Global expression profiling of yeast treated with an inhibitor of amino acid biosynthesis, sulfometuron methyl

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Jia, Melissa H., Robert A. LaRossa, Jian-Ming Lee, Antoni Rafalski, Ellen DeRose, Greg Gonye, and Zhixiong Xue. Global expression profiling of yeast treated with an inhibitor of amino acid biosynthesis, sulfometuron methyl. Physiol Genomics 3: 83–92, 2000.—The expression pattern of 1,529 yeast genes in response to sulfometuron methyl (SM) was analyzed by DNA microarray technology. SM, a potent herbicide, inhibits acetolactate synthase, a branched-chain amino acid biosynthetic enzyme. Exposure of yeast cells to 0.2 μg/ml SM resulted in 40% growth inhibition, a Gcn4p-mediated induction of genes involved in amino acid and cofactor biosynthesis, and starvation response. The accumulation of intermediates led to the induction of stress response genes and the repression of genes involved in carbohydrate metabolism, nucleotide biosynthesis, and sulfur assimilation. Extended exposure to SM led to a relaxation of the initial response and induction of sugar transporter and ergosterol biosynthetic genes, as well as repression of histone and lipid metabolic genes. Exposure to 5 μg/ml SM resulted in >98% growth inhibition and stimulated a similar initial expression change, but with no relaxation after extended exposure. Instead, more stress response and DNA damage repair genes become induced, suggesting a serious cellular consequence. Other salient features of metabolic regulation, such as the coordinated expression of cofactor biosynthetic genes with amino acid biosynthetic ones, were evident from our data. A potential link between SM sensitivity and ergosterol metabolism was uncovered by expression profiling and confirmed by genetic analysis.

DNA microarray; sulfometuron methyl; metabolic regulation

Cells adjust to changes in their environment in several ways, including alteration of gene expression patterns. In yeast, it is well established that regulation of gene expression occurs primarily via transcriptional control through interactions between promoter and transcription regulators (16, 26, 50). Transcript abundance, a function of transcript synthesis and degradation, provides an estimate of the gene product titer. Thus, accurate measurement of mRNA levels could reveal expression alterations caused by environmental challenges. Such information is vital in furthering our understanding of cellular regulations.

In the past, Northern blot hybridization and promoter-reporter fusion constructs have allowed simultaneous measurement of small numbers of mRNA species (1). Two-dimensional gel electrophoresis, on the other hand, provides direct detection of hundreds of polypeptides. However, it requires considerable effort to consistently and quantitatively measure each polypeptide. Moreover, the correspondence among genes, polypeptides, and function is often unclear (5, 38). Thus large-scale simultaneous measurement of gene expression profiles, made possible by DNA microarray technologies (33, 46), represents a significant advance. These techniques, for the first time, permit analysis of comprehensive expression profile changes in response to environmental challenges, for instance, nutrient availability and presence of inhibitory chemicals (9, 24, 34).

The yeast, Saccharomyces cerevisiae, is an ideal organism for studying gene expression alterations caused by environmental insults. The organism has a relatively small sequenced genome of about 6,000 genes (15), allowing the construction of DNA microarrays that include a large percent of its genes, or the entire genome (9, 55). More importantly, rapid and tractable genetic and biochemical manipulations of the organism are possible. Thus conclusions drawn from expression analysis can be quickly verified by followup gene disruption and amplification analysis. Finally, the conservation existing between different organisms makes yeast an ideal model system for investigating the effect of chemicals that are of pharmaceutical (4, 6) or agricultural (28) importance.

In this study, we monitored the expression profile changes of 1,529 yeast genes caused by treatment with the herbicide sulfometuron methyl (SM). SM is an inhibitor of acetolactate synthase (ALS), a branched-chain amino acid biosynthetic enzyme (13, 29, 43, 44). ALS catalyzes the second step in isoleucine and the first step in valine biosynthesis, the formation of α-acetoacetyl-α-ketoacids and their derivative α-ketoacids (51). Studies of Salmonella typhimurium and Escherichia coli have suggested that both an imbalance among α-ketoacids and end product deficiency contributed to inhibitor efficacy (12, 30). Since α-ketoacids and their derivative acetyl-CoA molecules are quantitatively predominant in biosynthetic metabolism (30a) and since similar imbalances are found in inborn errors of human metabolism
(1a) and in overproduction of certain proteins (3), it is of general interest to define the cascade emanating from ALS inhibition. We choose to further study the effect of SM inhibition of ALS in yeast, using DNA microarray technology. By monitoring the change of mRNA levels of 1,529 yeast genes in response to SM treatment, we provide a panoramic view of the response of yeast to branched-chain amino acid depletion and α-ketoacid imbalance.

**EXPERIMENTAL PROCEDURES**

**Strains, media, and chemicals.** Yeast strains BY4743, 30249, 30590, and 32667 were purchased from Research Genetics (Huntsville, AL). W1362-20A and W1362-17A were obtained from Dr. Rodney Rothstein, Columbia University, New York. The genotype of each strain is listed below: W1362-20A, Mata; W1362-17A, Mata and ara-3-1; BY4743, Matαα, his3Δ1α/ his3Δ1α, leu2Δ0α/ leu2Δ0α, lys2Δ0α/ lys2Δ0α, met15Δ0α// met15Δ0α, and ura3Δ0α// ura3Δ0α, same as BY4743 except gen4Δ:: KanMX4α/ gen4Δ::KanMX4α; and 30249, same as BY4743 except erg5Δ::KanMX4α/ erg5Δ::KanMX4α; and 30590, same as BY4743 except erg5Δ::KanMX4α/ erg5Δ::KanMX4α. Plasmids pRS313, 315, 316, and 426 were obtained from the American Type Culture Collection.

Standard media preparation and yeast cell culture were carried out according to Sherman et al. (47). Yeast transformations were done using the lithium acetate procedure (23). SM was obtained from DuPont Agricultural Products, Newark, DE. Reagents for cDNA labeling were purchased from Research Genetics, using Clontech (Palo Alto, CA) advantage cDNA PCR Kit. PCR products were purified using Qiagen (Valencia, CA) QiaQuick 96 PCR purification kit. PCR products were dissolved in 6 M NaSCN, and 0.03 mg/ml salmon sperm DNA). RNA was used as template to generate a DNA probe. Between 0.5 and 1.0 μg of poly(A)+ RNA was used as template to generate a labeled cDNA probe by reverse transcription. The reaction was carried out in a final volume of 20 μl. The reaction mixture contains 0.4 μM anchored dT25, 50 μM dATP, dGTP, and dTTP, 25 μM dCTP, 15 μM Cy3-labeled dCTP, and 22 μM Cy5-labeled dCTP, and 200 U of Superscript reverse transcriptase (GIBCO BRL, Life Technologies, Rockville, MD). Briefly, RNA sample and dT25 were heated to 70°C for 5 min and then allowed to anneal at room temperature for 10 min. Deoxynucleotides, appropriate buffer, and enzyme were added, and the reaction carried out at 42°C for 2 h. After the completion of reaction, RNA was removed by alkaline hydrolysis, and cDNA probes were purified using a Qiagen PCR purification kit. Purified cDNA probes were dried and resuspended in hybridization buffer (5 × SSC, 50% formamide, 0.1% SDS, and 0.03 mg/ml salmon sperm DNA). Hybridization and washing. DNA microarrays were treated with isopropanol for 10 min and boiling water for 5 min and dried under a stream of nitrogen. A volume of 30 μl of hybridization buffer, containing an equal amount of Cy3- and Cy5-labeled cDNA probes (30–40 pmol dye equivalent each, based on an absorbance of 1.5 × 10^{-5} M at 550 nm for Cy3 and 2.5 × 10^{-5} M at 650 nm for Cy5), was applied to an array, and a coverslip was placed on top. Arrays were incubated at 42°C for 16–18 h, washed twice with 2× SSC, 0.1% SDS at 45°C, twice with 0.1× SSC, 0.1% SDS at 37°C, and once with 0.1× SSC at 23°C (5 min for each wash). Washed arrays were rinsed with deionized H2O and dried under nitrogen.

**Scanning and data analysis.** A Molecular Dynamics GenIII laser scanner was used to acquire hybridization signals. Array images were analyzed with ArrayVision 4.0 software (Image Research, Toronto, Canada). The absolute intensities of each spot were then normalized using the default parameters of the software. The equation for normalization is:

\[
N_{D=A} = 1.529(D \times A - \text{Background})/(2D \times A - \text{Background})
\]

D is the signal density, and A the area of each spot. Thus one normal (1 N) is defined as the sum of the absolute intensity minus background for all the spots divided by 1,529.

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CA). 33P-labeled probes for individual yeast genes were generated with PCR-amplified yeast ORFs, using RadPrime DNA Labeling system (GIBCO BRL). Hybridized filters were washed with 0.5

3 SSC, 0.1% SDS at 59°C for 20 min, dried and exposed to X-ray film (Kodak). The size of the transcripts were estimated by comparison to RNA standards (0.24–9.5 kb, GIBCO BRL).

RESULTS

Global expression profile analysis of yeast cells treated with SM. The 1,529 ORF array was used to analyze expression profile changes in yeast cells treated with SM (see EXPERIMENTAL PROCEDURES). A wild-type strain W1362-20A was used for this experiment. We analyzed the expression profile changes at several time points after SM addition (15 min, 30 min, 1 h, and 4 h) to determine the kinetics of the cellular response.

It has been shown that the two Cy dyes used in microarray experiments do not label cDNAs equally (54). We examine the effect of dye swap on data quality with two control RNA samples. Each RNA sample was labeled with both Cy3 and Cy5 in two separate reactions. On one array, control sample labeled with Cy3 is mixed with test sample labeled with Cy5, and the mixture used for hybridization. On the second array, control sample labeled with Cy5 is mixed with test sample labeled with Cy3. In Fig. 1A, the average of two sets of normalized Cy3 signal intensity, from a single slide, was plotted vs. the average of the two sets of Cy5 signal intensity from the same slide (no dye swap). In Fig. 1B, the average of Cy3 intensities from the first slide and Cy5 from the second slide were plotted vs. the average of Cy5 from the first slide and Cy3 from the second slide (dye swapping). A comparison of Fig. 1A and 1B showed that the quality of data increased significantly with dye swap (see also Ref. 54). Under the conditions used, we could reliably measure normalized fluorescence intensities above 0.1, which corresponds to a mRNA level of 0.1 to 0.2 copies per cell (18, 53). Typically, when control samples are compared with each other, we observed a mean ratio of 1.0 to 1.1 with a standard deviation of 0.2 to 0.3. Thus changes in expression levels above twofold are highly significant.

Northern blot analysis. Total RNA samples (20 μg each) were separated by electrophoresis on a 1% formaldehyde agarose gel, transferred onto Hybond nylon membrane (Schleicher & Schuell, Keene, NH), and fixed by ultraviolet (UV) cross-linking using a Stratalinker (Stratagene, La Jolla, CA). 33P-labeled probes for individual yeast genes were generated with PCR-amplified yeast ORFs, using RadPrime DNA Labeling system (GIBCO BRL). Hybridized filters were washed with 0.5× SSC, 0.1% SDS at 59°C for 20 min, dried and exposed to X-ray film (Kodak). The size of the transcripts was estimated by comparison to RNA standards (0.24–9.5 kb, GIBCO BRL).

In Fig. 2, the normalized signal intensities of the SM samples are plotted against the normalized signal intensities of the controls (see EXPERIMENTAL PROCEDURES). Data points in the top left region represent transcripts that were induced by SM treatment, and those in the bottom right region represent transcripts that were repressed by SM. As shown in Fig. 2, a number of genes were induced or repressed significantly after a 15-min SM treatment. High (5 μg/ml) and low (0.2 μg/ml) SM concentrations resulted in similar expression profile changes (compare Fig. 2A and 2B). Similar results were also observed with a 30-min exposure to SM (data not shown). At the lower SM concentration, the level of induction and repression lessened as a function of time. After a prolonged exposure (4 h), the number of genes induced or repressed became much smaller, even though cell growth continued to be inhibited at the same level (Fig. 2C, see also below). In contrast, at high SM concentration (>98% inhibition) no such relaxation was observed. In fact, the change in expression profile became more pronounced after a 4-h SM treatment (Fig. 2D).

Analysis of the SM induced early expression profile changes. After 15 min of treatment with 0.2 μg/ml SM, 125 out of 1,529 genes (8%) were induced by more than twofold. Supplementary Table 1 lists these genes, sorted by their cellular function, and observed level of induction from two experiments, performed on different days under the same condition. (Tables 1–7 have been published online as Supplementary Material and can be viewed at the Physiological Genomics web site.)

It is clear (from Table 1) that the observed induction was highly reproducible. Furthermore, most of these genes were also induced after a 30-min (114 genes, 7%) or a 60-min (63 genes, 4%) treatment, although the
level of induction was progressively less for many of them (data not shown). A large number of these genes are involved in amino acid metabolism (e.g., biosynthesis, transport, attachment to tRNA) or in the biosynthesis of cofactors such as biotin, lipoic acid, riboflavin, nicotinic acid, and pyridoxine (10, 39, 40) (see Table 1). In addition, certain starvation response or cell cycle regulated genes were highly induced. For example, \texttt{APG1} and \texttt{PCL5} were induced 17- and 12-fold (35, 36, 49). Other genes that were induced include those implicated in the stress responses and transport (see Table 1).

Eighty genes (5.2\%) were repressed more than two-fold by the same treatment (see Table 2 of the Supplementary Material). The most striking repression was observed for genes involved in sulfur assimilation and single carbon metabolism. Many genes involved in carbohydrate metabolism and nucleotide biosynthesis were also significantly repressed. Other groups that were repressed included stress response genes (\texttt{HSP70} family and genes encoding cyclophilins), sugar transporter (\texttt{HXT2}, 3, and 4), and mitochondrial proteins (\texttt{ATP2}, \texttt{CBP4}, \texttt{COR1}, etc.). After 30 min or 60 min of treatment, 50 (3.3\%) or 59 (3.9\%) genes were repressed more than twofold.

Remarkably, a 25-fold increase in SM concentration only led to relatively moderate differences in expression profile change. Even though 183 genes (12\%) were induced and 111 genes (7\%) repressed by more than twofold, the effect on most metabolic processes appeared similar (supplementary Tables 1 and 2). Almost all the genes induced by 0.2 \(\mu\)g/ml SM were also induced by 5 \(\mu\)g/ml SM. High concentration of SM affected sterol biosynthesis more significantly, inducing a number of \texttt{ERG} genes. There was also a more pronounced effect on DNA damage repair and nitrogen metabolic genes. In addition, more pronounced effects on protein synthesis, modification, cell wall structure, and small molecule transport were seen with 5 \(\mu\)g/ml SM treatment.

SM treatment of 5 \(\mu\)g/ml repressed a number of genes more severely (see Table 2), such as those involved in fatty acid and phospholipid metabolism, respiration, proline isomerases (\texttt{CPR}s), and heat shock proteins (\texttt{HSP}s). Some of these genes are repressed by 0.2 \(\mu\)g/ml SM after a longer exposure (see below).

\textbf{Analysis of the SM-induced late expression changes.} After an extended treatment with 0.2 \(\mu\)g/ml SM (4 h), a total of 79 genes were induced by more than twofold, and 36 of them were involved in amino acid metabolism (see supplementary Table 3). The degree of induction was much less for most of the amino acid biosynthetic genes. For example, the expression of \texttt{ARG1}/10

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Expression profiles of yeast cells treated with sulfometuron methyl (SM). W1362-20A yeast cells were grown in synthetic minimal medium and treated with SM for indicated times. RNA isolation, probe generation, and array hybridization were performed as described in \textit{EXPERIMENTAL PROCEDURES}. All the plots are generated with data from 2 arrays, with dye swap. A: yeast cells treated with 0.2 \(\mu\)g/ml SM for 15 min. B: yeast cells treated with 0.2 \(\mu\)g/ml SM for 4 h. C: yeast cells treated with 5 \(\mu\)g/ml SM for 15 min. D: yeast cells treated with 5 \(\mu\)g/ml SM for 4 h.}
\end{figure}
was induced by 3.8-fold, compared with 21-fold after 15 min of treatment. Among the highly induced genes were \textit{AGP1}, a general amino acid permease (22), and the putative pyridoxine biosynthetic genes \textit{SNO1} and \textit{SNZ1}, although they were expressed at a lower level compared with the 15-min treatment. New classes of induced genes include sugar transporters (\textit{HXTs}), and genes involved in ergosterol biosynthesis (\textit{ERG1}, 3, and 5; see Table 3).

Only 39 genes were repressed by more than twofold (see supplementary Table 4). Compared with the 15-min treatment, genes involved in carbohydrate metabolism were no longer heavily affected. Genes involved in fatty acid and phospholipid metabolism (e.g., \textit{FAS1} and 2, \textit{ITR1}, \textit{OPI3}, and \textit{CHO1}) became highly repressed (see Table 4). It is interesting that these genes were repressed by a 15-min treatment with 5 \(\mu\)g/ml SM (see Table 2). Several histone genes were also repressed by a factor of greater than 2. Together, the induction and repression pattern indicated that cells may have adapted to the presence of SM in the medium and adjusted the expression pattern accordingly.

When yeast cells were treated with 5 \(\mu\)g/ml SM for 4 h, we observed an extended response at gene expression level, with a total of 241 genes (15.7\%) induced more than twofold (see Table 3) and 121 (8\%) repressed more than twofold (see Table 4). Most of the genes induced at the 15-min time point remained highly induced. In addition, a large number of genes involved in small molecule transport (especially sugar transport), stress and DNA damage response, and carbohydrate metabolism became induced. Genes encoding key enzymes in glycolysis, nucleotide biosynthesis, methionine biosynthesis, and single carbon metabolism remained highly repressed after 4 h of treatment with 5 \(\mu\)g/ml SM. A striking feature is that many genes encoding histones, ribosomal proteins, and cell cycle control proteins became significantly repressed. One possibility is that this is related to the severe growth inhibition under the condition.

**Confirmation of induction and repression by Northern blot analysis.** To independently verify the observed induction and repression seen with microarray, we directly determined the mRNA levels of \textit{ARG1/10}, \textit{PCL5}, \textit{SNO1}, and \textit{SAM1} by Northern blot (see EXPERIMENTAL PROCEDURES). A fifth gene, \textit{RPS10A}, served as a control, since the microarray analysis suggested that its mRNA level remain unchanged during 0.2 \(\mu\)g/ml SM treatment (sample/control ratio of 0.9 at 15 min and 4 h). As shown in Fig. 3, the mRNA levels of \textit{ARG1/10}, \textit{PCL5}, and \textit{SNO1} increased dramatically in yeast cells treated with SM for 15 min. In cells treated for 4 h, we observed a reduced level of induction for \textit{ARG1/10} and \textit{SNO1} (in Fig. 3, compare lanes B and D for \textit{ARG1/10} and \textit{SNO1}). The mRNA level of \textit{SAM1}, in contrast, decreased significantly after 15 min of SM treatment but was unchanged in cells treated for 4 h. The mRNA levels of \textit{RPS10A} were, as expected, the same in all samples. Hence Northern blot analysis agreed with results obtained with the microarray.

**Expression profile of \textit{gcn4} yeast cells after SM treatment.** \textit{Gcn4p} is the key transcription factor regulating those genes involved in amino acid biosynthesis that are under general control (19, 20). Analyzing the expression profile of yeast cells lacking this regulator after a SM treatment should reveal transcript changes unrelated to branched-chain amino acid depletion. We thus measured the expression profile change of a \textit{gcn4} strain 30249 (\textit{gcn4} diploid) in response to a 15-min or a 4-h SM treatment. As a control, we also obtained expression profiles of strain BY4743, the isogenic control (\textit{GCN4/GCN4} diploid). For 15-min samples, both strains were grown in SD medium and treated with 0.2 \(\mu\)g/ml SM as described earlier (see also EXPERIMENTAL PROCEDURES). Under this condition, the growth of BY4743 cells was inhibited by 35–40% and 30249 cells more than 70\%. In Fig. 4, fluorescence intensities of the control and the SM-treated samples were plotted vs. each other. It is clear that data obtained from BY4743 (\textit{GCN4/GCN4}) cells were similar to those from W1362-20A (\textit{GCN4}). Analysis showed that the same set of genes was induced and repressed in both strains (data not shown). However, data from 30249 cells indicated very little change induced by SM (compare Fig. 4, A and B, with Fig. 1C). After a 30-min SM treatment, only eight genes were consistently induced by more than 2-fold (see supplementary Table 5), although 16\% of the genes (253) were induced or repressed between 1.5- and 2-fold, compared with ~5\% observed in control/control comparison (see EXPERIMENTAL PROCEDURES). This observation suggests that most genes highly induced by SM in wild-type yeast cells were under the control of Gcn4p, either directly or indirectly. We further analyzed the DNA sequences of the promoter region of genes highly induced by SM in a wild-type strain (more than 4-fold), but not involved in amino acid biosynthesis. Potential Gcn4p binding sites were identified in a number of them (see supplementary Table 6), suggesting that these genes are under the direct control of Gcn4p.

SM represses the expression of 22 genes by twofold or more in \textit{gcn4} cells (see Table 5). As in the case of \textit{GCN4} cells, genes involved in sulfur assimilation...
(MET3, 6, 14, and 17), single carbon metabolism (SAM2), and purine nucleotide biosynthesis (ADE17) were repressed. However, the degree of the observed repression was less. Proteins involved in glycolysis were no longer significantly repressed in gcn4 cells.

When gcn4 yeast cells (strain 30249) were treated with 5 \( \mu \text{g/ml} \) SM for 4 h, we observed that 45 genes were induced and 42 repressed by more than twofold. The most striking induction is observed with genes involved in stress response and amino acid metabolism. In contrast, there was no systematic induction of amino acid and cofactor biosynthetic genes observed. Interestingly, we now see a significant repression of genes involved in glycolysis and nucleotide metabolism but not histone and ribosomal proteins. Compared with GCN4 cells, the effect of SM appears to be more modest on gcn4 cells at the gene expression level, supporting the idea that many observed gene expression changes were indirectly mediated by Gcn4p, perhaps through a rapid accumulation of \( \alpha \)-ketoacids.

Deletion of GCN4, ERG3, and ERG5 or overexpression of APG1 and PCL5 leads to SM hypersensitivity. The genes expressed at an altered level could result in either increased cellular resistance or inhibited growth. Deletion or overexpression of SM-induced or repressed genes should provide additional clues to their role in cellular response to SM. We tested the SM sensitivity of three deletion strains, 30249 (gcn4 homozygous diploid), 30590 (erg5 homozygous diploid), and 32667 (erg3 homozygous diploid), with a filter disc assay (see EXPERIMENTAL PROCEDURES). ERG3 encodes C-5 sterol desaturase, and ERG5 encodes a cytochrome P-450. Both are involved in desaturation steps in ergosterol biosynthesis. All three deletion strains were hypersensitive to SM (see supplementary Table 7). These results pointed to the key role that Gcn4p plays in mediating yeast response to SM. They also revealed a previously unknown link between SM sensitivity and membrane sterol composition.

We also tested the effect of overexpressing the SM-induced genes, APG1 and PCL5, on the SM sensitivity of yeast. These genes, heavily induced by SM, might be part of the defensive response of yeast to a SM challenge. Yeast strain W1362-17A was individually transformed with plasmids pAPG1 (overexpressing APG1) or pPCL5 (overexpressing PCL5). The sensitivity of transformants to SM was tested by the filter disc assay (see EXPERIMENTAL PROCEDURES). Surprisingly, overexpression of APG1 or PCL5 led to hypersensitivity (see Table 7).

**DISCUSSION**

ALS is the cellular target of the commercially important herbicide sulfonylurea, of which SM is the most studied example. The impact of ALS inhibition upon cellular metabolism has been a subject of many studies in yeast, *S. typhimurium*, *E. coli*, and plants (13, 29, 43). The links between altered metabolism and SM treatment, however, remain unclear. In this work we examined expression profile changes of 1,529 yeast genes in response to SM challenges. Our results showed that expression profile changes reflected the effects of both the limitation of amino acids and the accumulation of \( \alpha \)-ketoacids. Furthermore, new regulatory roles for the general control regulator Gcn4p are revealed by our analysis.

Addition of SM to the growth medium triggered a rapid response at the gene expression level. Fifteen minutes after the addition of a low concentration of SM (0.2 \( \mu \text{g/ml} \)), we observed the induction (>2-fold) of 125 genes and the repression (>2-fold) of 80 genes, out of 1,529 tested, in wild-type yeast cells. Higher concentration of SM, although it induced and repressed more genes (183 and 111, respectively), affected most cellular processes in a similar way. The most prominent group of induced genes are those involved in amino acid biosynthesis and transport. In contrast, only eight genes were induced significantly by a 15-min treatment with 0.2 \( \mu \text{g/ml} \) of SM, in cells lacking Gcn4p. Five of these genes (*CUP1A, CUP1B, SSE2, HSP104, and STI1*) are stress response genes, two (*SLT2* and *YPK2*) are protein kinases, and the eighth is involved in trans-
loration initiation (SIS1). This result indicates that one of the primary effects of SM is a rapid depletion of branched-chain amino acids isoleucine and valine, which triggers the Gcn4p-mediated general response (19, 20).

A comparison of the results from Gcn4 and gcn4 strains led to an important conclusion. That is, Gcn4p clearly regulates cofactor biosynthesis, in addition to amino acid and nucleotide biosynthesis. A significant GCN4-dependent induction was observed for genes involved in the biosynthesis of biotin, riboflavin, nicotinic acid, lipoic acid, and pyridoxine (BIO3, 4, and 5; BNA1; LIP5; RIB1, 3, and 5; SNO1; and SNZ1). Potential Gcn4p binding sites can be identified in the 5′ region of BIO3, BIO5, BNA1, SNO1, and SNZ1, suggesting that they are under the direct control of Gcn4p (see Table 6). In support of this, it has recently been shown that Gcn4p also regulates the expression of lipoamide dehydrogenase in yeast (56).

Other genes showing strong Gcn4p-dependent induction are APG1 and PCL5. APG1 encodes a protein kinase involved in the induction of autophagy after nutrient limitation (35, 49). Interestingly, in E. coli, some stationary phase genes, under the control of the global regulator σ7, are induced in response to amino acid limitation caused by treatment with sublethal levels of SM (52). This response appears to be part of the relA-dependent stringent response mediated by ppGpp (14, 27). Thus in both E. coli and yeast, the regulator controlling the response to amino acid limitation also regulates stationary phase response. PCL5, a G1 cyclin, has a potential Gcn4p binding site in its 5′ region. Pcl5p is known to associate with Pho85p, but its exact cellular role is not clear (36).

We tested the SM sensitivity of yeast cells overexpressing APG1 or PCL5. Overexpression of both genes led to an increased sensitivity toward SM (see Table 7). In the case of APG1, this is somewhat surprising since one expects that overexpression should lead to a better adaptation to amino acid limitation and increased resistance. Perhaps the balance of various metabolic pathways is important, and artificial overexpression of APG1 upsets such balance. The result with PCL5 overexpression suggests that one of the cellular functions of Pcl5p might be to coordinate changes in different metabolic pathways in response to nutrient limitation or cell cycle requirement.

A pronounced repression of genes involved in glycolysis was observed in wild-type cells, after a 15-min SM treatment (see Table 2). It is known that in E. coli and S. typhimurium, SM inhibition of ALS leads to accumulation of pyruvate and α-ketobutyrate (12, 30). However, it was unclear whether the same is also true in yeast. Accumulation of pyruvate could have a negative impact on glycolysis. Our results suggest that accumulation of α-ketoacids does occur in yeast. Strikingly, in gcn4 cells, glycolic genes are significantly repressed only after 4 h of SM treatment. This is most likely due to a slower accumulation of α-ketoacids, since the absence of Gcn4p prevents the rapid induction of branched-chain amino acid biosynthetic genes, resulting in delayed accumulation of intermediate α-ketoacids.

We also observed a significant induction of many genes involved in stress response and DNA damage repair. Although it is known that Gcn4p activates HIS1 and HIS4 upon UV irradiation in a RAS-dependent fashion (11), this activation is independent of DNA damage. Most stress response and DNA damage repair genes do not have potential Gcn4p binding sites in their promoter region, making it unlikely that they are directly regulated by Gcn4p. Rather, the induction likely reflects the effect of α-ketoacid accumulation. This suggests that the stress induced by SM comes partly from the accumulation of intermediates, rather than solely from amino acid depletion. In gcn4 cells, the effects should be less severe due to a slower accumulation of α-ketoacids. Consequently, no significant induction was observed until after 4 h of exposure.

An interesting and surprising observation from our experiment is the severe repression of several genes involved in sulfur assimilation, methionine biosynthesis, and S-adenosyl-methionine (AdoMet) production. MET3, MET14, SAM1, and SAM2 are each repressed more than 10-fold by both 0.2 and 5 μg/ml SM. This repression is also evident in gcn4 cells, although it is much less severe (3- to 4-fold, compared with >10-fold in wild-type cells). One possibility is that the accumulated α-ketobutyrate is converted to norleucine through the action of the leucine biosynthetic enzymes (3). Norleucine, a methionine analog, could serve as a corepressor of some methionine biosynthetic genes (37). An alternative explanation is that sulfur assimilation and AdoMet production consume the adenine portion of ATP. The lower level of ATP in SM-treated cells, due to repression of glycolysis and respiration, may have led to a repression of the MET and SAM genes. However, His7p also consumes the adenine moiety of ATP to produce AICAR, as part of the histidine biosynthesis pathway (25). We observed a significant induction of HIS7 expression (see Table 1).

A second surprising observation is the repression of nucleotide biosynthetic genes by SM in wild-type cells. These genes are regulated by Bas1p, Bas2p, and Gcn4p (7, 8, 45), and one would have expected an induction upon amino acid depletion. Indeed, HIS1 and HIS4, two genes known to be coregulated with purine biosynthesis genes (7, 8), were induced by two- and fivefold. Our results can be explained if additional transcription regulators participate in the regulation of nucleotide biosynthetic genes. It has been suggested that ADP or one of its derivatives is the effector in the adenine repression of purine nucleotide biosynthesis (17). In SM-treated cells, we expect a higher ADP level due to the repression of glycolysis and respiration. Thus the observed repression could be a reflection of the increased cellular ADP concentration. In agreement with this, we observed repression of these genes in gcn4 cells after 4 h of SM treatment (see Table 5). These cells are expected to have higher concentrations of ADP due to the repression of glycolysis at this time. It is interesting to note that in plants the first macromolecular biosynthetic process inhibited by SM is DNA syn-
thesis (43). This is in agreement with our observation of nucleotide biosynthetic gene repression in yeast.

The similarity between expression profile changes in response to 15-min treatment with low and high concentrations of SM suggests that 0.2 μg/ml of SM, although inhibiting cell growth by only 40%, already fully stimulated most metabolic processes affected by SM. There are a few exceptions. A number of genes involved in ergosterol biosynthesis, DNA damage repair, cell wall function, and small molecule transport were induced by 5 μg/ml, but not 0.2 μg/ml SM. Also, a much more severe repression of genes involved in fatty acid and lipid metabolism, respiration, and heat shock response was observed with 5 μg/ml SM (see Table 2; see also below).

After a prolonged exposure to 0.2 μg/ml SM (4 h), we observed a much reduced response at the gene expression level. The expression level of most genes highly induced or repressed at early time points had returned to near normal. This indicates that cells have adapted to the new conditions and adjusted the expression profile accordingly. Interestingly, although the expression of most amino acid biosynthetic genes had either returned to normal or much less induced levels, branched-chain amino acid biosynthetic genes were still highly induced. Likely the pathway-specific regulation controls branched-chain amino acid biosynthetic genes at this time (20). We also observed the induction and repression of new classes of genes, such as ergosterol biosynthetic genes and sugar transporters (ERG's and HXT's, induced) or fatty acid and phospholipid biosynthetic genes and histones (repressed). As discussed below, expression of ERG3, ERG5, and ERG6 is important to cellular resistance toward SM.

In contrast, a 4-h treatment with 5 μg/ml SM resulted in a much different picture. What we see is the pleiotropic effects caused by the depletion of amino acids and the accumulation of α-ketoacids. There was no relaxation of the early induction or repression; rather, there were additional responses in other cellular processes. For example, many genes involved in glyoxylate cycle, glycogen metabolism, and tricarboxylic acid cycle were induced, likely as a result of the continued accumulation of pyruvate. A large number of genes involved in oxidative stress response and DNA damage repair were also induced, suggesting that the accumulation of α-ketoacids and other intermediates is causing multiple stresses to the cell. Small molecule transporters, especially sugar transporters, are heavily induced, perhaps as a result of repression of glycolysis.

A repression of ribosomal proteins, histones, and tubulin genes (TUB1 and 2), as well as genes involved in cell cycle control (e.g., CDC16, 23, and 43; CIN2; PCL5; CDC5 and 12; CLN1 and 2; and CLB2) are observed. It is tempting to propose that a lack of branched-chain amino acids led to a slow down or stop in protein synthesis, which in turn triggers the repression of ribosomal proteins. A general slow down in growth could trigger the repression of histones and cell cycle genes. However, we did not observe a significant repression of these genes in gcn4 cells treated with 5 μg/ml SM for 4 h, even though these cells should be depleted of branched-chain amino acids and were not growing.

It has been shown that, similar to SM, rapamycin represses glycolic enzymes and ribosomal proteins (17a). The Tor proteins are proposed to mediate this repression. The expression of the Gcr1p, which regulates most glycolic enzymes, is induced by rapamycin but not diauxic shift (17a). Similar to the situation in diauxic shift, SM does not change the expression level of Gcr1p. It is interesting to note that in the cases of both diauxic shift and SM treatment, a real change in the levels of glycolic substrate or product happened. More studies are needed to dissect the regulatory elements involved in this control.

Several yeast deletion strains were tested for their SM sensitivity, in an attempt to establish a link be-

Fig. 5. Schematic diagram of the response of yeast cells to SM treatment. AA, amino acid; ALS, acetolactate synthase.

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between changes in metabolic pathways and cellular resistance to SM. We found that, as expected, gcn4 yeast cells were hypersensitive to SM, suggesting that Gcn4p-regulated broad-based induction is important for cellular resistance to SM. Deletion of ERG3 or ERG5 led to hypersensitivity toward SM. Previous data from this lab also showed that deletion of ERG6 led to SM hypersensitivity (S.-Y. Choi and Z. Xue, unpublished results). The erg3 and erg5 strains do not have ergosterol in their membrane, but a mixture of intermediates. The sensitivity of these cells to a variety of chemicals is altered. An explanation is that altered membrane composition affects the ability of SM to enter or exit the cells (32, 41).

Figure 5 summarizes the results presented in this study. Low concentrations of SM inhibit ALS and cause a depletion of isoleucine and valine and an accumulation of α-ketoacids. This leads to a Gcn4p-mediated elevation of activities in amino acid biosynthesis, cofactor biosynthesis, and starvation response, and the reduction of activities in nucleotide biosynthesis, methionine biosynthesis, sulfur assimilation, and glycolysis. The observed expression profile change provided clear evidence that Gcn4p regulates beyond amino acid and nucleotide biosynthetic genes. The expression profile relaxes back toward a new steady state after prolonged exposure. Cells adapted to the presence of low concentrations of SM by keeping branched-chain amino acid biosynthetic genes induced. Transporters for small molecules were also induced, together with genes involved in ergosterol biosynthesis. The latter appears to increase the resistance of yeast cells to SM. Histone, fatty acid, and phospholipid biosynthetic genes were repressed because of a reduced need at the slower growth rate.

At higher concentrations of SM, changes induced by the general control mechanism were unable to compensate for the inhibition of ALS, and cells were unable to grow. Consequently, we observed no relaxation after prolonged exposure. Instead, secondary effects caused by the accumulation of intermediates led to the induction and repression of even more genes, particularly those involved in stress response, DNA damage repair, cell cycle regulation, and protein synthesis. The accumulation of α-ketoacids and the associated secondary effects may be more harmful to the cell than simple amino acid depletion and contribute to the potency of SM (30).

In this study, the use of 1,529 genes, mostly of known function, allowed us to monitor many cellular processes and gain a picture of global cellular response to an amino acid biosynthesis imbalance. It is clear from our data that inhibition of amino acid biosynthesis leads to many changes in multiple metabolic processes, and the regulator of amino acid biosynthetic genes, Gcn4p, has a broader regulatory function than previously known. These changes either help cells to fight the effects of the inhibitor or are part of the detrimental effects of the inhibitor. Our data contribute to understanding the chain of cellular events resulting from inhibition of an amino acid biosynthetic enzyme.

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