Dissection of Transient Oxidative Stress Response in *Saccharomyces cerevisiae* by Using DNA Microarrays[□]

Marian Groot Koerkamp,* Martijn Rep,[†] Harmen J. Bussemaker,^{†‡} Guy P.M.A. Hardy,* Adri Mul,* Kasia Piekarska,* Cristina Al-Khalili Szigyarto,* Joost M. Teixeira de Mattos,[§] and Henk F. Tabak*^{||}

*Department of Biochemistry, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands; [†]Department of Plant Pathology, Swammerdam Institute for Life Sciences, University of Amsterdam, 1098 SM Amsterdam, The Netherlands; and [§]Department of Microbiology, University of Amsterdam, 1018 WV Amsterdam, The Netherlands

Submitted February 7, 2002; Revised May 27, 2002; Accepted June 5, 2002
Monitoring Editor: Thomas D. Fox

Yeast cells were grown in glucose-limited chemostat cultures and forced to switch to a new carbon source, the fatty acid oleate. Alterations in gene expression were monitored using DNA microarrays combined with bioinformatics tools, among which was included the recently developed algorithm REDUCE. Immediately after the switch to oleate, a transient and very specific stress response was observed, followed by the up-regulation of genes encoding peroxisomal enzymes required for fatty acid metabolism. The stress response included up-regulation of genes coding for enzymes to keep thioredoxin and glutathione reduced, as well as enzymes required for the detoxification of reactive oxygen species. Among the genes coding for various isoenzymes involved in these processes, only a specific subset was expressed. Not the general stress transcription factors Msn2 and Msn4, but rather the specific factor Yap1p seemed to be the main regulator of the stress response. We ascribe the initiation of the oxidative stress response to a combination of poor redox flux and fatty acid-induced uncoupling of the respiratory chain during the metabolic reprogramming phase.

INTRODUCTION

Aerobic life is associated with the production of reactive oxygen species (ROS) by various metabolic processes. ROS can modify lipids, proteins, and nucleic acids and can particularly cause mutations in DNA, which might contribute to tumor formation. Normally, ROS production is kept at bay by a variety of detoxifying enzymes, some of which derive their reducing power from glutathione (GSH) or thioredoxins (TRXs) (Hohmann and Mager, 1997; Jamieson and Storz, 1997; Grant *et al.*, 1998). However, in certain pathological conditions caused by tissue damage or during treatment with certain pharmaceuticals, this protection fails,

probably due to a compromised redox state: [NAD(P)H/NAD(P)]. Although the mitochondrial respiratory chain is an important source of ROS, peroxisomal metabolism is another contributor in this respect. For instance, in rodents, application of hypolipidemic drugs resulted in enlargement of the peroxisome compartment, and long-term treatment even caused cancer (Lock *et al.*, 1989; Reddy and Mannaerts, 1994).

Peroxisomes house a number of oxidative enzymes producing ROS, such as H₂O₂, which is formed during the β -oxidation of fatty acids (Beevers, 1969; Van den Bosch *et al.*, 1992). In the classic view, the raison d'être of the organelle is to provide a boundary to keep ROS confined within a compartment where they can be quickly detoxified. Several considerations indicate that this concept may be too simple (Tabak *et al.*, 1999). H₂O₂ can easily permeate through membranes and loss of peroxisomal catalase remains without symptoms. Is this due to the fact that other detoxifying enzymes come to the rescue? There are indeed suggestions that peroxisomes harbor additional GSH or thioredoxin-dependent detoxifying enzymes (Jeong *et al.*, 1999; Lee *et al.*, 1999b), but it may also be that cytosolic enzymes are recruited.

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E02-02-0075. Article and publication date are at www.molbiolcell.org/cgi/doi/10.1091/mbc.E02-02-0075.

[‡] Present address: Department of Biological Sciences, Columbia University, 1212 Amsterdam Ave., MC 2441, New York, NY 10027.

^{||} Corresponding author. E-mail address: h.f.tabak@med.uu.nl.

[□] Online version of this article contains complete data sets. Online version available at www.molbiolcell.org.

An opportunity to study the role of peroxisomes in relation to ROS metabolism arose from our work with *Saccharomyces cerevisiae* as a model to study on a genome-wide scale how cells adapt to growth on a fatty acid as sole carbon source. In the experimental setup that we chose to carry out these experiments, steady-state growth of cells in a glucose-limited chemostat and shifting them subsequently to oleate, we observed that the cells experienced a transient oxidative stress response. Remarkably, this stress response was very specific in terms of the enzymes that were recruited and was completely independent of factors usually considered to be part of the general stress response. This was inferred from our observations that targets of the Yap1p transcription factor were up-regulated, whereas targets of the transcription factors Msn2p and Msn4p were down-regulated during this stress period. These findings differ from prevalent opinions on the behavior of yeast cells experiencing stress and provide arguments for a role for Msn2p and Msn4p in reprogramming cellular metabolism.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions

S. cerevisiae strain DBY7286 (MATa, ura3-52, GAL2; Spellman *et al.*, 1998) was grown in chemostat cultures of 700 ml (L.H. Fermentation, Stoke Poges, Buckinghamshire, United Kingdom). Cells were cultured under glucose limitation at a constant dilution rate (*D*) of $0.1 \pm 0.01 \text{ h}^{-1}$, resulting in a doubling time of 6.9 h. At steady state, the optical density at 600 nm was 5 and the dry weight 2.2 mg/ml. The growth medium contained salt medium as described previously (Evans *et al.*, 1970), with the following modifications and additions: 2 mM nitrilo-triacetic acid replaced citrate as chelator, 3 g/l yeast extract, 5 g/l bactopecton, uracil (20 $\mu\text{g/l}$), and vitamins—myo-inositol (0.55 mM), calcium-D(+)-pantothenate (0.2 mM), pyridoxin-HCl (0.013 mM), thiamin-HCl (0.006 mM), D(+)-biotin (0.05 mM), nicotinate (0.16 mM), and 2.5 g/l glucose. This rich medium was chosen by necessity because *S. cerevisiae* cannot be grown in minimal medium with oleate as sole carbon source. The pH was kept at 5.8 ± 0.1 by titrating with sterile 2 M NaOH. Temperature was set at 28°C. Silicone antifoam was added to prevent foaming. Chemostat cultures were flushed with air at a flow rate of 1.05 l/min. The culture was stirred at 1250 rpm. Culture purity was routinely monitored by phase-contrast microscopy and by plating on YPD and selective ura⁻ plates. O₂ and CO₂ concentrations were determined in the effluent gas with an oxygen analyzer (paramagnetic O₂ transducer; Servomex, Crowborough, United Kingdom) and an I.R. gas analyzer (Servomex), respectively. Dry weight was measured by the procedure of Herbert *et al.* (1971). Growth in steady state was limited by glucose availability as shown by various controls: 1) the initial glucose concentration in the medium was 13 mM but undetectable (<0.5 mM) in the chemostat chamber, whereas the presence of alcohol could not be demonstrated; 2) when the glucose concentration in the medium was doubled biomass increased correspondingly, and 3) this is in line with extensive chemostat studies (Van Hoek *et al.*, 1998), which indicate that below growth rates of 0.3/h, glucose is the limiting factor determining the increase in biomass. Media and culture supernatants were analyzed by high-performance liquid chromatography with an HPX 87H Aminex ion exchange column (30 × 7.8 mm; Bio-Rad, Hercules, CA) at 60°C. The column was eluted with 5 mM H₂SO₄ at a flow rate of 0.6 ml/min. Fatty acids were quantitatively analyzed in supernatants by capillary gas chromatography (Dacremont *et al.*, 1995).

Time Course Experiments, RNA Isolation, and cDNA Synthesis

Starting from steady-state cultures in the chemostat, two time-course experiments were set up and sampled. In an oleate pulse (OP) experiment, at time point 0, oleic acid (Merck, Darmstadt, Germany) was added to a final concentration of 0.12% and the pump supplying nutrients, including glucose, was stopped. Samples of 30 OD units of cells were taken at various times during 90 min. A control experiment was carried out in which the addition of glucose was stopped but the supplementation with oleate was omitted.

Samples were taken by collecting 7.5 ml of cells (30 OD units) with a QuickSampler in an equal volume of ethanol at −80°C. The transfer time of the sample from the culture to ethanol was <0.1 s (Lange *et al.*, 2001). The cells were concentrated at −20°C, resuspended, and stored as pellets at −80°C.

Cell content was released from the frozen cell samples by disruption in a Braun Microdismembrator S (B. Braun Biotech International GmbH, Melsungen, Germany). RNA was extracted from the frozen powder by using TRIzol and was further purified according to the instructions from the supplier (Invitrogen). ³²P-Labeled cDNA probes were made using oligo(dT) and reverse transcriptase on 5–20 μg of total RNA as described previously (Hauser *et al.*, 1998).

Microarray Setup

Gene pairs (6219) were provided by Research Genetics (Huntsville, AL) and were used to amplify open reading frames (ORFs) from yeast DNA, including start and stop codons. All primers contained tags that allow a second round of amplification with one set of primers: RG1, 5' GGAATTCAGCTGACCACC 3'; and RG2, 5' GATCCCCGGGAATTGCCATG 3'.

Forward and reverse oligonucleotides were mixed and diluted using a Biomek 2000 robot (Beckman Coulter, Fullerton, CA). Polymerase chain reaction (PCR) reactions were set up in 384-well plates by using the same robot; 20- μl reactions contained 4 ng of yeast DBY7286 genomic DNA, 8 pmol of each primer, and 0.4 U of *Taq* polymerase (Takara, Kyoto, Japan). Thirty amplification cycles (annealing at 55°C; 5-min extension) were run in a PTC200 (MJ Research, Waltham, MA), containing an automatic 384-well block. All products were supplied with sucrose (1.5%) and cresol red (0.025%) and were tested on 1% agarose gels to be verified for quantity and correct length. ORFs that failed to amplify in the first round were included in a second attempt at amplification. The final success rate was 97% (6013 ORFs in total, including 18 amplification products whose sizes visibly differed from expected sizes and another 14 for which an additional "contaminating" product was seen on the gel). Reamplifications were carried out with the common tag oligonucleotides on a dilution of the primary products. Typical reactions yielded 5 μg of product, as was estimated from gel electrophoresis. The PCR reactions were diluted into sucrose (1.5%) and cresol red (0.025%) to a concentration of 0.1–0.5 $\mu\text{g}/\mu\text{l}$, and this mixture was used for spotting.

Microarrays were generated using a home-built arrayer (see <http://cmgm.stanford.edu/pbrown/mguide/>), with some home-made modifications. The print-head contains 12 adjustable custom-made quilted pins. The machine is programmed to yield filter arrays with duplicate spot patterns of each gene product. The amount of DNA delivered to the filter was 60–80 nl/spot (6–40 ng). With spot sizes of ~700 μm and a heart-to-heart distance of 1 mm, ³²P can be used without the risk of overlapping signals.

Filter arrays were generated using Hybond N⁺ (Amersham Biosciences, Piscataway, NJ). Besides all duplicate gene products, external controls were spotted (Holstege *et al.*, 1998) as well as cornerspots to facilitate the image analysis.

After drying, the filters were denatured and renatured according to standard protocols (Sambrook *et al.*, 1989), and cross-linked using

a Stratalinker (position auto cross-link; Stratagene, La Jolla, CA). Filters were reused several times after melting off the labeled target, cDNA (Hauser *et al.*, 1998).

Hybridization with labeled target was performed in bottles in a hybridization oven in 5× SSC, 5× Denhardt, and 0.1% SDS at 65°C for at least 40 h. A stringent wash was done at 0.2× SSC, followed by exposure and scanning at 50-μm resolution on a Storm 860 (Molecular Dynamics, Sunnyvale, CA).

Image and Data Analysis

The Storm image file (16-bit tif) was analyzed using Bioimage high-density grid analyzer (Genomic Solutions, RMLuton, Inc., Jackson, MI). Resulting report files containing integrated intensities for each spot were then subjected to further data analysis.

Normalization was carried out using total integrated intensities per filter, thus assuming a constant amount of mRNA per cell throughout the time course of the experiment. As an alternative, external spots were taken for normalization (Holstege *et al.*, 1998), but no significant differences in results were seen between those methods in this experimental setup.

Duplicate spots were averaged, and values with a SD (SD/average × 100%) >50% were ignored for further analysis (mostly very low values, usually 400–600 spots/filter). The reproducibility of the microarray analysis was assessed by correlation of datasets. Duplicate spots on one filter correlated >0.99; different labeling on identical RNA samples correlated >0.95.

Normalized intensities were converted to ratios by using the zero time point, the steady state, as the reference and were transformed to ²log-ratios to be used in all subsequent computational analyses. Using the computer program REDUCE each time point was analyzed separately for regulatory motifs in the 600-base pair upstream region of all ORFs (allowing no overlap with other ORFs) (Bussemaker *et al.*, 2001). For changes in gene categories defined by the Gene Ontology Project (Ashburner *et al.*, 2000), the program QUONTOLOGY (Bussemaker and Lascaris, unpublished data) was used. The magnitude of the Pearson correlation between the log-ratio for a gene and the relevant feature (i.e., motif count in the promoter region for REDUCE; belonging to specific Gene Ontology Project category for QUONTOLOGY) was represented by a Z-score equal to the deviation from zero in units of the SD for random log-ratios drawn from a normal distribution.

Ratio data sets of time courses as described above were used for filtering genes by applying ²log calculation, >95% presence, and a more than twofold change as described by Cluster (Eisen *et al.*, 1998). Expression data of selected ORFs were used as input for GeneMaths (Applied Maths, Sint-Martens-Latem, Belgium) to be subjected to several clustering algorithms. Hierarchical clustering was performed by Euclidian distance metric clustering and UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering.

GFP Labeling of Transcription Factors Yap1p and Msn2p

The coding sequences of YAP1p and MSN2 were cloned to yield an in-frame fusion of each ORF with the green fluorescent protein (GFP) behind a weak promoter (PEX5) in YCPLac33. The fusion constructs were then subcloned in YIPlac211 and integrated into yeast strain DBY7286. Resulting strains were subjected to the chemostat culturing conditions and time-course experiments described above. Samples were drawn and unfixed cells were as soon as possible photographed using a fluorescence microscope (Axiophot 2; Carl Zeiss, Thornwood, NY), supplemented with phase-contrast light.

RESULTS

Full transcriptome analysis is an excellent method to study physiological adaptation processes in yeast cells experiencing

a sudden change in carbon source. In most laboratory protocols, a shift in carbon source involves manipulations such as collecting cells by centrifugation and resuspending them in a new culture medium, followed by batch-wise growth. Such procedures prevent analysis on a short time scale and may also interfere with the objectives of the study itself. Instead, we chose a chemostat for our experimental setup because 1) cells can be cultivated under well-defined growth conditions, keeping biological noise to a minimum; 2) the steady state can be easily reproduced and thus a renewable reference source can be created for multiple experiments, and 3) under conditions of carbon-limited growth, neither medium nor cells contain residual carbon source, which makes it possible to change to a new carbon source immediately and effectively.

Yeast cells were grown in the chemostat on rich medium containing glucose (for rationale, see MATERIALS AND METHODS). The amount of glucose supplied (2.5 g/l) and the dilution rate (0.1 h⁻¹) in the chemostat were chosen to limit growth of the cells by the availability of glucose and to obtain a generation time of 7 h. This rather long generation time was dictated by the relatively slow growth rate of yeast on oleate and our wish to compare the cells under equal conditions on both carbon sources. After reaching steady state, the addition of glucose-containing medium was stopped and oleate was added to bring the concentration of oleate in the chemostat vessel to 0.12%. This selective change in carbon source without altering the other components of the medium is a typical advantage of the chemostat setup. At various time points aliquots of the culture were taken for mRNA profiling. Time courses were determined in two independent chemostat experiments. For each time point two independent RNA isolations were carried out and ³³P-labeled cDNA was synthesized. Labeled cDNA was hybridized with nitrocellulose filters containing duplicate DNA spots of 6013 PCR-amplified yeast ORFs (for details, see MATERIALS AND METHODS).

Global Analysis of Changes in mRNA Abundance

To get insight into the physiological changes that took place after the shift from glucose to oleate, we analyzed our data by using three different bioinformatics tools. Using the steady state as a reference, the mRNA abundance measured at different times was first converted to log-ratios. The program REDUCE (Bussemaker *et al.*, 2001) was then used to perform simultaneous analyses of genome sequence and expression data. Based on an unbiased search, REDUCE selects those sequence motifs whose occurrence in the upstream region of a gene correlates with a change in expression. Multivariate analysis is then applied to infer changes in the activity of all relevant transcription factors. This allows a compact and informative representation of the transcriptional response and takes into account the combinatorial nature of transcriptional regulation. Many of the significant motifs we found corresponded to binding sites of known transcription factors. The activity time courses for six known DNA control elements are shown in Figure 1A.

We also used the program QUONTOLOGY (Bussemaker and Lascaris, unpublished data), which scores functional categories (Gene Ontology) based on expression of the genes they contain, in close analogy with REDUCE. This provides global information about the biological processes that are

Figure 1. Dissection of the transcriptional response of yeast cells to a shift from glucose to oleate as new carbon source as analyzed by DNA microarrays and three bioinformatics tools. (A) REDUCE analysis of the oleate induction time course. The log-ratios data set of an oleate induction time course were correlated with DNA sequence elements in promoter regions using the program REDUCE. Six relevant regulatory motifs are shown. Complete results are available at <http://bussemaker.bio.columbia.edu/papers/oleate>. (B) QUANTOLOGY analysis of the same oleate induction time course. Time courses corresponding to the average log-ratio for genes in five significantly changing functional categories from Gene Ontology. (C) Hierarchical clustering of expression profiles for genes whose expression changed significantly. Three time-course experiments were analyzed: one oleate induction experiment with double RNA isolations (OP1 and OP2), and one independent oleate induction experiment from a separate chemostat (OP3). All datasets were gathered and filtered as described in MATERIALS AND METHODS. Filtering resulted in 269 significantly regulated genes (more than twofold induced or repressed). This set was used as input for GeneMaths (www.applied-maths.com) to perform Euclidean correlation combined with UPGMA clustering. Enlarged are 1) an early up-regulated cluster, containing many Yap1-regulated genes (red squares) or containing a Yap1-control element (TTASTAA); and 2) a cluster containing peroxisomal matrix genes (green squares) and/or an ORE element. Also, down-regulation of glycolysis, the significance of a (promoter) element with unknown function (CGATGAG) and STRE elements (AAGGGG) are indicated.

associated with the transcriptional response. Time courses of categories that were significantly induced or repressed at one or more time points are shown in Figure 1B.

Finally, we scrutinized the data by hierarchical clustering, after first filtering out the genes whose expression did not change significantly during the time course (Eisen *et al.*, 1998; Tamayo *et al.*, 1999) (Figure 1C). Approximately five clusters of genes could be discerned: 1) a group that was up-regulated in the beginning of the time course, most genes of which contained the Yap1p DNA target sequence (TTASTAA) in their promoter; 2) a group that was increasingly down-regulated during the time course, comprising many genes coding for glycolytic enzymes; 3) a group that was up-regulated after a delay of ~30 min, most genes of which contained an oleate response element (ORE) motif in their promoter; 4) a group that was first up- but later down-regulated, with a common motif of unknown function (CGATGAG) in the region upstream of the genes; and 5) a group that was down-regulated in the beginning of the time course, which is rather enriched in STRE (AAGGGG) elements, which are reported to mediate a general stress re-

sponse. Thus, the oxidative and general stress responses appeared in opposite clusters.

Taken together, the three different approaches complemented each other and allowed us to delineate the most important physiological changes that took place after the change in carbon source.

Genes Involved in Peroxisomal Functions

As expected, the shift to oleate activated genes encoding enzymes involved in fatty acid degradation, allowing efficient use of the new carbon source. These enzymes are housed in peroxisomes and indeed also genes were expressed coding for PEX proteins, involved in the maintenance of these organelles and required for the increase in their number and volume during growth on oleate. It is known that the levels of the β -oxidation enzymes increase due to a dramatic and coordinate induction of the corresponding genes (Kal *et al.*, 1999). This is achieved via the action of two transcription factors, Oaf1p and Pip2p, that recognize a *cis*-acting sequence called ORE in the promoter of these genes (Einerhand *et al.*, 1993; Luo *et al.*, 1996; Rot-

Figure 1. Continued

tensteiner *et al.*, 1996). Indeed, REDUCE analysis indicated the use of the ORE UAS (Figure 1A) and QUANTOLOGY reported a strong correlation of peroxisome functional categories with the expression data (Figure 1B). This picture was reinforced by hierarchical clustering that grouped a set of genes, most of which are dependent on ORE for their expression (Figure 1C).

An Oxidative Stress Response Precedes Metabolic Adaptation

Expression of genes coding for peroxisomal functions did not start immediately after the addition of oleate (Figure 1A). Instead, their expression increased ~20–30 min later. From control elements and corresponding transcription factors that became active immediately after the addition of oleate, we could deduce that the cells had encountered a temporary crisis. Genes under the control of the transcription factor complex MCF (Swi6p + Mbp1p) targeting the MCB site were down-regulated, which indicates that cells stopped multiplying and arrested during the G1 phase (Spellman *et al.*, 1998). Genes controlled by the transcription factor Pdr3p (DeRisi *et al.*, 2000) were up-regulated. Among other things, this transcription factor controls the expression of genes coding for multidrug transporters located in the plasma membrane, suggesting that oleate might initially be experienced as an unwanted compound due to its detergent-like properties. A clue as to the nature of the stress was given by the up-regulation of genes controlled by Yap1p (Figure 1A), a transcription factor involved in oxidative stress (Stephen *et al.*, 1995; Delaunay *et al.*, 2000). Remarkably, the targets of the transcription factors Msn2p and Msn4p, which are considered to be factors that operate during general stress (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996), were in fact down-regulated during this period (Figure 1A) and also the functional category “stress” displayed a negative Z-score during the first 50 min (Figure 1B).

The occurrence of oxidative stress deduced from the behavior of the Yap1p-binding element could be inferred from a marginally positive identification of an oxidative stress functional group (Figure 1B). The low Z-score for this group may be due to the fact that this category is still rather small and heterogeneous and that some of the enzymes belonging to this group are still dispersed in other functional groups of the Gene Ontology database. This annotation problem was particularly obvious for the functional group “gluconeogenesis,” which group showed a negative Z-score increasing with time (our unpublished data). This was unexpected because for growth on oleate gluconeogenesis is indispensable. Indeed, expression of the genes required for gluconeogenesis, *FBP1* and *PCK1*, was strongly induced. However, because this functional group also contains genes coding for glycolytic enzymes, it was not surprising that this group was found to be down-regulated. In addition, of the genes coding for isoenzymes/proteins listed in the oxidative stress group only some were induced (see below), which affected the Z-score of the functional group as a whole. This neatly illustrates the problems underlying the definition of functional groups.

Figure 2. GFP-Yap1p and GFP-Msn2p localization after oleate induction. A yeast strain containing an integrated copy of GFP-Yap1p (A) and GFP-Msn2p (B) was subjected to glucose-limited growth in a chemostat, followed by oleate induction. At indicated times samples were taken and immediately inspected under a fluorescence microscope and photographed. A full oxidative stress response for Yap1p is shown in panel CCCP in which cells were treated with the uncoupler CCCP.

Movement of Yap1p and Msn2p between Cytosol and Nucleus

Independent control over the active state of the transcription factors Yap1p and Msn2/Msn4p is made possible by their property to move from cytosol to nucleus upon activation (Beck and Hall, 1999; Delaunay *et al.*, 2000). To visualize this process, we constructed an expression cassette of the *YAP1* and *MSN2* genes fused to the GFP gene behind the weak promoter of the *PEX5* gene. These cassettes were integrated in the *URA3* locus, and the corresponding stable transformed strains were grown in the chemostat under the conditions specified above. During the steady state the Yap1p-GFP fusion protein was present in the cytosol and cells showed an overall green fluorescence (Figure 2). After stopping the supply of glucose and the subsequent addition of oleate, cells temporarily showed green fluorescent nuclei indicating the transfer of Yap1p to the nucleus. The number of fluorescent nuclei peaked early after the addition of oleate. Thereafter the fluorescence returned to the cytosol, which confirms the transient nature of the oxidative stress response. As a control we treated steady-state grown cells with the uncoupler carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP). This resulted in much brighter fluorescent nuclei confirming the relatively mild nature of the oleate-induced oxidative stress. The opposite was noted for Msn2p-GFP. During the steady state Msn2p-GFP resided both in the cytosol and in the nucleus as expected for glucose-starved cells (Gorner *et al.*, 2002), moved to the cytosol after the addition of oleate, to return to the nucleus later. These qualitative results show the contrasting behavior of Yap1p

Figure 3. Components of the redox/ROS stress response. Isoenzymes are shown that are possibly involved in maintaining cellular redox balance and detoxification of ROS.

and Msn2p and confirm our interpretation deduced from the quantitative microarray data.

Dissection of Oxidative Stress Response

In view of the difficulties with annotation and grouping in functional categories mentioned above, we decided to carry out a more detailed analysis of the apparent oxidative stress during the carbon shift, based on the expression of individual genes. The most important agents that keep the cytosol in a reduced state are GSH and the TRXs 1–3, which are all kept in reduced state by NADPH (Grant *et al.*, 1998). Reduced GSH and thioredoxins are used as cofactors by a number of enzymes in various detoxification reactions, whereas other ROS-removing enzymes use alternative hydrogen donors (Figure 3). For all these enzymes, we inspected how their mRNAs are expressed during the first hour after the switch to oleate.

We found that mRNAs of *TRR1* and *TRX2* rose quickly after the switch (Figure 4A). *TRR1* encodes thioredoxin reductase, which keeps thioredoxin reduced at the expense of NADPH; *TRX2* encodes cytosolic thioredoxin. The genes coding for the enzymes required for synthesis of GSH (*GSH1* and *GSH2*) and keeping it reduced (*GLR1*) were also induced but to 10–20-fold lower levels (Figure 4B). These data suggested that a redox imbalance developed shortly after the switch to oleate, causing induction of primarily the thioredoxin system. At the same time, mRNAs for ROS-removing enzymes accumulated (Figure 5). A very transient rise in mRNA was observed for a glutathione peroxidase (*GPX2*). Peaking at 20–30 min were mRNAs coding for cytochrome *c* peroxidase (*CCP1*), a typical H_2O_2 scavenger associated with mitochondria in yeasts (Verduyn *et al.*, 1991), for thioredoxin peroxidase (*TSA1*), and for cytosolic superoxide dismutase (*SOD1*). Remarkably, catalases were not called upon in coping with the stress: mRNA for cytosolic catalase (*CTT1*) remained very low. To validate this last result *CTT1* mRNA was also probed by Northern blot analysis (our unpublished data), which confirmed the DNA microarray data. *CTT1* is an Msn2/4p-controlled gene and indeed behaved similarly to genes belonging to the “STRE” group and the “general stress” group (Figure 1, A and B). The mRNA for peroxisomal catalase (*CTA1*) followed the

Figure 4. Expression of individual redox genes in oleate induction. Absolute expression levels of selected redox genes were taken from the microarray data of an oleate induction time course (A). POT1 was included as a marker for peroxisomal induction. Glutathione-related genes show 10-fold lower expression levels (B).

normal expression pattern of peroxisomal enzyme-encoding genes, indicating that also this catalase did not contribute to combating the oxidative stress. Recently, it was reported that *AHP1* codes for a thioredoxin or glutathione-dependent ROS-detoxifying enzyme (Geraghty *et al.*, 1999; Lee *et al.*, 1999b). In *Candida boidinii* the orthologous protein is located in peroxisomes (Horiguchi *et al.*, 2001) and on the basis of its PTS1-like C-terminal tripeptide in *S. cerevisiae*, it was proposed to be a peroxisomal enzyme in this organism, too. Surprisingly, however, expression of *AHP1* followed exactly the early oxidative stress response preceding the appearance of mRNAs typical for peroxisomal enzymes. In addition, Ahp1p, NH-tagged at its N terminus, appeared in the cytosolic fraction upon biochemical fractionation of a cell homogenate (our unpublished data). It will therefore be of interest to find out whether Ahp1p is indeed a peroxisomal enzyme in *S. cerevisiae*.

Interestingly, mRNA coding for Yap1p also increased slightly, shortly after the shift from glucose to oleate. However, Skn7p, another transcription factor involved in oxidative stress (Morgan *et al.*, 1997; Lee *et al.*, 1999a), was not significantly altered in its expression (our unpublished data).

Figure 5. Expression of ROS genes after oleate addition. Absolute expression levels of selected ROS genes were taken from the microarray results. POT1 was taken as a marker of peroxisome induction.

Possible Toxicity of Oleate

At the moment the carbon source was changed, the cells experienced no glucose repression because glucose is the limiting factor in the chemostat and therefore cells are in a derepressed state. Such cells express *PEX* genes for peroxisomal maintenance functions (see above) and genes coding for peroxisomal enzymes are expressed at low levels. Although full adaptation to growth on oleate as single carbon source requires much higher levels of peroxisomal enzymes than the derepressed state and reprogramming of cellular metabolism must take place, it is likely that some redox flow can be maintained to produce ATP, NADH, and NADPH. Moreover, the cells were not completely deprived of nutrients because they were grown in rich medium, which to some extent provides alternative carbon sources to oleate.

If the time to reprogram metabolism was the critical factor producing the oxidative stress, one would predict that stopping the supply of glucose and providing no new carbon source would bring the cells in an even worse condition. The opposite was found, however: no indication for an oxidative stress response was observed (Figure 7C). We analyzed the results of this time-course experiment with the algorithm REDUCE (Figure 6) and compared them with the data shown in Figure 1A. Withholding glucose did not lead to

Figure 6. REDUCE analysis of a glucose-stop time course, performed as control for the oleate-response experiment presented in Figure 1. Activity profiles are shown here for the six promoter elements in Figure 1A.

down-regulation of MCF-controlled genes or stimulation of stress-related processes controlled by Yap1p or Pdr3p. Predictably, there was no transcriptional activation of genes controlled by Pip2p/Oaf1p or Adr1p. Interestingly, the transcription factor Msn2p/Msn4p behaved completely different from the glucose-to-oleate shift. Shortly after stopping the addition of glucose genes controlled by Msn2p/Msn4p were strongly up-regulated. This was a transient effect, however, because after 30 min it was no longer observed. We also carried out an experiment in which the final concentration of oleate was much lower (0.0002 compared with 0.12%). Under these conditions, which also led to production of mRNAs coding for peroxisomal enzymes, the cells did not mount a stress response either. Together, the findings of these experiments suggest that the oxidative stress response is related to the sudden exposure of the cells to relatively high levels of oleate that are commonly used in the field to start batch cultures, rather than to withdrawal of glucose.

Table 1. The uncoupling effect of oleate addition (0.12%)

Percent oleate	q_{O_2} [mmol $\times$ (g biomass $\cdot$ h) $^{-1}$]	Percent increase in biomass concentration	Doubling time (h $^{-1}$)
Steady state	2.0 ($\pm$ 0.2)		6.9
0.12	2.0 ($\pm$ 0.2)	0	0
0.0002	1.4	3.7	9
0	1.1 ($\pm$ 0.1)	3.7 ($\pm$ 0.4)	9 ($\pm$ 0.5)

Oxygen consumption was monitored continuously according to MATERIALS and METHODS. The doubling time was calculated from viable counts and optical density measurements of samples taken every 5 min during 30 min. Data for steady state are the averaged values of six experiments, two experiments for 0.12%, one experiment for 0.0002%, and two experiments for 0%. (STDEV)

Figure 7. RNA levels of indicative genes for peroxisome induction and stress-response are plotted from time courses of 0.12% oleate addition (A), 0.0002% oleate addition (B), and the glucose stop experiment (C).

Oleate-induced Uncoupling

We explored the possibility that the transient toxicity of oleate was due to its ability to uncouple the respiratory chain (Polcic *et al.*, 1997; Skulachev, 1998). This is indeed in line with several observations. We have compared O_2 consumption, CO_2 production and increase in biomass under the same three conditions as specified above: 1) glucose

addition was stopped and oleate was added to 0.12% (conditions as in Figure 1), 2) glucose addition was stopped and a lower dose of oleate to 0.0002% was added, and 3) glucose addition was stopped and no new carbon source was supplied. In all cases, there is comparable O_2 consumption after the change in carbon source, but only in 2 and 3 is there an increase in biomass (Table 1). Supplementation of 0.12% oleate results in O_2 consumption without increase in biomass, a typical feature of uncoupled respiration.

We also compared the changes in mRNAs coding for representative genes of the stress response with peroxisomal thiolase (*POT1*) mRNA as reference marker for peroxisomal enzyme formation. Figure 7 shows that the reported stress response upon exposure to 0.12% oleate (Figures 1 and 7A) does not take place in the other two conditions (Figure 7, B and C). The nature of the oxidative stress response combined with the physiological growth parameters presented in Table 1 suggests the occurrence of a redox imbalance in the cells due to a transient uncoupling of the respiratory chain by oleate.

DISCUSSION

Cellular life is frequently endangered by adverse conditions. Particularly unicellular organisms experience many forms of unfavorable changes to which they react with various stress responses. In the experimental setup described herein we observed the development of a transient oxidative stress response of short duration. It occurred when we exposed yeast cells grown in a chemostat on limiting glucose (derepressed state) to the fatty acid oleate as new carbon source and analyzed the alterations in mRNA content by DNA microarrays. Global insight into the changing patterns of gene expression was obtained by applying the algorithm REDUCE (Bussemaker *et al.*, 2001). REDUCE uses expression patterns to determine which promoter elements are responsible for the observed dynamic transcriptional response, and to infer the activity of the associated transcription factors. With an estimated number of 250 transcription factors and >6000 genes in yeast, the complexity of the data can be much reduced. Annotated UAS elements were found as well as elements for which binding factors still need to be identified, including the motifs AAAATTTT, TGAAAAA, and CGATGAG. The last motif correlated particularly well with the expression data upon hierarchical clustering (Figure 1C). REDUCE predicted that Yap1p, a transcription factor of oxidative stress, would be active shortly after the change from glucose to oleate.

The clues provided by REDUCE were followed up by inspecting the time course of expression of the individual genes that belong to the target genes of the corresponding transcription factor, and by inspecting the behavior of genes that belong to the same functional category. This analysis showed that there was a rise in mRNAs coding for proteins upholding the redox state of the cell (cytosolic thioredoxin [*TRX2*], thioredoxin reductase [*TRR1*]) and to a lesser extent in the mRNAs coding for the enzymes required for the synthesis of glutathione (*GSH1*, *GSH2*) and glutathione reductase (*GLR1*), which keeps glutathione reduced. At the same time we saw a rise in mRNAs coding for ROS-detoxifying enzymes. Within 0.5 h the cells apparently recuperated from this stressful period and resumed normal metab-

olism, as evidenced by the steep rise in mRNAs coding for enzymes of peroxisomal fatty acid metabolism and mitochondrial enzymes, and the decrease in levels of mRNAs of genes regulated by Yap1p and Pdr3p.

A striking aspect of the oxidative stress response observed herein is its specificity. A rise in thioredoxin mRNA was preferred to a rise in the level of mRNAs involved in glutathione biosynthesis. Furthermore, there was a remarkable specificity in the choice of expression of genes coding for isoenzymes or isoproteins. Among the thioredoxins *TRX2* was preferred to *TRX1* and *TRX3*, and among the glutaredoxins *GRX2* was preferred to *GRX1*, *GRX3*, and *GRX4*. Thioredoxin reductase type 1 (*TRR1*) was preferred to thioredoxin reductase type 2. The same is true for the enzymes capable of detoxifying ROS. Contributing to the specificity of the detoxifying response may be substrate preference toward the type of ROS produced. However, this cannot be the full explanation. For instance, for the removal of H_2O_2 cytochrome *c* peroxidase (*CCP1*) was strongly preferred to cytosolic catalase (*CTT1*), which was not expressed at all.

The observed specificity differs in a number of respects from reports in the literature. Reviews emphasize the (partial) coinduction of the proteins that are part of various stress responses and the interconnections between the control circuits responsible for their induction (Hohmann and Mager, 1997; Jamieson and Storz, 1997; Causton *et al.*, 2001). This difference in results is best illustrated by the results of REDUCE shown in Figure 1A, which indicate that during the stress period targets of the transcription factors Msn2p and Msn4p are down-regulated. These transcription factors are considered to be main actors in responding to various forms of stress, including oxidative stress, and to be activators to induce responses to environmental change (Hohmann and Mager, 1997; Beck and Hall, 1999; Causton *et al.*, 2001). Surprisingly, in our experimental setup they only came into action when the oxidative stress period was over and the other transcription factors needed for adaptation to the environmental change became active (Adr1p, Pip2p, and Oaf1p). Similar behavior of Msn2p/Msn4p was observed when we evaluated the course of events occurring after stopping the supply of glucose and providing no new, alternative carbon source. Under these conditions, no stress response was mounted but Msn2p/Msn4p was very active during the first 30 min of this experiment (Figure 6). This again indicates that Msn2p/Msn4p was particularly active during events requiring metabolic reprogramming. Such a role has also been proposed before for Msn2p and Msn4p under conditions of diauxic shift (DeRisi *et al.*, 1997; Boy-Marcotte *et al.*, 1998). An explanation for the observed differences is offered by recent work indicating that the nuclear localization of Msn2p can be achieved in two independent ways: 1) by controlling the phosphorylation state of the nuclear import signal in Msn2p by cAMP-dependent protein kinase in response to glucose availability; and 2) by affecting the functional state of the nuclear export signal, located in a different part of Msn2p, in response to various forms of environmental stress (Gorner *et al.*, 2002).

We ascribe the cause of the oxidative stress not only to the sudden shift in carbon source but also to the fact that this carbon source is a fatty acid. When a carbon source is scarce or when there is a temporary inability to degrade it due to absence of the corresponding catabolic enzymes, lack of

reduction equivalents ensures that redox flow through the respiratory chain stops. Yet, under our experimental conditions the respiratory chain as the main producer of ROS remained active. Fatty acids can uncouple the respiratory chain (Polcic *et al.*, 1997; Skulachev, 1998). Indeed, all our observations pointed into the direction of an idling respiratory chain that compromised the redox state of the cell by producing the ROS that elicited the oxidative stress response.

After the recovery from oxidative stress, other transcription factors became active: Oaf1p, Pip2p, Adr1p, Msn2p, and Msn4p. Oaf1p and Pip2p are transcription factors that form a heterodimer capable of binding to OREs (Einerhand *et al.*, 1993; Luo *et al.*, 1996; Rottensteiner *et al.*, 1997). OREs are found in genes coding for proteins directly or indirectly involved in fatty acid metabolism. They are considered to be the main factors in reprogramming the cells for growth on fatty acids. The contribution of Adr1p is less clear. Adr1p was originally found as a transcription factor controlling the expression of the *ADH2* gene coding for alcohol dehydrogenase (Thukral *et al.*, 1991). Adr1p target sites are present in a variety of genes, suggesting that it is a globally acting factor. In certain genes such target sites are also located together with OREs (Gurvitz *et al.*, 2001). An interesting aspect is that Adr1p is important for expression of the *POX1* gene coding for acyl-CoA oxidase, the first enzyme of the β -oxidation pathway (Baumgartner *et al.*, 1999). Several observations suggest that fatty acids or their oxidized derivatives form a signal to start the genetic program leading to peroxisome proliferation via activation of Oaf1p (Baumgartner *et al.*, 1999; Van Roermund *et al.*, 2000). Failure to start *POX1* expression could then prevent formation of the signal derived from fatty acids, resulting in stalling of Oaf1p-mediated gene expression. Such a scenario would be in line with the global role of Adr1p in gene expression and would explain how the cells wait for the oxidative stress to pass before preparing themselves for growth on the new carbon source.

One of the benefits of genome-wide expression studies is that the response of unknown genes can be linked to groups of genes with known functions, for instance, by hierarchical clustering analysis. In this way informative functional clues can be obtained. An interesting example is *OYE2/3* (Karplus *et al.*, 1995). This gene clustered within the oxidative stress category and was expressed very strongly shortly after the shift to oleate. *OYE2/3* codes for "old yellow enzyme," the first enzyme found to contain flavin as prosthetic group. It displays NADPH oxidase activity but the natural hydrogen acceptor is not known, despite the fact that much has been learned about the physical and chemical properties of the enzyme over the past 65 years. Recently, it was found that quinones can serve as efficient substrates for the enzyme (Xu *et al.*, 1999). In this context, it is important to note that semiquinones are excellent free radical generators, initiating a redox cycle that results in the formation of superoxide (Pius *et al.*, 2000). It is thus conceivable that in a period of redox imbalance Oye2/3p acts to keep quinones fully reduced to prevent the damaging consequences of the presence of semiquinones.

The events that we observed as responses to a drastic shift in nutrients combined with recent findings by others suggest the existence of a delicately tuned control circuit for redox

homeostasis (Delauny *et al.*, 2000; Carmel-Harel *et al.*, 2001). Insufficient flux of reduction equivalents compromises the cytosolic redox balance of the cell and affects the reduction state of glutathione and thioredoxin. Particularly thioredoxin plays a key role in relaying this state of alarm to compensatory action in the nucleus by controlling the oxidative state of crucial cysteine residues in Yap1p (Kuge *et al.*, 2001). As a result Yap1p moves from the cytosol into the nucleus where it activates its target genes. This may be an example of a more general principle. Recently it was reported that the cellular redox environment in *S. cerevisiae* influences the DNA-binding activity of the Hap3p subunit of the HAP transcription complex, and the importance of cysteine residues in Hap3p in this process was demonstrated (Yao *et al.*, 1999). A similar picture was drawn for the mammalian homolog of Hap3p, NF-YB (Nakshatri *et al.*, 1996); cysteine residues in NF-YB were shown to be important for DNA binding of the heterotrimeric NF-Y complex. Together, the results suggest that a number of transcription factors tune in to the redox state of the cell to exert their activity and that this principle may extend from yeast to man.

ACKNOWLEDGMENTS

We thank Drs. D. Botstein and P.T. Spellman (Stanford University, Stanford, CA), Drs. J.D. Hoheisel and N.C. Hauser (DKFZ, Heidelberg, Germany), and Dr. F. Holstege (University Medical Center, Utrecht, the Netherlands) for support in setting up a microarray facility. We also thank G. Klingers, H. Ijzendoorn, and J. Pijnenborg (Department of Engineering) for building the DNA spotter and Dr. F. Baas and N. Ponne (Department of Neurology) for support with robotic procedures. C. Al-Khalili Szgyarto was funded by the Swedish Research Counsel through a postdoctoral fellowship. The project was financially supported by the Netherlands Foundation for Chemical Research (Scheikundig Onderzoek Nederland).

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