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# Early Expression of Yeast Genes Affected by Chemical Stress†

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**The variety of environmental stresses is probably the major challenge imposed on transcription activators and the transcriptional machinery. To precisely describe the very early genomic response developed by yeast to accommodate a chemical stress, we performed time course analyses of the modifications of the yeast gene expression program which immediately follows the addition of the antimitotic drug benomyl. Similar analyses were conducted with different isogenic yeast strains in which genes coding for relevant transcription factors were deleted and coupled with efficient bioinformatics tools. Yap1 and Pdr1, two well-known key mediators of stress tolerance, appeared to be responsible for the very rapid establishment of a transient transcriptional response encompassing 119 genes. Yap1, which plays a predominant role in this response, binds, in vivo, promoters of genes which are not automatically up-regulated. We proposed that Yap1 nuclear localization and DNA binding are necessary but not sufficient to elicit the specificity of the chemical stress response.**

Cellular organisms develop a myriad of strategies to maintain specific internal conditions constantly challenged by the varying drug environment. The complexity of the yeast cell system for detecting and responding to environmental variations is only beginning to come to light. It has been reported previously (13) that a large set of yeast genes (about 900) showed a similar drastic response to a large variety of environmental changes including temperature shocks, hydrogen peroxide, menadione, diamide, dithiothreitol, hyper- or hypoosmotic shock, amino acid starvation, nitrogen source depletion, and progression into stationary phase. Since these pioneering studies were reported, many observations of the global effects of a large variety of drugs on gene expression have been made. In most of these studies, a binary comparison (i.e., control versus stress-exposed cells) was carried out, whereas in some cases, time course experiments over rather long periods (several hours) were conducted. Although much valuable information has been collected in these studies, the heterogeneity in the protocols followed precludes a simple comparison between the different drug responses. In particular, it is extremely difficult to identify the different regulatory networks and to establish their chronological relationships. Time series experiments soon appeared and were much more informative than simple binary experiments. Such approaches were a particularly valuable source of information in the case of cell cycle analyses (24, 27); however, they were less suitable to describe the chronology of transcriptional events in the case of environmental stress responses of the cell. This is probably due to the natural complexity of the genomic response which has to cope with a large variety of chemical environments. In *Saccharomyces cerevisiae*, a large panel of transcription factors, from the general factors like Yap1 or Msn2/Msn4 to the more specialized factors like the Pdr family, offers a large spectrum of genomic responses to new environmental conditions. Yap1 is better known for its role in the regulation of the transcriptional response to oxidative stress (22). In this context, Yap1 activates two groups of genes which are both required in the presence of $H_2O_2$, the reactive oxygen species (ROS)-removing enzymes (*SOD1-2*, *CTT1*, *TSA1*, etc.) and the REDOX group, which keeps the cytosol in reduced state by NADPH (*TRR1-2*, *GLR1*, *GRX1*, etc.) (9). Researchers have argued that the thiol peroxidase Gpx3 is required for formation of the disulfide bridge between the N-terminal and the C-terminal cysteine-rich region (c-CRD) of the factor in the presence of $H_2O_2$. This modified form of Yap1, released from Crm1p-mediated nuclear export, accumulates in the nucleus and can activate artificial reporter genes (6, 18, 31). In addition, Yap1 can regulate the yeast response to several unrelated chemicals and metals (31). It was previously observed that Yap1 discriminates between oxidative stress elicited by the oxidative $H_2O_2$ and chemical stresses like diamide. More recently, these chemicals could be assigned to regulation of nuclear localization of the factor through the c-CRD (1, 18).

We have focused our studies on the very early genomic events which characterize the chemical stress response. We used DNA microarrays to analyze changes in transcript abundance in yeast cells responding to the presence of benomyl. Benomyl is known to activate Yap1 (23) by a process which probably implicates its c-CRD region (1). Time series whole-genome expression data were generated to unfold the very early transcriptional changes which occur soon after the addition of benomyl. These data revealed that cells experienced a transient transcriptional response which partly mimics the oxidative stress response. This stress response, which depends mainly on Yap1, was very specific in terms of the enzymes that were recruited. Genes coding for redox-controlling enzymes

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† Supplemental material for this article may be found at http://mcb.asm.org/.

(*TRR1*, *TRX2*, etc.), which keep the cytosol in a reduced state, were up-regulated, whereas genes coding for ROS enzymes (*CTT1*, *SOD1*, etc.) remained unchanged. This specificity was studied by time course chromatin immunoprecipitation (tChIP) experiments. tChIP experiments suggested that in vivo Yap1 binding to the relevant promoters is an essential step in the benomyl-controlled activation process but that it is not sufficient to discriminate between up-regulated (like *FLR1*) or invariant (like *CTT1*) genes.

## MATERIALS AND METHODS

**Yeast strains and growth conditions.** All strains were from the Euroscarf collection. They were derived from *S. cerevisiae* strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ*). Cells were grown in YPD medium (1% [wt/vol] Bacto yeast extract, 2% [wt/vol] Bacto peptone, 2% [wt/vol] glucose). Drug resistance assays were performed by tests with serial dilutions in YPD liquid medium in order to establish the most appropriate concentration of benomyl.

**Time course experiments and microarray experiments.** Different strains were grown in YPD liquid medium to an optical density at 600 nm of 0.5. Addition of benomyl or dimethyl sulfoxide (DMSO) to 20 μg/ml was performed for various times (30 s, 2 min, 4 min, 10 min, 20 min, and 40 min), starting with the same initial culture. Cells were harvested instantaneously by centrifugation, flash frozen in liquid nitrogen, and stored at −80°C. Total RNA was isolated, purified, and used to synthesize and label cDNA as described on our website (http://www.transcriptome.ens.fr/sgdb). For each time of exposure to benomyl and for each strain analyzed, we performed two independent microarray experiments and two technical repetitions. Microarrays containing oligonucleotides to probe most of the open reading frames of *S. cerevisiae* were produced in our laboratory with an Omnigrid II Biorobotics robot (http://www.transcriptome.ens.fr/sgdb). They were based on the principle of 40-mer oligonucleotides from MWG covalently deposited onto Corning glass slides coated with pure gamma amino propyl silane. The microarray protocol used is described on our website (http://www.transcriptome.ens.fr/sgdb). A total of 20 μg of total purified RNA was used for each experiment. In each experiment, the cDNA corresponding to cells treated with benomyl was labeled with Cy5-dUTP, and cDNA from control cells treated with DMSO was labeled with Cy3-dUTP. Dye swap experiments were carried out for each analysis. The arrays were read using a Genepix 4000A scanner (Axon) and analyzed with Genepix 3.0 software. Artefactual, saturated, or low-signal spots were eliminated from the analysis.

Linear regression of Cy5 against Cy3 channels (21) was applied to data in order to normalize fluorochrome channels. Duplicate spots were averaged, and nonreproductive values were not used for the rest of the analysis.

**(i) Missing value handling.** Gene expression patterns where the minimum percentage of existing values was less than 80% were eliminated from the rest of the analysis. The remaining missing values were replaced by using the KNN-imputation method (29).

**(ii) Selection of up- and down-regulated genes.** $\text{Log}_2$(ratio) data sets of times courses were used for filtering genes for which a change of more than twofold was observed for at least two successive times during the experiments.

**Chromatin immunoprecipitation analyses (ChIP assays).** The *YAP1* gene with an N-terminal Myc tag on pRS316 plasmid (8) was introduced into a wild-type yeast strain producing Yap1 under the control of the its own promoter. Cells were grown on YPD medium to an optical density of 0.5. Addition of benomyl or DMSO to 20 μg/ml was performed for 5, 15, 40, and 60 min, starting with the same initial culture. The full protocol as well as the various primer sets used for amplification of the 14 promoters can be found in the supplemental material (see Fig. S1 in the supplemental material and www.biologie.ens.fr/lgmgml/publication/benomyl/).

**Bioinformatic analyses. (i) Principal component analysis (PCA).** Complete interpretation of the biplots, given different transformation of the data expression matrix, can be found elsewhere (12). The analysis was completed with the statistical computing and graphics environment R (http://cran.r-project.org/).

**(ii) Knockout gene cluster analysis.** The differential gene expression ratio induced by benomyl between wild-type and *yap1Δ* strains was plotted as $x$ [$\log_2$(ratio) value observed in the wild type strain] versus $y$ [$\log_2$(ratio) value observed in the *yap1Δ* strain] for the different time points (see Fig. 3A).

When all the times were plotted on the same graph, lines could be drawn between points corresponding to the same gene. The resulting profiles were therefore characteristic of the differential expression of the genes under consideration throughout the time course analysis. Similarity measure between the differential gene expression profiles could be calculated, and cluster analysis was conducted. More precisely, each gene can be given a coordinate of two expression vectors defined as $A_g(T_m)$ and $A'_g(T_m)$, where $A_g(T_m)$ is the logarithm to base 2 of the ratio for the gene $g$ in the wild-type strain, measured at $T_m$, the time point of the kinetic [$T_m$ which is included in $T$(30 s, 2 min, 4 min, 10 min, 20 min)], and $A'_g(T)$ is the logarithm to base 2 of the ratio for the gene $g$, measured for the $T_m$, the time point of the kinetic, in the *yap1Δ* strain.

Thus, considering two genes, $g_1$ and $g_2$, the similarity measure between them is computed as follows:

$$D(g_1,g_2) = \sum_{T_m \in T} \sqrt{[A_{g_1}(T_m) - A_{g_2}(T_m)]^2 + [A'_{g_1}(T_m) - A'_{g_2}(T_m)]^2}$$

Next, a classical hierarchical cluster analysis was performed by using the distance matrix. Initially, each object is assigned to its own cluster and then the algorithm proceeds iteratively, with the two most similar clusters being joined at each stage and continuing until the analysis reaches a single cluster. The resulting tree is finally split into several groups of genes (see Fig. 3B) joined together (mean value) in the two-dimensional graph (see Fig. 3C).

Graphical representation and distance computation as well as hierarchical clustering were performed with a script to utilize library function in R (http://cran.r-project.org/).

**(iii) REDUCE algorithm.** REDUCE was used as described previously (26). REDUCE is a motif-based regression method for microarray analysis. This algorithm uses unbiased statistics to identify oligonucleotide motifs whose occurrence in the regulatory region of a gene correlates with the level of mRNA expression. Regression analysis is used to infer the activity of the transcriptional module associated with each motif. REDUCE is available online at http://bussemaker.bio.columbia.edu/reduce/ (26).

## RESULTS

**Time course transcript profiling with benomyl conditions which do not significantly alter yeast growth.** We turned to transcript profiling to provide a genome-wide view of the cellular response to benomyl in conditions in which the cell can still adapt to environmental conditions. Since the purpose of this work was to identify the very early events triggered by addition of benomyl to the growth medium, we had to define appropriate growth conditions (time and drug concentration). Thus, to depict a precise view of chronological events after the addition of benomyl (20 μg/ml), we carried out a series of transcriptome analyses from 30 s to 40 min. Parallel experiments were conducted in different genetic contexts. The wild-type strain was thus compared with strains in which the following genes coding for different transcription factors connected to the drug response were deleted: Yap1, Pdr1, Pdr3, Yrr1 (19), Pdr8 (15), and Yrm1 (20). Only the strains in which *yap1* and *pdr1* were deleted are considered in the rest of this work; deletions of the other genes did not lead to significant alterations of the genome-wide transcription response to benomyl under the conditions of this study.

**Global analysis of changes in mRNA abundance.** To gain insight into the physiological changes that followed the benomyl addition, we analyzed our data using different bioinformatics tools. We first applied PCA to our gene expression data as described previously (4). PCA is a well-established technique in multivariate statistics; the objective is to determine a new coordinate system such that the first coordinate explains the maximal amount of variance in the data and successive coordinates explain maximal variance while being orthogonal to the first. Explaining the majority of the variance in the data, the first and the second principal components can be represented simultaneously as a biplot (Fig. 1A). The horizontal axis corresponds to the first principal component (PCA1), which

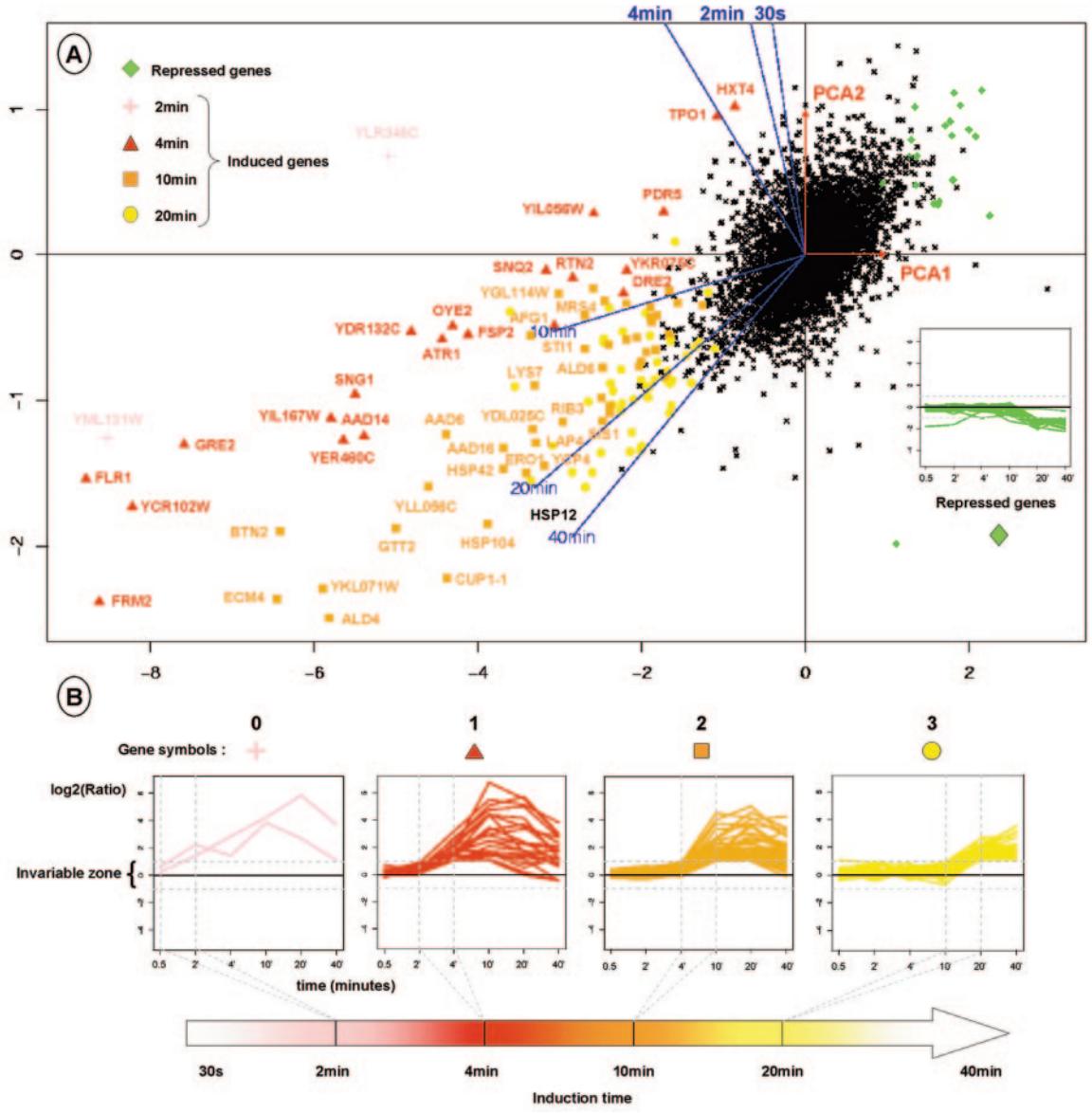


FIG. 1. PCA analysis of the time course response of the wild-type strain to benomyl. Microarray results for the kinetics of benomyl action were analyzed by PCA as described previously (4). (A) Biplot of the results of PCA. The first principal component (PCA1, horizontal axis) and the second principal component (PCA2, vertical axis) account for 42 and 20%, respectively, of the global variance. Initial time vectors are represented in blue. This method clearly distinguished groups of up-regulated genes which appear in different "sectors" from 30 s to 40 min. (B) Expression profiles of 119 genes whose expression is up-regulated twofold relative to untreated controls at least two successive times during the course of the experiments. Four groups of up-regulated genes, groups 0 to 3, can be distinguished by the delay in the response to the presence of benomyl, which goes from 2 min (group 0; two genes) to 20 min (group 3, 51 genes). Genes contained in each group are shown as symbols (group 0, +; group 1, red triangle; group 2, orange square; group 3, yellow circle) which can also be identified on the PCA diagram (A). Complete gene lists with *Saccharomyces* Genome Database (4) annotation are available on the related publication website.

accounts for 42% of the total variance in the data. The second principal component (PCA2) corresponds to the vertical axis and explains 20% of the total variance. Initial time vectors are also represented (Fig. 1A), and the resulting sectors reflect classes of similar expression profiles (Fig. 1B). For instance, the first two up-regulated genes appeared extremely rapidly, between 30 s and 2 min after the addition of benomyl (Fig. 1B, group 0). Within the 40 min following the addition of benomyl, three classes of expression profiles represent the up-regulated genes (Fig. 1B, groups 1, 2, and 3). Classes 1 and 2, which include most of the genes, differed only by the delay in the start of induction (4 and 10 min after benomyl addition, respectively). On the other hand, they both exhibited a typical transient activation process, and 40 min after benomyl addition, most of the genes had returned to a near-basal expression ratio. Genes affected by this transient activation phenomenon constitute the core of this study.

We also scrutinized the data corresponding to diverse knockout strains by a specific cluster analysis based on the direct comparison between data on expression of wild-type and

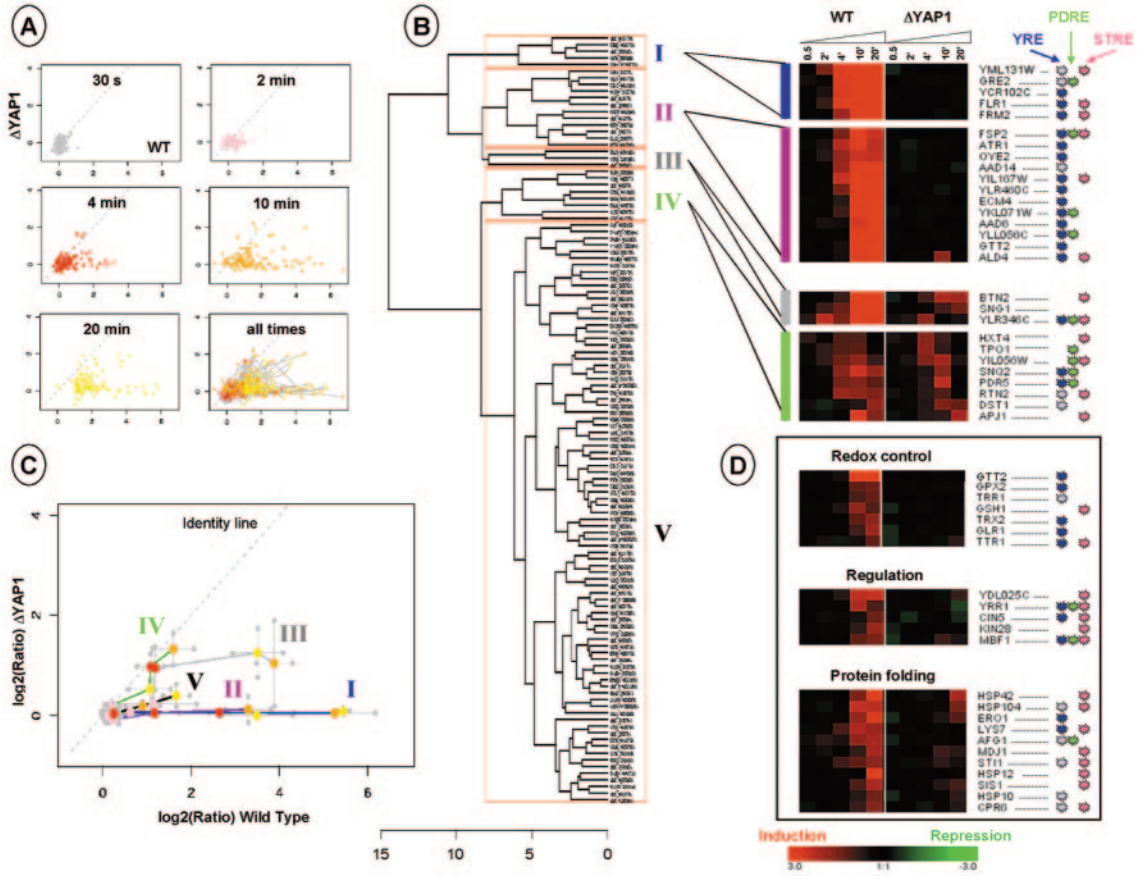


FIG. 2. Knockout gene cluster analysis: comparison of wild-type (WT) and *yap1* strains. A bioinformatics tool was developed (see Materials and Methods) to infer gene network organization from perturbed expression profiles. Two sets of microarray expression data corresponding to the time course effects of benomyl on gene expression of wild-type and *yap1*Δ strains were compared. (A) Typical graphs corresponding to different time points represent $\log_2$(ratio) values for wild type (*x*) and *yap1*Δ (*y*) strains for each gene significantly up-regulated in the wild-type strain. (B) A hierarchical clustering analysis could be carried out from the two sets of data (see Materials and Methods). The resulting tree was split into five groups, I, II, III, IV, and V. Clusters I, II, III, and IV were enlarged in order to display complete gene lists. For each gene, their expression profiles in the wild type and *yap1*Δ time course experiments are represented by using the common color code (green for repressed and red for induced). PDRE, pleitotropic drug resistance element. (C) The mean differential expression profiles for clusters I, II, III, IV, and V are represented. The mean values were calculated for each time point, and the standard deviation is represented as a gray line along the *x* and *y* axes. (D) Only subsets of genes were extracted from cluster V (see the supplemental material for the complete list). Those genes correspond to pertinent functional categories, redox control, regulation, and protein folding, which characterize the benomyl response (see the text). A systematic search for the regulatory motifs of the transcription factor Yap1 (TTANTAA), Pdr1/Pdr3 (TCCGYGGA), and STRE (AGGGG) was performed in the upstream sequence of all these genes.

knockout strains. This approach, described in Materials and Methods, relies on the time course comparison of differential expression of each gene when wild-type and *yap1*Δ strains are analyzed. It allows a quantitative assessment of the consequence of gene deletion on global gene expression. Snapshots of differential gene expression at different times (30 s to 20 min) show (Fig. 2A) that among the 119 genes that are significantly up-regulated in the wild-type strain, groups of genes can be distinguished. A similar measurement can be derived from the "differential expression profiles," and these groups of genes appeared to be connected via their dependence on the presence of Yap1 (Fig. 2C). For instance, groups I and II are comprised of genes which are completely dependent on Yap1, while groups III, IV, and V are comprised of genes whose up-regulation does not rely only upon Yap1. Interestingly, this rigorous analysis of deletion strain properties revealed gene groups which could not be simply deduced from classical visual examination of the expression profiles.

We also tried to connect time course expression data with molecular data contained in the promoter sequences of the expressed genes. It is well established that specific DNA sequence elements that act as binding sites for transcription factors coordinate the expression of genes in whose regulatory region they appear. To perform simultaneous analysis of genome sequence and expression data, we used the REDUCE program (2, 26) and thus quantified the extent to which some regulatory sequence elements can explain changes in genome-wide expression data. REDUCE works by fitting a multivariate predictive model to a single genome-wide expression pattern. The expression level of a gene is therefore modeled as a sum of independent contributions from all transcription factors for which binding sites occur in the promoter region. Single-motif

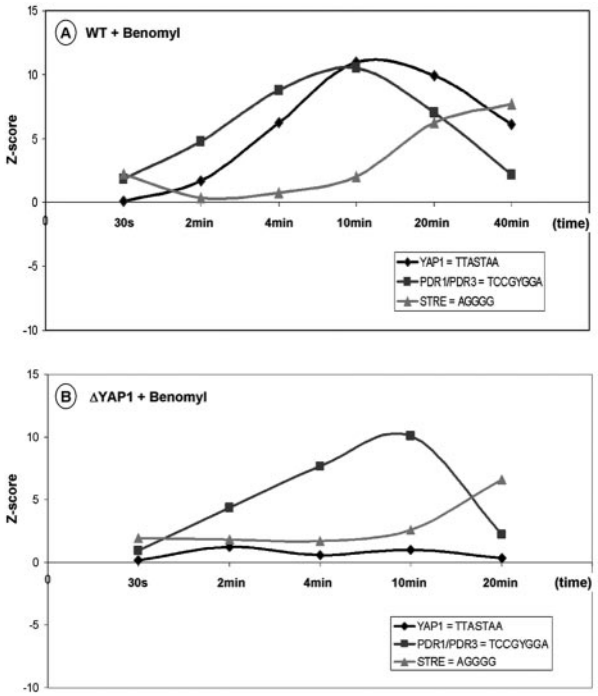


FIG. 3. Dissection of the transcriptional response of yeast cells to the addition of benomyl in the growth medium. The program REDUCE (2) was used to correlate DNA sequence elements in promoter regions with $\log_2$(ratio) data sets of the benomyl induction time course in the wild-type strain (WT) (A) and the *yap1*Δ strain (B). Three relevant regulatory motifs are shown. Complete results can be found in the supplemental material. The transient programs of genes containing Yap1 and Pdr1 motifs are remarkably similar to the transient expression profiles depicted in Fig. 1B.

fits were performed for each time point in the whole data set of the benomyl time course. The 20 strongest motifs detected for the 10-min time points, which have the most significant *P* values, are shown in the supplemental material. The first motifs that were found to be clearly the most significant are compatible with the regulatory region of the transcription factor Yap1 (TTASTAA) and Pdr1/Pdr3 (TCCGYGGA). In addition to these two motifs, the model computed the emergence, at 40 min, of the well-known stress response element (STRE) (AGGGG or CCCCT), which suggests that the transcription factors Msn2/Msn4 may be involved. The magnitude of the correlation between the $\log_2$(ratio) for a gene and the relevant motifs can be represented by a *Z* score reflecting the relative reduction in the error between the experimental data and the linear model based on a single motif. We plotted the *Z* score values for the motifs *YAP1*, *PDR1*/*PDR3*, and STRE as a function of time for both wild-type and *yap1*Δ strains (Fig. 3).

This independent analysis of the transcription control is in full agreement with the above-described studies. Moreover, when REDUCE was applied to the expression data obtained with the *yap1*Δ strain, it showed (Fig. 3B) that, as expected, the Yap1 motif-containing genes are totally absent, whereas the other signals are unaffected. This result suggests that independent transcription programs control Yap1 and Pdr1 networks.

**Early up-regulated genes. (i) Yap1-dependent genes.** It is interesting that gene clustering analyses conducted by independent approaches on wild-type and *yap1*Δ cells led to comparable gene clusters. Especially clear are groups I and II (Fig. 2B and C) which include genes that were plotted along the direction of the 10-min vector in the wild-type cell (Fig. 1). This reflects the fact that the transcription factor Yap1 plays a predominant role in the early events governing the benomyl response of the cell. Indeed, in the absence of *YAP1*, the benomyl-controlled up-regulation of these genes is totally abolished. This result is in agreement with the presence of at least one Yap1 response element, YRE (TTANTAA), in the promoter of these genes (Fig. 2B). This suggests that the up-regulation phenomenon actually results, at least in part, from a direct action of Yap1 with the relevant promoters. Chromatin immunoprecipitation experiments conducted with some of these promoters emphasize this point (see below).

**(ii) Pdr1-dependent genes.** Shortly after gene activation of groups I and II, genes of groups III and IV are up-regulated (Fig. 2B and C). These different early expressed clusters differ in their dependence on the transcription factors. While Yap1 controls groups I and II exclusively, genes of groups III and IV are poorly affected in the *yap1*Δ strain. On the contrary, deletion of *PDR1* completely abolishes the up-regulation process for half of these genes (TPO1, YIL056W, PDR5, and APJ1) and clearly alters the up-regulation process of the others (see the supplemental material). Again, the presence of typical PDR elements (TCCGA/GCGGA; [10]) in these promoters strongly suggests that a direct interaction of a benomyl-activated form of Pdr1 recognizes the regulatory signals of the relevant genes. It should be remembered that the set of yeast genes possibly activated by Pdr1 is well identified and concerns a set of 25 genes which mainly encode membrane proteins (11). Groups III and IV constitute a subset of the genes which are up-regulated when Pdr1 is derepressed by a gain-of-function mutation or by an artificial fusion with a Gal4 activation domain (11). As in the case of Yap1, which is discussed below, the benomyl-directed activation of Pdr1 confines the transcription factor specificity. Factors involved in the specific restriction of the transcription factor activity spectra are thus far unknown.

**(iii) The early activated genes mainly control membrane properties.** Among the 14 early up-regulated genes (Fig. 1B, group 1) whose function is known (see the supplemental material), 9 genes code for proteins which are directly involved in the plasma membrane organization and 6 genes function as active transporters of either ABC (*SNQ2* and *PDR5*) or MFS types (*FLR1*, *SNG1*, *TPO1*, and *ATR1*). The case of *FLR1* deserves special mention. Our results are in agreement with those of previous studies (23, 28), which pointed out the specific role of Yap1 in the regulation of *FLR1* in the response of the cell to diverse drugs, oxidants, and alkylating agents.

**Late up-regulated genes.** A second wave of up-regulated genes can be distinguished from the previous early activated genes (Fig. 1B, groups 2 and 3). These 96 genes, like the early induced genes, predominantly exhibit a transient expression profile, which in this case starts at about 10 min after the addition of benomyl. Most of these genes are included in the genes induced in the environmental stress response (ESR) described previously (13). Also, whereas early induced genes mainly concern gene coding for membrane remodeling proteins, this last group of genes contains relatively few membrane protein-coding genes like transporters (*YCF1*, *PCA1*, and

*MRS4*) or proteins involved in cell wall structure (*ECM4*). In fact, the three main characteristics of this cluster which distinguish it from the other environmental stress response already studied (13) are presented in the functional clusters of Fig. 2D.

**(i) Protein folding control.** An important group of genes coding for protein folding chaperones operating in diverse cellular compartments (endoplasmic reticulum, mitochondria, cytoplasm, and nucleus) are up-regulated 10 min after the addition of benomyl. Most of them have a Yap1 binding signal in their promoter, and their benomyl-dependent up-regulation process is severely affected by the deletion of *YAP1*. However, remnant transcriptional activation suggests that other transcription factors are involved in their regulation.

**(ii) Dissection of the oxidative stress response.** Shortly after benomyl addition, most of the genes coding for REDOX-controlling enzymes were up-regulated, whereas expression of the genes coding for ROS-removing enzymes was unchanged. Remarkably, neither catalases nor superoxide dismutases were called upon in coping with the benomyl stress; the expression levels of these genes remained unchanged. *GSH* and *TRX1* to *TRX3*, the most important agents that keep the cytosol in a reduced state and which are all kept in a reduced state by NADPH (14), were produced. We observed that mRNAs of *GTT2*, *GPX2*, *TRR1*, *GSH1*, *TRX2*, *GLR1*, and *TTR1* rose quickly after benomyl addition. *TRX2* and *TRR1* encode thioredoxin and thioredoxin reductase, respectively; *GSH1* and *GLR1* encode glutathione synthetase and glutathione reductase, respectively. This is a strong indication that a redox imbalance developed shortly after the addition of benomyl.

**(iii) Regulatory control.** This study focuses on the description of the very early regulatory events in response to benomyl; no more than 200 genes are involved. Clearly, Yap1 and Pdr1 are the first transcription factors to be involved in the genome adaptation to the drug. Analyses of strains in which genes coding for diverse transcription factors known to be involved in the pleiotropic drug response, like *PDR3*, *YRM1* (20), and *PDR8* (15), are deleted did not provide any evidence that these factors respond to benomyl under the experimental conditions. On the other hand, several transcription factor-encoding genes are themselves activated in the course of this early benomyl response. This is the case with *CIN5*, also named *YAP4*, whose function is not clear and which was already shown to be induced under conditions of either oxidative or osmotic stress (see reference 25 for a review). *YRR1*, which codes for a zinc finger transcription (7, 19), is also activated by benomyl, probably via the activation of Yap1. The presence of Yap1 recognition elements in the promoters of both *CIN5*/*YAP4* and *YRR1* suggests a direct interaction of Yap1 with these promoters. It will be interesting to study the putative role of these transcription factors in the regulation cascade induced by benomyl.

**Benomyl and DNA-binding properties of Yap1.** The observation (see above) that the early genomic response to benomyl concerns only a subset of the genes generally involved in the oxidative stress response is intriguing. To better understand this phenomenon, we analyzed the in vivo Yap1 binding properties to promoters of ROS and REDOX genes, the latter group being the only one to be up-regulated by benomyl. For this purpose, we used a version of Yap1 tagged at its C terminus with *myc* epitopes, which was previously shown to be fully active while having no effect on cell growth (8). After addition of benomyl to the growth medium, ChIP analyses were conducted at different times (tChIP) to assess the variations of *myc*-Yap1 binding on different promoters (tChIP analyses). Based on the results of three independent experiments, the enrichment of *myc*-Yap1 occupancy, with reference to time zero before benomyl addition, was assessed on 15 different promoters containing Yap1 response elements. Promoters of *ACT1* and *COX6* which do not have an apparent Yap1 binding site and do not exhibit any significant enrichment after benomyl addition (Fig. 4) were used as negative controls. The most salient feature of this analysis is that all the promoters containing at least one Yap1 recognition element exhibit a significant time course variation in the level of promoter occupancy after addition of benomyl. It is striking that this variation for the gene *FLR1*, for instance, reflects the variation in the mRNA level previously observed. Taken at face value, this finding suggests a close connection between Yap1 DNA-binding properties and gene transcription. However, it is not that simple, since *SOD1*, *CCP1*, and *TSA1*, which show similar benomyl-dependent *myc*-Yap1 promoter occupancies, do not exhibit any increase in their mRNA levels during this period. Thus, benomyl is likely to act at the level of Yap1 nuclear localization rather than on the specific binding process (see Discussion). Reciprocally, when the *myc*-Yap1 promoter occupancy decreases at the end of the transient phenomenon, a negative regulatory process is supposed to limit Yap1 availability independently of gene expression properties. In other words, it looks as if Yap1 promoter binding is necessary but not sufficient to power transcription of the adjacent gene.

## DISCUSSION

**The early drug response.** Cellular life is frequently endangered by various adverse conditions, and the speed of the adaptative process is a clue to survival. The aim of this work was to decipher the early gene expression program following chemical, in this case benomyl, stress. Time course analyses of yeast transcriptome shortly after addition of benomyl to the growth medium, bioinformatics analyses of the set of data, and use of transcription factor deletion strains allowed us to accurately describe the first groups of coregulated genes. Several complementary approaches were conducted to assess the validity of the results. For instance, time course expression data in wild-type and Yap1-deleted strains were combined to generate differential expression profiles for each gene, and genes could be clustered according to similarities in their profiles. Also, global insight into the changing patterns of gene expression was obtained by applying the algorithm REDUCE (2). Annotated upstream activation elements corresponding to Yap1 and Pdr1 were found for groups of genes activated shortly after the addition of benomyl. The clues provided by REDUCE were confirmed by microarray analyses of the strains deleted in Yap1 and Pdr1. These different approaches demonstrated that two parallel pathways involving both Yap1 and Pdr1 are required for acquisition of early benomyl tolerance in the budding yeast *S. cerevisiae*.

**Generality of the stress response: the transient gene activation process.** Many transcriptional response analyses have been conducted for a large variety of stresses. However, simple

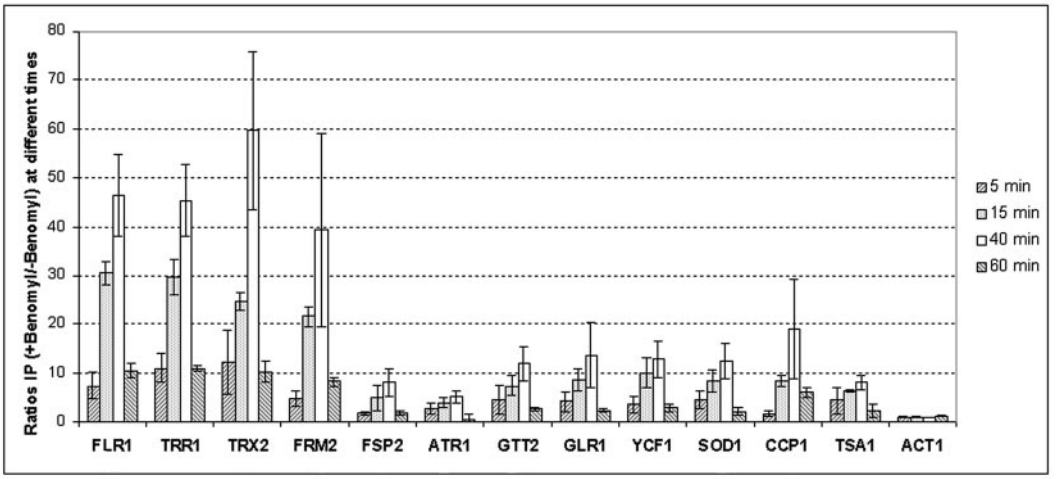


FIG. 4. Time course analysis of Yap1 promoter occupancy after addition of benomyl to the growth medium of the wild-type strain. Growth conditions in the presence of benomyl were strictly identical to those used to follow genome expression. Cells were harvested, treated with formaldehyde, and lysed with glass beads; the extracts were sonicated to produce chromatin fragments of about 500 bp. Aliquots were immunoprecipitated with hemagglutinin antibodies, and DNA was extracted from the immunoprecipitate (IP) after reversing the cross-links. DNA was extracted directly from another aliquot of chromatin to serve as the input control. A 1,000-fold dilution of the input and the undiluted immunoprecipitate samples were PCR amplified using primers for the different promoters (see Materials and Methods and the supplemental material). Quantitative PCR amplifications were conducted as indicated in Materials and Methods. Enrichment ratios were calculated by taking the time zero addition of benomyl as a reference. Three totally independent experiments were conducted for each point, and the mean values with standard errors are represented. Note that the negative control genes which do not contain the Yap1 motif in their promoter *ACT1* exhibit a weak signal which is time independent. On the other hand, all the other genes contain a Yap1 motif, but some of them (*SOD1*, *CCP1*, and *TSA1*) are not up-regulated during the transient benomyl activation.

direct comparison of these results is difficult because of the heterogeneity of the experimental conditions. For instance, it was previously observed that variegated responses are likely to be found by varying the intensity of environmental stresses (5). It was nevertheless proposed that a general stress response program called ESR could account for the observed cross-resistance to various stresses (13). However, while the ESR shows stereotypical expression changes, its regulation is nevertheless both gene specific and condition specific (13). Probably one of the best examples of the stereotypical behavior of the ESR is the repression of genes involved in protein synthesis and cellular growth which is also observed after benomyl action (Fig. 1 and see the supplemental material). Also, since the ESR is a graded response whose amplitude corresponds to the severity of the environmental stress, we reasoned that initiation of the ESR in suboptimal environmental perturbations may provide a useful operational characterization of the specificity or generality of the environmental response. Indeed, this approach allowed us to point out a small group of up-regulated genes which mainly code for proteins involved in plasma membrane properties (Fig. 1). Probably, the main consensus feature of all these ESRs is the transient aspect of the early genomic response. Very soon after the addition of benomyl, the membrane-controlling genes are up-regulated during a short period of 30 s to 40 min before adjusting to a new steady state. A similar transient response has been observed for many different stress conditions (13, 17), whereas the sets of genes responding to different stresses are different. The raison d'être of this transient expression is not clear. Although it is intuitive that, in the case of benomyl, changes in membrane properties might make the cell more resistant to the drug, it was hypothesized that in fact the transient changes in transcript levels would be accompanied by relatively small alterations in the corresponding protein levels (13). Another interesting aspect of this transient expression phenomenon is the necessary negative regulation which leads to a decrease in Yap1 activity, which was found to correlate with a decrease in promoter occupancy (Fig. 1 and 4). Nuclear export control of Yap1, probably in connection with specific protein modifications, is likely to have an active role in this negative regulation (18). In addition, it was recently proposed (3) that the thioredoxin system might play a crucial role in the reduction of Yap1 genome-wide activity.

The early transient period is followed by other transcriptional waves which are clearly visible in Fig. 1B (expression profile 3). Interestingly, the REDUCE analysis pointed out (Fig. 3) a group of up-regulated genes which have an STRE signal in their promoter. This finding suggests that 40 min after benomyl addition, Msn2/Msn4 transcription factors might have a role in the transcriptome modifications. A Yap1 (and/or Pdr1)-controlled up- or down-regulation of genes encoding regulatory proteins like kinases might lead to Msn2/Msn4 activation. Several candidate genes in group V (Fig. 2) are being studied to test this hypothesis. In addition, Yap1 activates the transcription of at least two other transcription factors, Yap4 (Cin5) and Yrr1, which probably shape the further evolution of the transcriptome. More work is required to present an accurate view of the regulatory network cascade following Yap1 and Pdr1 regulation.

**Regulation of the early stress response: a specific role for Yap1.** Yap1 is clearly the main coordinator of the early transcriptional response to benomyl stress. All the sequential steps of this transient response are recapitulated in Fig. 5 and concern Yap1 in different ways. (i) Yap1 first acts as the only

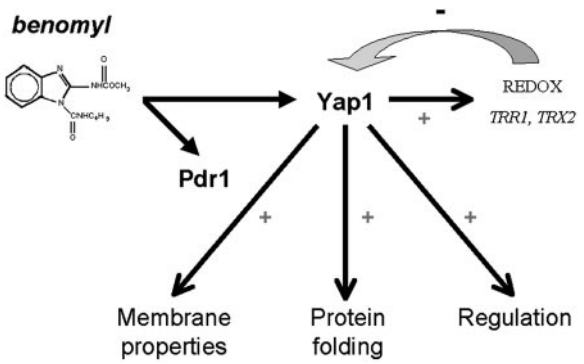


FIG. 5. Model for the early genomic response to benomyl. Very soon after addition of benomyl, Yap1 and Pdr1 transcription factors are involved in the activation of genes which code for proteins controlling plasma membrane properties (transporters, lipid synthesis, etc.). Yap1 also controls the transient activation of many (about 119) genes involved in metabolism, protein folding, and regulation (the complete list can be found in the supplemental material). Yap1 also up-regulates genes of the REDOX system (*TRR1*, *TRX2*, *GSH1*, *GLR1*, etc.) but leaves unchanged expression of genes of the ROS detoxification system (*CTT1*, *SOD1*, *CCP1*, etc.), which makes a clear distinction between benomyl and $H_2O_2$ responses. The transient transcription response implies a negative regulation. It was previously suggested (3, 16) that the thioredoxin system might negatively control Yap1 activity.

transcriptional activator to elicit the up-regulation of 17 genes of groups I and II (Fig. 2). (ii) Yap1 and Pdr1 up-regulate the transcription of 11 genes of groups III and IV (Fig. 2). (iii) Yap1 and other unidentified transcription activators up-regulate the transcription of 87 genes of group V (Fig. 2). Yap1 is already known as an essential factor in the response to oxidative stress (22, 25). Its nuclear localization mediated by its C terminus cysteine-rich domain is an essential feature of its regulation (18). The $H_2O_2$-induced nuclear accumulation of Yap1 is dependent on two proteins, Gpx3 (9) and Ybp1 (30). Interestingly, it was previously shown that Yap1 discriminates between oxidative stress elicited by the oxidants $H_2O_2$ and diamide (31), and it was recently proposed that Yap1 has two distinct molecular redox centers, one triggered by ROS (hydroperoxides and the superoxide anion) and the other triggered by chemicals with thiol reactivity (electrophiles and divalent heavy metal cations) (1). This last class of chemical Yap1 activators does not require the presence of Gpx3 or Ybp1 to trigger the oxidative response. In that respect, benomyl seems to elicit a diamide-like response since we observed (data not shown) that neither Gpx3 nor Ybp1 is required to trigger the transcriptome response presented in this work. Moreover, our study demonstrates that the spectrum of Yap1-up-regulated genes differs according to the chemical nature of the signal which activates Yap1. This is especially clear if one considers the oxidative stress response. Schematically, in the presence of an $H_2O_2$ stress, two classes of important genes are immediately up-regulated: those which code for enzymes involved in the detoxification of ROS species (*SOD1*, *SOD2*, *CTT1*, *CCP1*, and *TSA1*) and those which control the REDOX balance (*GSH1*, *GLR1*, *GRX1*, *TRX2*, and *TRR1*). Strikingly (Fig. 1), shortly after the addition of benomyl, only the genes of the second group which keep the cytosol in a reduced state (*GSH1*, *TRX2*, etc.) were up-regulated; the mRNA level of genes of the first group, like *CTT1*, did not significantly change. Furthermore, there was a remarkable specificity in the choice of expression of genes coding for isoenzymes; *TRX2* was preferred to *TRX1* or *TRX3*, *TRR1* was preferred to *TRR2*, etc. A similar bias has already been observed when a glucose-to-oleate switch was imposed in the growth medium (17).

**DNA-binding properties of Yap1 activated by benomyl.** In our study, tChIP experiments were crucial to better analyze the cell response to chemical stress and the specific role of Yap1. When benomyl was added to the growth medium, the in vivo promoter occupancy by Yap1 strictly followed the transcription activity of the corresponding promoter binding. This is especially clear for genes like *FLR1* which completely depend upon Yap1 for their benomyl activation. This Yap1 promoter occupancy probably reflects, at least in part, an active nuclear retention of an activated form of Yap1 (18). However, the level of promoter occupancy for each gene does not correlate with the mRNA level increase of the different genes. This is especially striking for genes of the ROS system (*SOD1*, *CTT1*, etc.) which showed no increase in mRNA after the addition of benomyl in spite of the fact that their promoter is recognized by Yap1. In other words, the benomyl-activated Yap1 form is able to bind Yap1 promoters, but it does not discriminate the genes which will be transcriptionally activated. This observation is in agreement with the proposition (18, 22) that although important, the regulated nuclear localization of Yap1 is not the only regulation process of the oxidative stress. It was already clear that mutants exhibiting a constitutive nuclear localization of Yap1 do not show greater resistance to $H_2O_2$ (18), suggesting that processes other than the simple nuclear localization of the transcription factor control the REDOX response.

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## REFERENCES

1. **Azevedo, D., F. Tacnet, A. Delaunay, C. Rodrigues-Pousada, and M. B. Toledano.** 2003. Two redox centers within Yap1 for H2O2 and thiol-reactive chemicals signaling. Free Radic. Biol. Med. **35:**889–900.
2. **Bussemaker, H. J., H. Li, and E. D. Siggia.** 2001. Regulatory element detection using correlation with expression. Nat. Genet. **27:**167–171.
3. **Carmel-Harel, O., R. Stearman, A. P. Gasch, D. Botstein, P. O. Brown, and G. Storz.** 2001. Role of thioredoxin reductase in the Yap1p-dependent response to oxidative stress in *Saccharomyces cerevisiae*. Mol. Microbiol. **39:** 595–605.
4. **Chapman, S., P. Schenk, K. Kazan, and J. Manners.** 2002. Using biplots to interpret gene expression patterns in plants. Bioinformatics **18:**202–204.
5. **Chen, D., W. M. Toone, J. Mata, R. Lyne, G. Burns, K. Kivinen, A. Brazma, N. Jones, and J. Bahler.** 2003. Global transcriptional responses of fission yeast to environmental stress. Mol. Biol. Cell **14:**214–229.
6. **Coleman, S. T., E. A. Epping, S. M. Steggerda, and W. S. Moye-Rowley.** 1999. Yap1p activates gene transcription in an oxidant-specific fashion. Mol. Cell. Biol. **19:**8302–8313.
7. **Cui, Z., T. Shiraki, D. Hirata, and T. Miyakawa.** 1998. Yeast gene *YRR1*, which is required for resistance to 4-nitroquinoline *N*-oxide, mediates transcriptional activation of the multidrug resistance transporter gene *SNQ2*. Mol. Microbiol. **29:**1307–1315.
8. **Delaunay, A., A. D. Isnard, and M. B. Toledano.** 2000. $H_2O_2$ sensing through oxidation of the Yap1 transcription factor. EMBO J. **19:**5157–5166.
9. **Delaunay, A., D. Pflieger, M.-B. Barrault, J. Vinh, and M. B. Toledano.** 2002.

A thiol peroxidase is an $H_2O_2$ receptor and redox-transducer in gene activation. Cell **111:**471–481.
10. **DeRisi, J., B. van den Hazel, P. Marc, E. Balzi, P. Brown, C. Jacq, and A. Goffeau.** 2000. Genome microarray analysis of transcriptional activation in multidrug resistance yeast mutants. FEBS Lett. **470:**156–160.
11. **Devaux, F., P. Marc, C. Bouchoux, T. Delaveau, I. Hikkel, M. C. Potier, and C. Jacq.** 2001. An artificial transcription activator mimics the genome-wide properties of the yeast Pdr1 transcription factor. EMBO Rep. **2:**493–498.
12. **Gabriel, K. R., and C. L. Odoroff.** 1990. Biplots in biomedical research. Stat. Med. **9:**469–485.
13. **Gasch, A. P., P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen, G. Storz, D. Botstein, and P. O. Brown.** 2000. Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell **11:** 4241–4257.
14. **Grant, C. M., G. Perrone, and I. W. Dawes.** 1998. Glutathione and catalase provide overlapping defenses for protection against hydrogen peroxide in the yeast *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **253:**893–898.
15. **Hikkel, I., A. Lucau-Danila, T. Delaveau, P. Marc, F. Devaux, and C. Jacq.** 2003. A general strategy to uncover transcription factor properties identifies a new regulator of drug resistance in yeast. J. Biol. Chem. **278:**11427–11432.
16. **Izawa, S., K. Maeda, K. Sugiyama, J. Mano, Y. Inoue, and A. Kimura.** 1999. Thioredoxin deficiency causes the constitutive activation of Yap1, an AP-1-like transcription factor in *Saccharomyces cerevisiae*. J. Biol. Chem. **274:** 28459–28465.
17. **Koerkamp, M. G., M. Rep, H. J. Bussemaker, G. P. M. A. Hardy, A. Mul, K. Piekarska, C. A. Szigyarto, J. M. Teixeiros de Mattos, and H. F. Tabak.** 2002. Dissection of transient oxidative stress response in *Saccharomyces cerevisiae* by using DNA microarrays. Mol. Biol. Cell **13:**2783–2794.
18. **Kuge, S., N. Jones, and A. Nomoto.** 1997. Regulation of yAP-1 nuclear localization in response to oxidative stress. EMBO J. **16:**1710–1720.
19. **Le Crom, S., F. Devaux, P. Marc, X. Zhang, W. S. Moye-Rowley, and C. Jacq.** 2002. New insights into the pleiotropic drug resistance network from genome-wide characterization of the YRR1 transcription factor regulation system. Mol. Cell. Biol. **22:**2642–2649.
20. **Lucau-Danila, A., T. Delaveau, G. Lelandais, F. Devaux, and C. Jacq.** 2003. Competitive promoter occupancy by two yeast paralogous transcription factors controlling the multidrug resistance phenomenon. J. Biol. Chem. **278:** 52641–52650.
21. **Marc, P., and C. Jacq.** 2002. Arrayplot for visualization and normalization of cDNA microarray data. Bioinformatics **18:**888–889.
22. **Moye-Rowley, W. S.** 2003. Transcriptional control of multidrug resistance in the yeast Saccharomyces. Prog. Nucleic Acid Res. Mol. Biol. **73:**251–279.
23. **Nguyen, D.-T., A.-M. Alarco, and M. Raymond.** 2001. Multiple Yap1p-binding sites mediate induction of the yeast major facilitator *FLR1* gene in response to drugs, oxidants, and alkylating agents. J. Biol. Chem. **276:**1138–1145.
24. **Pramila, T., S. Miles, D. GuhaThakurta, D. Jemiolo, and L. L. Breeden.** 2002. Conserved homeodomain proteins interact with MADS box protein Mcm1 to restrict ECB-dependent transcription to the M/G1 phase of the cell cycle. Genes Dev. **16:**3034–3045.
25. **Rodrigues-Pousada, C. A., T. Nevitt, R. Menezes, D. Azevedo, J. Pereira, and C. Amaral.** 2004. Yeast activator proteins and stress response: an overview. FEBS Lett. **567:**80–85.
26. **Roven, C., and H. J. Bussemaker.** 2003. REDUCE: an online tool for inferring *cis*-regulatory elements and transcriptional module activities from microarray data. Nucleic Acids Res. **31:**3487–3490.
27. **Spellman, P. T., G. Sherlock, M. Q. Zhang, V. R. Iyer, K. Anders, M. B. Eisen, P. O. Brown, D. Botstein, and B. Futcher.** 1998. Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. Mol. Biol. Cell **9:**3273–3297.
28. **Tenreiro, S., A. R. Fernandes, and I. Sá-Correia.** 2001. Transcriptional activation of *FLR1* gene during *Saccharomyces cerevisiae* adaptation to growth with benomyl: role of Yap1p and Pdr3p. Biochem. Biophys. Res. Commun. **280:**216–222.
29. **Troyanskaya, O., M. Cantor, G. Sherlock, P. Brown, T. Hastie, R. Tibshirani, D. Botstein, and R. B. Altman.** 2001. Missing value estimation methods for DNA microarrays. Bioinformatics **17:**520–525.
30. **Veal, E. A., S. J. Ross, P. Malakasi, E. Peacock, and B. A. Morgan.** 2003. Ybp1 is required for the hydrogen peroxide-induced oxidation of the Yap1 transcription factor. J. Biol. Chem. **278:**30896–30904.
31. **Wemmie, J. A., S. M. Steggerda, and W. S. Moye-Rowley.** 1997. The *Saccharomyces cerevisiae* AP-1 protein discriminates between oxidative stress elicited by the oxidants $H_2O_2$ and diamide. J. Biol. Chem. **272:**7908–7914.