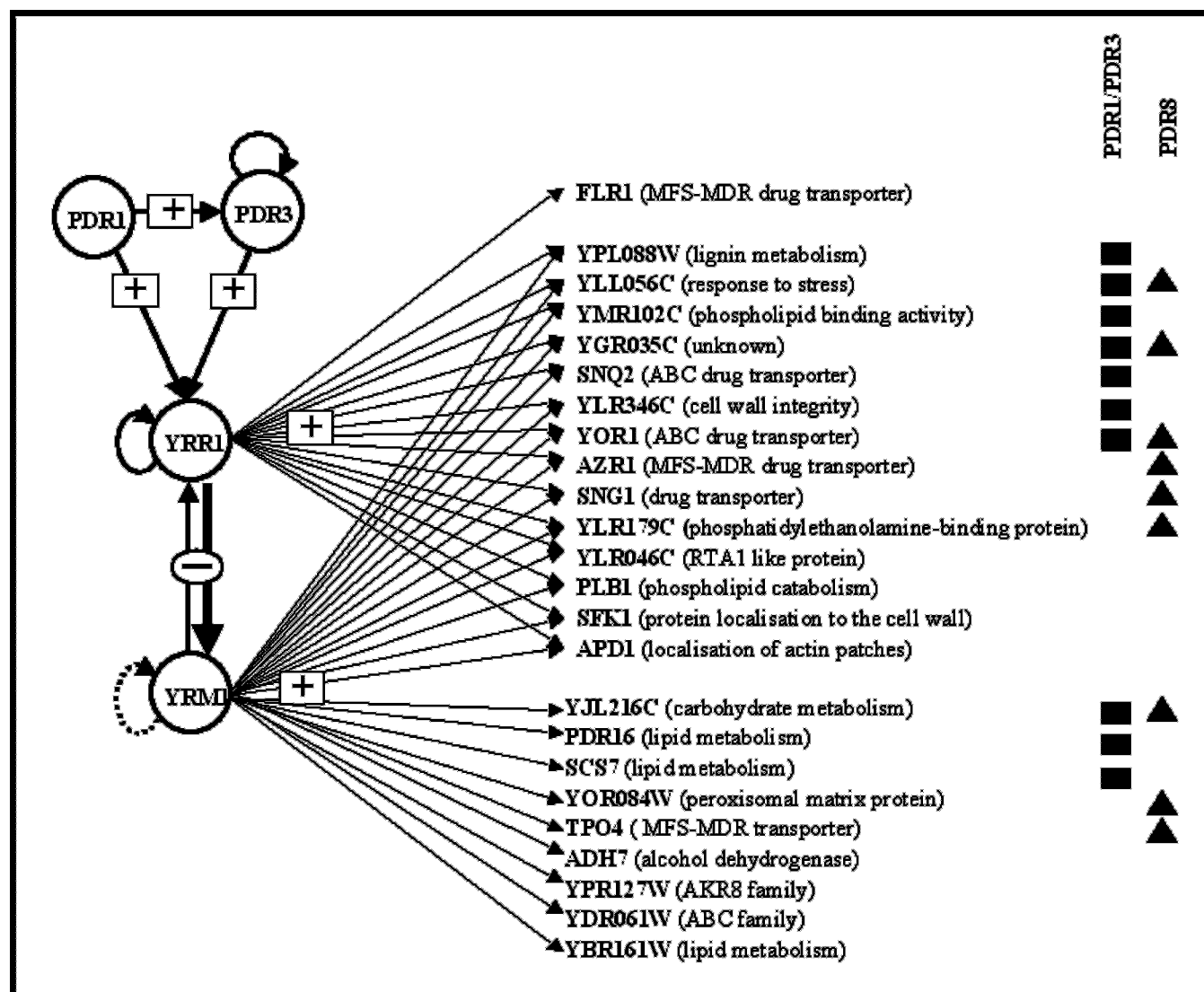


FIG. 5. The yeast multidrug resistance phenomenon is controlled by a pair of paralogous transcription factors, which are themselves interconnected. The square plus indicates transcriptional regulation whereas the circle minus indicates post-transcriptional regulation. *PDR1/PDR3* were the first example of paralogous transcription factors acting on the drug resistance phenotype to be identified. They positively regulate a set of genes, most of which encode for plasma membrane proteins (3, 4), but they also regulate the expression of *YRR1*. The target genes directly regulated by *YRR1/YRM1* and by *PDR1/PDR3* are indicated by a black square (right). The genes directly regulated by *PDR8* (32) and by *YRR1/YRM1* are indicated by black triangles. Note that *FLR1* is regulated by *YRR1* only.

ing is probably involved in the drug resistance program enabling the cell to grow in various chemical environments. The sets of genes directly regulated by *YRR1* and *YRM1* differ only in terms of the genes that may play a critical role in the properties of the cell. This is typically the case for *FLR1*, which is specifically activated by *YRR1* and plays a critical role in the azole resistance phenotype. This switch program may result from transcriptional regulation of the two transcription factors or from different transduction pathways controlling the balance between the negative cross-control of the two transcriptional activities. It is known that *YRR1* expression can be increased by gain-of-function mutations in *PDR1* or *PDR3*. No regulated expression of *YRM1* has been observed to date (www.transcriptome.ens.fr/ymgv/), but it is worth noting that the comparative genomic analysis of the three *Saccharomyces*-related strains (34) has revealed conserved motifs in the promoters of the orthologous forms of *YRM1* that differ from those in the promoter of *YRR1* (data not shown), suggesting that the two genes differ in their regulation processes. Interestingly, this post-transcriptional negative cross regulation between *YRR1* and *YRM1* constitutes the first example to be described

of such regulation between transcription factors involved in the multidrug resistance phenomenon. However, this regulation was anticipated as the cell clearly needs to know how to reprogram gene expression after changes in the chemical environment.

Current approaches to the analysis of gene expression data make it possible to identify groups of co-expressed genes, which, in turn, provide an opportunity to describe the organization of a regulatory module network in the genome (35). We describe here a complementary experimental approach based on full description of the set of genes directly regulated by functionally related transcription factors. Our results demonstrate that the real situation may be more complicated than a simple regulator-module relationship. Our data (Fig. 5) suggest that the drug resistance regulatory network presents a major overlap between the various transcription modules controlled by different transcription factors. This suggests that the systems involved in drug resistance are highly modular, possibly due to the necessity to adapt to a variety of different environments. It should also be borne in mind that the development of resistance by microbes as an evolutionary response to the se-

lective pressure exerted by antimicrobial drugs is probably highly complex and may involve many other sophisticated regulatory pathways. Detailed analyses of genome response programs to the presence of specific drugs are underway to integrate regulatory networks such as those of Yrm1 and Yrr1 into a global hierarchic response.

Acknowledgments—We thank our collaborators from the ENS Transcriptome Platform (www.transcriptome.ens.fr/sgdb/), Stéphane Le Crom, Véronique Tanty, and Corinne Blugeon. We are grateful to Philippe Marc for many helpful discussions, and André Goffeau and Scott Moye-Rowley for useful advice.

REFERENCES

- Zeitlinger, J., Simon, I., Harbison, C. T., Hannett, N. M., Volkert, T. L., Fink, G. R., and Young, R. A. (2003) *Cell* **113**, 395–404
- Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W., Murakami, Y., Philippsen, P., Tettelin, H., and Oliver, S. G. (1996) *Science* **274**, 546–547
- DeRisi, J., van den Hazel, B., Marc, P., Balzi, E., Brown, P., Jacq, C., and Goffeau, A. (2000) *FEBS Lett.* **470**, 156–160
- Devaux, F., Marc, P., Bouchoux, C., Delaveau, T., Hikkel, I., Potier, M. C., and Jacq, C. (2001) *EMBO Rep.* **2**, 493–498
- Delahodde, A., Delaveau, T., and Jacq, C. (1995) *Mol. Cell. Biol.* **15**, 4043–4051
- Hallstrom, T. C., and Moye-Rowley, W. S. (2000) *J. Biol. Chem.* **275**, 37347–37356
- Devaux, F., Carvajal, E., Moye-Rowley, S., and Jacq, C. (2002) *FEBS Lett.* **515**, 25–28
- Hallstrom, T. C., Katzmman, D. J., Torres, R. J., Sharp, W. J., and Moye-Rowley, W. S. (1998) *Mol. Cell. Biol.* **18**, 1147–1155
- Gautschi, M., Lilie, H., Funfschilling, U., Mun, A., Ross, S., Lithgow, T., Rucknagel, P., and Rospert, S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3762–3767
- Dohrmann, P. R., Voth, W. P., and Stillman, D. J. (1996) *Mol. Cell. Biol.* **16**, 1746–1758
- Rutherford, J. C., Jaron, S., Ray, E., Brown, P. O., and Winge, D. R. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 14322–14327
- Blaiseau, P. L., Lesuisse, E., and Camadro, J. M. (2001) *J. Biol. Chem.* **276**, 34221–34226
- Rutherford, J. C., Jaron, S., and Winge, D. R. (2003) *J. Biol. Chem.*
- Hiesinger, M., Roth, S., Meissner, E., and Schuller, H. J. (2001) *Curr. Genet.* **39**, 68–76
- Haurie, V., Perrot, M., Mini, T., Jenö, P., Sagliocco, F., and Boucherie, H. (2001) *J. Biol. Chem.* **276**, 76–85
- Jacquet, M., Renault, G., Lallet, S., De Mey, J., and Goldbeter, A. (2003) *J. Cell Biol.*
- Keller, G., Ray, E., Brown, P. O., and Winge, D. R. (2001) *J. Biol. Chem.* **276**, 38697–38702
- Cui, Z., Shiraki, T., Hirata, D., and Miyakawa, T. (1998) *Mol. Microbiol.* **29**, 1307–1315
- Zhang, X., Cui, Z., Miyakawa, T., and Moye-Rowley, W. S. (2001) *J. Biol. Chem.* **276**, 8812–8819
- Le Crom, S., Devaux, F., Marc, P., Zhang, X., Moye-Rowley, W. S., and Jacq, C. (2002) *Mol. Cell. Biol.* **22**, 2642–2649
- Mamnun, Y. M., Pandjaitan, R., Mahe, Y., Delahodde, A., and Kuchler, K. (2002) *Mol. Microbiol.* **46**, 1429–1440
- Akache, B., and Turcotte, B. (2002) *J. Biol. Chem.* **277**, 21254–21260
- Moye-Rowley, W. S. (2002) *Antioxid Redox Signal* **4**, 123–140
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Marc, P., and Jacq, C. (2002) *Bioinformatics* **18**, 888–889
- Dysvik, B., and Jonassen, I. (2001) *Bioinformatics* **17**, 369–370
- Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 14863–14868
- Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor, NY
- Balzi, E., and Goffeau, A. (1995) *J. Bioenerg. Biomembr.* **27**, 71–76
- Servos, J., Haase, E., and Brendel, M. (1993) *Mol. Gen. Genet.* **236**, 214–218
- Katzmann, D. J., Hallstrom, T. C., Voet, M., Wysock, W., Golin, J., Volckaert, G., and Moye-Rowley, W. S. (1995) *Mol. Cell. Biol.* **15**, 6875–6883
- Hikkel, I., Lucau-Danila, A., Delaveau, T., Marc, P., Devaux, F., and Jacq, C. (2003) *J. Biol. Chem.* **278**, 11427–11432
- Schjerling, P., and Holmberg, S. (1996) *Nucleic Acids Res.* **24**, 4599–4607
- Kellis, M., Patterson, N., Endrizzi, M., Birren, B., and Lander, E. S. (2003) *Nature* **423**, 241–254
- Segal, E., Shapira, M., Regev, A., Pe'er, D., Botstein, D., Koller, D., and Friedman, N. (2003) *Nat. Genet.* **34**, 166–176