Transcriptional Remodeling and G₁ Arrest in Dioxygen Stress in Saccharomyces cerevisiae*

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Saccharomyces cerevisiae, which lack a functional SOD1 gene, encoding the cytosolic Cu,Zn-superoxide dismutase (SOD1), exhibit a variety of metabolic defects in aerobic but not in anaerobic growth. We test here the hypothesis that some of these defects may be due to specific transcriptional changes programmed for cell survival under dioxygen stress. Analysis of the budding pattern and generation time showed that the slower proliferation of an sod1Δ mutant strain under air was due to an increase from 42 to 89 min spent in the G1 phase of the cell cycle. This delay in G1 was not due to an overall decline in biosynthetic activity since total protein and mRNA synthesis was not reduced even under 100% O₂. However, rRNA synthesis was strongly decreased, e.g. by 80% in the mutant under 100% O₂ (in comparison to N₂). Under these conditions, the mutant permanently arrested in G1; this arrest was due to an inhibition of the Start function that prepares yeast for S phase. This Start arrest was due to an inhibition of transcription of the autoregulated G1 cyclins, CLN1 and CLN2; the transcription of the constitutive G1 cyclin, CLN3, was unaffected by the stress. Expression of a hyperstable Cln3 prevented the G1 arrest, indicating that it was due solely to the inhibition of cell cycle-dependent cyclin expression. This remodeling of transcription in oxidative stress was seen also in the inhibition of glucose derepression of SUC2 expression. In contrast, the signaling and activation of mating pheromone (FUS1) and copper-responsive (CUP1) promoter activity were not affected by dioxygen stress, while genes encoding other anti-oxidant enzymes (SOD2, CTT1 and CTA1) were strongly induced. The UBI loci, encoding ubiquitin, were particularly good examples of this pattern of negative and positive transcriptional response to the stress. UB1–UB13 expression was repressed in the mutant under 100% O₂, while expression of UBI4 was strongly induced. The data demonstrate that extensive remodeling of transcription occurs in yeast under a strong dioxygen stress. This remodeling results in a pattern of expression of gene products needed for defense and repair, and suppression of activities associated with normal proliferative growth.

Organismal response to stressors represents an adaptive response to environmental change. This change could be in temperature, nutrient supply, incident radiation, or degree of oxidative stress, for example. Transcriptional control of gene expression is a major mechanism by which the yeast, Saccharomyces cerevisiae, like most organisms, adapts to new environments (1, 2). For example, glucose, which is a hormone-like messenger and a rapidly fermented sugar, has a dramatic effect on yeast metabolism and also on growth rate (3). These effects are due both to induction of genes necessary for rapid growth, e.g. genes encoding ribosomal proteins, and to repression of genes involved in respiration and metabolism of alternative carbon sources. Stress-induced genes such as CTT1 (4), encoding cytosolic catalase T, and UBI4 (5), encoding a polyubiquitin polypeptide, are also nutrient-repressed (6). Changes in gene expression are also characteristic of the heat shock response (1, 2), but heat shock has the opposite effect in comparison to the glucose response; heat shock causes an up-regulation of stress genes and a down-regulation of genes encoding rRNA and ribosomal proteins (7). The effect of stress on rRNA synthesis is significant because of the close correlation between this synthesis and growth and proliferation (8).

Recent work has shown that yeast adapts to oxidative (peroxide) stress in a manner similar to its adaptation to heat shock, i.e. pre-exposure to a limited degree of stress protects from an otherwise lethal one (9–13). This adaptation to oxidative stress accompanies a change in the protein synthetic pattern (12, 14), a phenomenon seen also in response to heat shock (15). However, there are limited data on the transcriptional remodeling that presumably underlies the changes in protein synthesis under oxidative stress. Thus, although it is known that in yeast H₂O₂ induces the expression of CTT1 (14, 16), for example, and that the expression of SOD1, encoding the cytosolic Cu,Zn-superoxide dismutase, is slightly elevated in hyperoxic conditions (17), the overall transcriptional pattern associated with adaptation to oxidative stress, particularly due to dioxygen (rather than H₂O₂), has not been studied in detail.

The theory of superoxide-mediated oxygen toxicity postulates that the superoxide radical (O₂⁻) is pathogenic to cells due to the redox activity of O₂⁻ and other downstream reactive oxygen species (18). A eukaryotic cell lacking a primary defense against this cytotoxin, the cytosolic Cu,Zn-superoxide dismutase (SOD1), would be expected to be more sensitive to oxidative stress and therefore exhibit a stronger adaptive response. That is, by inactivating the "housekeeping" anti-oxidant enzyme in S. cerevisiae, we (19, 20) and others (21) have suggested that the aggravation of the stress due to both normo- and hyperoxia in an sod1Δ mutant will allow for a more definitive characterization of the oxidative stress response (22). In fact, the phenotypes of an sod1Δ null mutant strain are consistent with this suggestion, in that this mutant strain does exhibit metabolic defects in aerobic but not in anaerobic growth, e.g. lysine and methionine auxotrophy (20–22). Although such growth defects could be explained by reactive oxygen species inactivation of specific enzymes, as demon-
strated for 6-phosphogluconate dehydratase in Escherichia coli, for example (23), they could also reflect a programmed, protective down-regulation of the expression of genes encoding these enzymes. That is, the adaptive response of a sod1Δ mutant might include a suppression of some, otherwise normal metabolic activities.

Thus, in this study, we have sought to demonstrate that dioxygen stress in yeast does cause a change in the transcriptional pattern that extends beyond the induction of anti-oxidant enzyme defenses. In fact, the data presented do show that both positive and negative transcriptional changes occur in oxidative stress. While these changes do include the transcriptional activation of stress response genes, significantly they also include a repression of expression of G1 cyclins. This inhibition correlates to a cell cycle arrest in G1 or a stationary phase-like state in which anti-oxidant defenses are activated at the expense of expression of cell functions that promote growth and/or proliferation. Thus, this work provides new insight into the expense of expression of cell functions that promote growth.

The Material and Methods section of the document includes detailed experimental procedures and materials used in the study.

**MATERIALS AND METHODS**

**Strains and Plasmids**—The yeast strains used were DBY747 (MATa leu2-3, 112 his3 trp1-289 ura3-52 gal2), EG1 (DBY747 with sod1Δ:URA3 (21)), DTT3 (MATa trp1-1 leu2-3, 112 gal1 ura3-50 his4), and EG151 (DTT3 with sod1Δ:TRP1 (24)). The CLN3-2 allele was inserted at the ARS1 locus in strains DBY747 and EG1 by double homologous recombination of CLN3-2 DNA taken from plasmid YRpDaf1-1 as described (25). This created CLN3-2 dominant mutants in the wild type and sod1Δ backgrounds. Plasmid pSB234 is a high copy plasmid containing a FUS1 lacZ gene fusion; the fusion contains the 5′ FUS1 promoter plus sequences encoding the first 254 amino acids of FUS1 fused in-frame to the gene encoding β-galactosidase (26). Gene fragments used for obtaining probes for Northern analysis were: SOD1, EcoRI-NaeI (nucleotides −169 to 365); SOD2, SphI-NruI (nucleotides 565-1116); CTA1, EcoRV-HpaI (nucleotides 2263-2275); CTT1, AccI (nucleotides 830-2095); SUC2, BamHI-HindIII (80-base pair coding region, from M. Carlson); UBI1–UBI4, KpnI-EcoRI (200-base pair coding region, from D. Finley); CLN1, NdeI-BamHI (1.6 kb from pPB171-CLN1) and CLN2, BamHI (1.8 kb from pUC19-CLN2) (27); and CLN3, EcoRI-Khol (1.6 kb from pWJ310) (28).

**Culture Conditions**—Cells were grown in YPD (1% yeast extract, 2% peptone, and 2% glucose) or a synthetic complete medium (29) at 30 °C. Media used in glucose repression/derepression studies contained 4% (repression) or 0.1% glucose (derepression). Strains were maintained in YPD plates in triplicate and incubated under N2 for 3 days at 30°C. Log phase, then transferred to fresh medium that had been

**Viability (Colony Forming Ability) Assay**—Cells incubated under O2 were subjected to 7-fold serial dilutions in YPD medium under air. Samples (100 μl) from the final dilution were plated on N2-preserved YPD plates in triplicate and incubated under N2 for 3 days at 30 °C. Visible colony formation at that time was taken as the measure of cell viability. For determining cell numbers, cells from the same culture taken at each point were fixed immediately, washed, and air-dried. The fixed cells were sonicated briefly to disrupt any clumps and were counted by microscopy using a hemocytometer. At least 200 cells were scored for each determination.

**RNA Preparation and Northern Analysis**—Cells (10–100 ml of culture, A600 = 1.0) were harvested by centrifugation in the cold. Cells were broken using glass beads in cold breaking buffer in the presence of phenol-chloroform by vigorous vortexing. Following repeated extractions with phenol, chloroform, and isomyl alcohol, total RNA was precipitated with ethanol. Electrophoresis of RNA (15–20 μg of total RNA) in formaldehyde-agarose gels was performed as described (30). The RNA was blotted onto Immobilon N (Millipore) by capillary action. The membrane was hybridized with probes labeled by random priming. Typically, 5 × 10⁶ cpm/ml were added per filter. The membrane was then washed, dried, and exposed to x-ray film. Quantitation of transcripts was obtained by densitometric analysis of the film. Even loading of total RNA was verified by ethidium bromide staining of RNA bands; in addition, ACT1 mRNA was used as an internal control.

**Labeling of Cells**—Exponentially growing cells were transferred to [3H]leucine or [3H]uracil-containing SC medium for the determination of protein or RNA synthesis, respectively. Cell samples were directly quenched into cold 5% trichloroacetic acid solution, washed, dried, and counted. There was a linear incorporation into trichloroacetic acid-precipitable material during the time course of these experiments. For the determination of mRNA synthesis and degradation, the labeled cells were processed for RNA isolation and poly(A)+ RNA binding assay. To determine the rate of RNA degradation, thiololin, an inhibitor of all three yeast RNA polymerases (31) was added to 6 μg/ml (32) to the labeled cells prior to the addition of 100 μM cold uracil. The decline of radiolabeled RNA species with time following this chase was taken as the degradation rate.

**Poly(A)+ Binding Assay**—Poly(U) filters were prepared by spotting 0.1 μg of poly(U) in the center of Whatman GF/C filters and irradiating 3 min/side under a 30-watt germicidal UV lamp (33). Filters containing immobilized poly(U) were washed with binding buffer (0.1 M sodium phosphate, 0.12 M NaCl, 0.5% sodium dodecyl sulfate, and 0.01 M Tris, pH 7.3). RNA samples were re-suspended in binding buffer and applied to filters in a total volume of 200 μl. Samples were allowed to bind for 5 min, washed with the binding buffer and then with 5% cold trichloroacetic acid. The percentage of poly(A)+-containing RNA was corrected for nonspecific binding to control filters, which had not been treated with poly(U).

**Biochemical Assays**—β-Galactosidase activity (34) was measured using cell extracts and normalized by protein content which was determined by the Bradford assay (35). Cells (1 ml) containing the pSB234 lacZ fusion plasmid were harvested, washed, and resuspended in 100 μl of Z buffer (100 mM sodium phosphate, 10 mM KCl, and 1 mM MgSO4, pH 7.0). An equal volume of glass beads was added and vortexed vigorously at 4 °C until 90% of the cells were broken as determined by microscopic examination. Supernatant (50 μl) was added and incubated in ONPG buffer (0.7 mM p-nitrophenyl-β-galactopyranoside in Z buffer) at 28 °C for 5–60 min. The reaction was quenched by the addition of 0.3 ml of 1 M Na2CO3, and the absorbance was read at 420 nm.

**RESULTS**

**The Effect of Dioxygen-dependent Stress on RNA and Protein Biosynthesis, and RNA Turnover**—In all of the experiments that follow, cells were pregrown under N2 in synthetic medium to log phase, then transferred to fresh medium that had been preflushed with N2, or 100% O2. This protocol was designed to induce a strong oxidative stress; this stress was demonstrated by the strong induction of a variety of anti-oxidant and stress response genes as shown in later figures. However, we tested first if the alteration in gene expression under oxidative stress observed in these subsequent experiments reflected effects of oxygen on macromolecular synthesis and/or turnover in general. Thus, the incorporation of radiolabeled precursors into RNA and protein was measured under various oxygen tensions for the sod1Δ mutant and wild type strains. Trichloroacetic acid-insoluble radioactivity was used as a relative measure of biosynthesis.

Steady-state [3H]uracil incorporation into trichloroacetic acid-insoluble material was markedly sensitive to the degree of oxidative stress (Fig. 1). This [3H]uracil labeling of RNA in either the wild type in O2 (Fig. 1A, closed circles) or in the mutant in air (Fig. 1B, open triangles) was reduced to about the same extent, compared to the N2-grown samples for each strain (Fig. 1C). Four mutant samples were resuspended in the same buffer of the mutant cells to O2, there was almost complete inhibition of continued [3H]uracil incorporation after 40 min (Fig. 1B, open circles). The inhibition of RNA synthesis under this oxidative stress was

1 The abbreviation used is: kb, kilobase pairs(s).
stress imposed on the mutant strain correlated with the magnitude of the oxygen-mediated inhibition of growth since sod1Δ mutants do not grow under 100% O2 although they remain >90% viable for up to 3 h in this condition (Refs. 21 and 22, and data not shown).

In contrast to RNA synthesis, protein synthesis was not markedly inhibited over this initial 1-h period of oxidative stress (data not shown). Except for moderate inhibition of [3H]leucine incorporation in the mutant under O2 (<15% inhibition in comparison to labeling under N2), there were no significant differences in protein synthesis, as measured by this criterion in the mutant cells in air or in the wild type strain under either air or O2. Protein labeling did decline in the mutant after 3 h under O2; however, this correlated with a significant loss of viability as noted above.

The fact that overall protein synthesis does not change immediately following exposure of the cells to oxidative stress suggests that the steady state level of total mRNA species did not change either although total RNA synthesis was inhibited (Fig. 1). To test this inference, the total RNA was fractionated under the stress. In these experiments, the effect of oxidative stress on the pattern of RNA synthesis described above is similar to what is observed when S. cerevisiae is treated with chemical reagents (other than mating factor) that block performance of Start, the interval between the G1 and S phases of the yeast cell cycle (36). We tested the possibility that the sod1Δ strain, in particular, exhibited a similar Start delay or arrest in G1. Under air, this mutant does grow more slowly than wild type (21). Representative doubling times for wild type and mutant in rich medium under air (and N2) are given in Table II. To determine if the slower growth of mutant in air was characterized by a difference in cell budding morphology, we examined both cultures microscopically to assess the ratio of budded to unbudded cells in each. The fraction of the cells that are unbudded is a measure of the fraction of the culture that is in the G1 phase of the cell cycle. In fact, there were 50% more unbuds in the sod1Δ air-grown culture than in the wild type (Table II). As shown in the table, the growth rate and budding patterns were the same for these strains when grown under N2. Assuming that the unbudded fraction represented the fraction of the doubling time spent in G1, the time spent in G1 was calculated for both strains. This calculation indicated that the mutant strain grew more slowly than wild type under air because it spent twice as long in G1 (Table II).

We next examined the change in the budding pattern associated with the shift from N2 to O2 (or air) as in the labeling experiments above. At t = 0, cells proliferating under N2 were transferred to fresh media presaturated with air or 100% O2. The O2-exposed cultures were divided in half 3 h later, at which point one-half was transferred back to N2-air or N2-O2. When bud morphology was examined in these cultures, wild type responded to 100% O2 by slightly and transiently accumulating as unbudded cells (Fig. 2A, solid circles); the population of unbudded cells was not significantly changed under air (Fig. 2A, solid triangles).

The response of the sod1Δ mutant strain was markedly different. First, the shift from N2 to air caused a temporary increase in unbudded cells (Fig. 2B, open triangles), i.e. the pattern of arrest in the mutant under air was similar to that suggested that overall mRNA synthesis was less susceptible to inhibition under these conditions than was rRNA synthesis and that the severe inhibition of total [3H]uracil incorporation in O2-treated mutant cells seen in Fig. 1B was due to the specific inhibition of rRNA synthesis. RNA degradation under oxidative stress was examined as well. In these experiments, mutant cells were first labeled with [3H]uracil for 1 h under N2. The label was then chased during a 30-min incubation with cold uracil under N2-air, or 100% O2. Thiolutin, an inhibitor of all three yeast RNA polymerases (31), was added during this chase period. mRNA was again separated from total RNA as described above. The data showed that in the mutant there was no significant difference in the turnover of total RNA between the N2-air, and O2 samples (<5% turnover in 30 min). The rate of mRNA degradation under air or O2 was also not significantly different than under N2, with 50–55% loss of label in the 30-min chase period in all three samples. This result is consistent with the reported average rate of mRNA turnover in yeast (half-life, 20 min; Ref. 32). Thus, overall RNA degradation is not stimulated by oxidative stress in yeast. Taken together, these several results indicate that under oxidative stress there is a strong inhibition of rRNA synthesis and that overall mRNA synthesis is not inhibited, nor is RNA stability altered by the stress.

**Growth and Cell Cycle Progression in Oxidative Stress**—The effect of oxidative stress on the pattern of RNA synthesis described above is similar to what is observed when S. cerevisiae is treated with chemical reagents (other than mating factor) that block performance of Start, the interval between the G1 and S phases of the yeast cell cycle (36). We tested the possibility that the sod1Δ strain, in particular, exhibited a similar Start delay or arrest in G1. Under air, this mutant does grow more slowly than wild type (21). Representative doubling times for wild type and mutant in rich medium under air (and N2) are given in Table II. To determine if the slower growth of mutant in air was characterized by a difference in cell budding morphology, we examined both cultures microscopically to assess the ratio of budded to unbudded cells in each. The fraction of the cells that are unbudded is a measure of the fraction of the culture that is in the G1 phase of the cell cycle. In fact, there were 50% more unbuds in the sod1Δ air-grown culture than in the wild type (Table II). As shown in the table, the growth rate and budding patterns were the same for these strains when grown under N2. Assuming that the unbudded fraction represented the fraction of the doubling time spent in G1, the time spent in G1 was calculated for both strains. This calculation indicated that the mutant strain grew more slowly than wild type under air because it spent twice as long in G1 (Table II).

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seen with the wild type cells under 100% O₂. Furthermore, unlike wild type cells under any condition, in oxygen the mutant cell culture arrested permanently as large, unbudded cells (Fig. 2B, open circles). These cells remained viable for up to 3 h, since they completely recovered in 1 h if the stress was removed at that time (Fig. 2B, open squares). After 3 h under 100% O₂, however, the sod1Δ mutant culture began to lose viability (data not shown). These budding data indicate that dioxygen stress in yeast causes a cell cycle arrest in G₁ prior to Start that is either transient or permanent depending on the level of stress.

**FIG. 2. G₁ arrest and recovery in air or O₂ in wild type and sod1Δ mutant cells.** DYT3 wild type (A, solid symbols) and EG151 sod1Δ mutant (B, open symbols) growing exponentially (A₅₅₀ = 1.5) in YPD medium under N₂ were transferred at t = 0 to media saturated with air (A, ●) or O₂ (B, ○). Cells under O₂ were transferred back to N₂ (●, □) at 3 h and incubated for the times indicated. Samples were fixed in formaldehyde and the fraction of budded cells determined microscopically. The data are representative of three independent experiments; the values varied ±10%.

A culture of sod1Δ mutant strain, EG1, was grown exponentially under N₂ in SC-Ura medium. The culture was divided into three aliquots and labeled for 1 h ([³H]uracil, 5 µCi/ml) in medium which was presaturated with N₂, air, or O₂. Trichloroacetic acid-precipitable [³H]uracil in the RNA samples was measured and normalized to micrograms of total RNA precipitated. For poly(A’- isolated total RNA samples were incubated for 10 min in binding buffer (0.01 M Tris, 0.12 M NaCl, pH 7.5) at 25 °C and then bound to poly(U) filters. These were washed and counted. The counts were normalized to micrograms of total RNA loaded. Data are means ± S.D. for three experiments.

### TABLE I

**Effect of oxygen on poly(A’) and total RNA synthesis in sod1Δ mutant strain**

| Sample       | Poly(A’) cpm/µg RNA | %a | Total RNA cpm/µg RNA | %a | Poly(A’)/total RNA (%) |
|--------------|---------------------|----|----------------------|----|------------------------|
| N₂           | 115 ± 12b           | 100| 6995 ± 230c          | 100| 1.6 (100)a             |
| Air          | 124 ± 3             | 108| 6894 ± 765           | 99 | 1.8 (110)              |
| O₂           | 86 ± 8              | 75 | 1197 ± 147           | 17 | 7.2 (440)              |

- a % of controls, N₂-labeled cultures for each RNA pool.
- b Actual values (cpm/filter) ranged from 6000 to 14000. A range of 50–100 µg of total RNA was used for the three different experiments. Background counts were measured using regular glass filters loaded with corresponding unlabeled RNA samples. These blank values ranged from 5000 to 40,000; 5 µg of total RNA was used. Background counts were ≤200 cpm/filter.
- c Actual values (cpm/sample) ranged from 5000 to 40,000; 5 µg of total RNA was used. Background counts were ≤200 cpm/filter.

**TABLE II

**Generation time and G₁ duration in wild type and sod1Δ mutant strains**

| Wild type (DBY747) | sod1Δ mutant (EG1) |
|--------------------|--------------------|
| Generation time (min)b | Generation time (min)b |
| Under N₂          | 120                |
| Under air         | 120                |
| Percent unbudded cells | Percent unbudded cells |
| Under N₂          | 35 ± 3             |
| Under air         | 34 ± 4             |
| Length of G₁ (min)b | Length of G₁ (min)b |
| Under N₂          | 42                 |
| Under air         | 42                 |

- a Generation times were calculated by counting number of newly-budded cells appearing during the 8-h incubation under air. The average number of new cells in a single colony was 16.1 for wild type (999 cells, 62 colonies) and 6.7 for mutant (417 cells for 59 colonies).
- b Duration in G₁ = generation time (min) × fraction of unbuds.
Fig. 3. CLN2 gene expression in oxidative stress. DTY3 wild type and EG151 sod1Δ mutant growing exponentially (Δgeo = 1.5) in YPD medium under N2 were transferred to O2-saturated medium. At 3 h, cultures were transferred back to N2 and incubated for another 2 h. Cultures were harvested, and total RNA was extracted and characterized by Northern analysis using 32P-labeled probes for ACT1 (cyclin) and ACT1 (actin). The Northern blots are presented in panel A. Relative quantitation of the CLN2 mRNA was obtained by densitometric analysis of the film and the absolute values were normalized to ACT1 mRNA. These data for wild type are presented in panel B (O2, ○; N2, ■) and for the sod1Δ mutant in panel C (O2, ●; N2, □).

Fig. 2B followed from the abrupt disappearance of both autocatalytically regulated G1 cyclin messages. As the data in Fig. 2B indicated, when O2-treated mutant cells were returned to N2, the inhibition of Start performance was relieved. To test the possibility that resumption of Start performance was preceded by CLN2 expression, mutant cells incubated under O2 for 3 h were transferred back to N2 as above, and samples were removed every 15 min for assessment of CLN2 transcript abundance. As shown in Fig. 3 (A and C), a burst of CLN2 gene expression was observed after 45 min in N2, followed by a cycle of down- and up-expression. The initial burst of CLN2 transcripts resulted from the simultaneous recovery of the arrested cells, synchronized by oxidative stress at Start.

In contrast to the expression of CLN1 and CLN2, the level of the CLN3 transcript under oxidative stress in both strains was not changed. Northern data for the sod1Δ mutant are shown in Fig. 4. This differential regulation of CLN3 and CLN2 expression has been observed in a number of other stress conditions. For example, CLN2 transcription decreases in response to heat shock, nutritional starvation, and addition of mating factor, whereas CLN3 transcription does not (27, 28, 38). In addition, CLN1 and CLN2 expression is cell cycle-regulated, peaking at Start, while, as noted, CLN3 is expressed constitutively throughout the cell cycle (37, 40). In summary, the growth, budding, and Northern analyses indicate that oxidative stress inhibits Start through an inhibition of cell cycle-dependent cyclin gene expression and a resulting loss of sufficient G1 cyclin protein to perform the Start function.

We tested this latter conclusion by constructing wild type and sod1Δ mutant strains that carried a single copy of the CLN3–2 allele integrated at the ARS1 locus. This allele encodes a C-terminal truncation of the Cln3 protein that renders the protein proteolytically stable in the cell (25). Expression of this allele constitutively raises the steady-state level of G1 cyclin and blocks the G1 arrest caused by heat shock (27). In fact, this hyperstable cyclin blocks the cell cycle arrest caused by oxidative stress, also. These data, again for the sod1Δ mutant, are given in Fig. 5, which show the budding pattern under O2 for the mutant expressing only the wild type Cln3 (solid circles) and for the mutant expressing the hyperstable Cln3–2 protein as well (open circles). The fact that this protein was able to suppress the apparent Start arrest in the mutant upon transfer from N2 to O2 is consistent with the model that this arrest is due to the down-regulation of G1 cyclin expression in oxidative stress.

Transcriptional Remodeling in Oxidative Stress—Protein analyses show that a remodeling of protein synthesis occurs in response to oxidative stress in yeast (12, 14). This altered pattern of gene expression most reasonably results from changes in the transcriptional controls in the cell. The data above show that CLN expression is one example of this transcriptional remodeling. They also define the cell cycle stage in which adaptation to oxidative stress is occurring, i.e., primarily in G1. We therefore carried out two kinds of experiments to examine what the pattern of gene expression was in these apparently G1-arrested cells. First, the level of expression of other (than SOD1) oxidative stress response genes was determined by Northern analysis. Second, the expression of some specific and inducible genes was determined when cells were simultaneously exposed to the inducing stimulus and oxidative stress. The strategy in this experiment was to test the hypothesis that under oxidative stress (and/or in G1) the cell may lose the capacity to transcriptionally activate or express genes whose products are otherwise not essential to the adaptation to the stress.

As an example of the first of these two types of experiments, we analyzed the expression of the UBI1 genes. The UBI loci encode ubiquitin, which when conjugated to protein(s), targets them for turnover by the 26 S protease in an ATP-dependent process (41). In S. cerevisiae, UBI1, UBI2, and UBI3 are constitutively expressed, while UBI4 transcription is induced in heat shock (5). UBI4 encodes a polyubiquitin polypeptide which appears to play some role in stress response, since mutations at this locus cause sensitivity to hydrogen peroxide (42) and to heat and starvation (5), while homozygous mutant diploids are
sporulation-defective (43). Indeed, the pattern of expression of these four loci in the sod1Δ mutant and wild type strains under the oxidative stress imposed by 100% O₂ provided a strong example of an apparent programmed transcriptional response of yeast to this stress (Fig. 6). That is, upon shifting cells from N₂ to 100% O₂ in the wild type there was little decrease in the expression of the constitutive, “house-keeping” UBI genes, while slight activation of transcription from UBI4 was observed. In contrast, in the mutant strain this environmental change resulted in the nearly complete loss of UBI1–UBI3 mRNA concurrent with a strong transcriptional activation of UBI4. This result clearly illustrates a transcriptional remodeling in yeast under oxidative stress, which involves a pattern of both activation and repression. This result also suggests a likely role for polyubiquitin expression in the adaptation to oxidative stress in yeast whether due to O₂ (superoxide) or H₂O₂ (43).

We also wished to examine in the sod1Δ mutant the expression of genes encoding other anti-oxidant enzymes in S. cerevisiae such as SOD2, CTT1, and CTAl (Mn-superoxide dismutase, catalase T, and catalase A, respectively) and to compare this expression to wild type. The rationale for this experiment was that in the absence of SOD1, the expression of these other genes should be exaggerated. We also imposed the oxidative stress under conditions of glucose derepression to maximize the oxidative stress response. All of these genes exhibit some glucose repression (6). The Northern data in Fig. 7 show that induction of SOD2 by air and O₂ (in the absence of glucose) in the mutant (lanes 3 and 4) was markedly increased compared to the induction in wild type under the same conditions (lanes 7 and 8). Induction of this locus by glucose derepression alone (under N₂) was also observed, although it was relatively weak, particularly in wild type (cf. lanes 1 and 2 and lanes 5 and 6). However, activation of SOD2 expression in response to oxygen was strongly enhanced in the sod1Δ mutant (lane 4). This amplified induction by O₂ of oxygen-responsive genes in the mutant was observed for CTT1 and CTAl, as well.

In carrying out these experiments, we noted that the expected increase in transcription from these loci due to glucose derepression alone was diminished when glucose withdrawal was accompanied by oxidative stress. We wished to determine whether this effect, e.g. a silencing of glucose derepression, was a general transcriptional feature of oxidative stress. As a simple test of this possibility, we analyzed the levels of the SUC2 message in the same conditions. SUC2 encodes invertase, expressed and secreted under conditions of low glucose (44). Thus, cells grown in high glucose, N₂-saturated medium were transferred to low glucose medium presaturated with N₂, air or O₂. Cultures were harvested after 1 h, and total RNA was extracted. This RNA (15 μg) was used for Northern analysis using 32P-labeled probes for CTAl (catalase A), CTT1 (catalase T), SOD2 (MnSOD), and ACT1 (actin) mRNA. Ten-fold more radioactivity (5 × 10⁶ cpm/ml) than normally used was added for hybridization to the CTAl and CTT1 transcripts. Relative quantitation of the SOD2 mRNA was obtained by densitometric analysis, and the absolute values were normalized to ACT1 mRNA; the normalized values in high glucose under N₂ for each strain were used as the basal level, and the fold induction calculated is indicated.

**Fig. 6. Effect of oxidative stress on UBI mRNA levels.** Wild type DBY747 and EG1 sod1Δ mutant growing exponentially (A₅₅₀ = 1.0) in high glucose (4%) SC medium under N₂ were transferred to low glucose medium presaturated with N₂, air or O₂. Cultures were harvested after 1 h, and total RNA was extracted. This RNA (15 μg) was used for Northern analysis using 32P-labeled probes for CTAl (catalase A), CTT1 (catalase T), SOD2 (MnSOD), and ACT1 (actin) mRNA. Ten-fold more radioactivity (5 × 10⁶ cpm/ml) than normally used was added for hybridization to the CTAl and CTT1 transcripts. Relative quantitation of the SOD2 mRNA was obtained by densitometric analysis, and the absolute values were normalized to ACT1 mRNA; the normalized values in high glucose under N₂ for each strain were used as the basal level, and the fold induction calculated is indicated.

**Fig. 7. Anti-oxidant enzyme gene expression in oxidative stress.** Wild type DBY747 and EG1 sod1Δ mutant strains growing exponentially (A₅₅₀ = 1.0) in high glucose (4%) SC medium under N₂ were transferred to low glucose medium presaturated with N₂, air or O₂. Cultures were harvested after 1 h, and total RNA was extracted. This RNA (15 μg) was used for Northern analysis using 32P-labeled probes for CTAl (catalase A), CTT1 (catalase T), SOD2 (MnSOD), and ACT1 (actin) mRNA. Ten-fold more radioactivity (5 × 10⁶ cpm/ml) than normally used was added for hybridization to the CTAl and CTT1 transcripts. Relative quantitation of the SOD2 mRNA was obtained by densitometric analysis, and the absolute values were normalized to ACT1 mRNA; the normalized values in high glucose under N₂ for each strain were used as the basal level, and the fold induction calculated is indicated.
stress response and not just due to cell cycle arrest in general. To accomplish the first of these goals, we examined under oxidative stress the transcriptional activation by copper of \textit{CUP1}, which encodes yeast copper thionein, and the activation by mating factor of \textit{FUS1}, which encodes the membrane fusion protein required for yeast mating. In both cases, the stress had no effect on the level of transcriptional activation. That is, in the \textit{sod1Δ} mutant, when \textit{N}_{2}\textsuperscript{-}grown cells were switched to \textit{N}_{2}\textsuperscript{-}, \textit{air} -, or \textit{O}_{2}\textsuperscript{-} -saturated media containing 50 μM copper sulfate and total RNA was examined by Northern analysis for \textit{CUP1} mRNA after 1 h of treatment, the level of \textit{CUP1} induction was equivalent in all three conditions, i.e., 15–20-fold over the no added copper control. Similarly, the activity of the \textit{FUS1} promoter was the same in unstressed and stressed mutant cells. This was established using a reporter plasmid containing the \textit{FUS1} promoter upstream from the \textit{lacZ} gene (26). When mutant cells were switched to \textit{N}_{2}\textsuperscript{-}, \textit{air} -, and \textit{O}_{2}\textsuperscript{-} -saturated media containing α-factor and then β-galactosidase activities were measured in the cell extract after 30 min, all samples exhibited a 12–16-fold induction over control (no α-factor).

To link the transcriptional changes observed more directly to the stress as opposed to cell cycle arrest in general, we examined the induction of \textit{SUC2} transcription by glucose derepression in cells that were arrested at Start by pretreatment with α-factor for 2 h. At this time, assessment of the percent unbudded cells (85%) indicated that the culture was primarily in G1. The cells were then washed and resuspended in glucose-free medium containing α-factor; after 1 h of incubation, total RNA was prepared and analyzed for \textit{SUC2} mRNA as above. The results were negative in that the pretreatment with mating factor, and the cell cycle arrest that followed (as determined by the percentage of unbudded cells), did not inhibit the transcriptional activation of \textit{SUC2} when these arrested cells were switched to a glucose-free medium.

**DISCUSSION**

Treatment of \textit{S. cerevisiae} with sublethal doses of H\textsubscript{2}O\textsubscript{2} (12, 14) or of menadione (14), a superoxide generator, induces a marked change in the protein biosynthetic pattern in this organism. The synthesis of 15–20 proteins at the least is stimulated by either or both of these treatments, while the synthesis of several other proteins is depressed, at least by H\textsubscript{2}O\textsubscript{2} (12). In general, however, the underlying transcriptional changes that these protein gels reflect have not been characterized. Not surprisingly, the expression of some genes encoding anti-oxidant enzyme activities in yeast is stimulated by peroxide or menadione or paraquat (another superoxide generator) as indicated by Northern analysis (4, 17) or use of reporter plasmids constructed using promoter elements from these genes (4, 14, 17). Indeed, much study has gone into the identification of the \textit{cis} elements that drive expression of these oxidant-responsive stress genes. For example, an AP-1 response element has been identified in the promoter of \textit{TRX2}, one of the two genes that encode thioredoxin in \textit{S. cerevisiae}. This element binds and is activated by Yap1 in response to oxidative stress (45). Yap1 is the yeast homolog of the mammalian transcription factor, AP-1, a member of the Jun family of proteins (46). Deletion of either \textit{TRX2} or \textit{YAPI} makes yeast sensitive to peroxides (23, 45). \textit{GSHEL}, encoding γ-glutamylcysteine synthetase, is also regulated by Yap1 through an AP-1 response element (13, 47). A relationship between \textit{GSHEL} expression and defense against oxidative stress has not been established, however. Two sequences resembling AP-1 sites have been noted in the 5′ region of the \textit{SOD1} locus as well (1), but no role for them in \textit{SOD1} expression has been demonstrated. Another \textit{cis} element, designated stress response element, has been identified in several stress response genes including \textit{CTT1} (1, 16). Activation via this element also requires Yap1, although this protein does not bind to the stress response element sequence in vitro (48).

These studies have provided significant molecular insight about the transcriptional control, primarily by H\textsubscript{2}O\textsubscript{2}, of these stress response genes. In contrast, we sought to develop a more global picture of the transcriptional changes that occur in yeast in dioxygen stress, specifically, in order to provide a better understanding of how this more typically chronic stress actually impacts on the cell’s overall physiology. We felt that this more global picture of the dioxygen stress response would give some clues as to what selective advantages the cell can bring to the fore in order to adapt to and survive aerobiosis.

This and other work suggests the following about oxidative stress and anti-oxidant defense in yeast. First, the CuZn-superoxide dismutase activity due to \textit{SOD1} expression represents the dominant “housekeeping” anti-oxidant enzyme activity in this organism. This is indicated by the level of its expression relative to the others (14, 22) and the fact that in glucose-grown, log phase yeast it represents better than 95% of the total anti-oxidant enzyme activity with the balance contributed by \textit{SOD2}, the mitochondrial MnSOD in yeast (17, 49). Furthermore, neither catalase gene is expressed in glucose-grown, log phase cells. This can explain why, for example, menadione causes only a weak induction of \textit{SOD1} or \textit{SOD2} in a \textit{SOD1} wild type strain (14); apparently, there is already excess superoxide dismutation activity in the cell. This situation, however, explains also why \textit{sod1Δ} strains exhibit such strong growth phenotypes in comparison to \textit{sod2Δ} ones (22) and why, in the work here, conditions that in wild type fail to transcriptionally activate the other anti-oxidant enzyme genes (e.g. 100% O\textsubscript{2}), strongly activate them in the \textit{sod1Δ} background.

The most significant biologic advantage due to the presence of \textit{SOD1} in yeast is illustrated by the growth data for the \textit{sod1Δ} strain under air in rich, non-selective medium; it grows 50% slower than wild type. We show here that this increased doubling time appears to be due to an increased time spent in G1. Furthermore, this delay in performing Start can be exaggerated if the mutant is placed under a more acute stress as occurs in a switch from \textit{N}_{2} to either air or 100% O\textsubscript{2}. In both cases, there is an arrest in G1, apparently at Start, that under O\textsubscript{2} is permanent. This acute phase response includes a repression of expression of G1 cyclin genes that normally are autoactivated at this point in the cell cycle. This repression appears to underlie the Start arrest observed. Although we provide no data on this point, it seems reasonable to propose that the slow
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mutant growth under air is associated with a somewhat reduced rate of G1 cyclin expression as well, and that this condition represents the physiologic state of yeast in chronic oxidative stress due to lack of SOD1.

The strong inhibitory effect of acute dioxygen stress on rRNA synthesis in comparison to mRNA synthesis suggests that the cells are preparing to enter a stationary phase-like state. Veinot-Drebot et al. (37) showed that chemicals like o-phenanthroline and l-ethionine that were known to cause a cell cycle arrest prior to Start had a similar inhibitory effect on rRNA synthesis. This was in contrast to mating factor, which, while causing Start arrest, had no inhibitory effect on rRNA biosynthesis. Subsequent work by Barnes et al. (50) suggested that o-phenanthroline produced a stationary phase arrest, a finding that was consistent with the observation that this chemical caused induction of the general control response that is characteristic of nutrient-depleted stationary phase cultures. With respect to the dioxygen stress response studied here, arrest in a stationary phase-like condition is reasonable. Stationary phase cells are known to be generally more stress-resistant (1, 2, 7). The higher level of induction of SOD2, CTT1, and CTA1 in the sod1Δ mutant in this arrested state is consistent with this in that the first two of these genes are transcriptionally activated in the stationary phase induced by nutrient depletion (7). Stationary phase sod1Δ mutant cells also survive longer under air than do log phase ones (51). On the other hand, the recovery of mutant growth (budding) upon returning from O2 to N2 that we observed was somewhat faster than that typically seen for stationary phase cells returned to fresh medium (50).

Thus, it seems likely that although similar to stationary phase in some respects, the metabolic state of the mutant arrested by oxidative stress is also different.

We noted that glucose derepression of the other anti-oxidant enzyme genes was suppressed in oxidative stress and tested this more directly by analyzing SUC2 expression under these conditions. This analysis showed that oxidative stress inhibited completely the expression of this locus. Thus, SUC2, like the housekeeping UBI genes and CLN1 and CLN2, is an example of transcriptional down-regulation in oxidative stress, although this observation does not provide a mechanism for it. One possibility is that in this arrested state the lack of cell proliferation limited the cell’s capacity to deplete its reserves of glucose following the switch to the glucose-free medium. We cannot rule this explanation out, but do note that the cells, although unbudded, did continue to grow in size (as is true of slowly growing yeast) and continued to make RNA and protein.

In addition, cells arrested by α-factor did express SUC2 under glucose derepression. We suggest, therefore, that the lack of glucose derepression in oxidative stress is a direct result of the stress and/or is characteristic of the cell cycle arrest specific to the stress. That is, the data do not distinguish between a model in which the arrest and suppression of glucose derepression are independent phenotypes of oxidative stress or one in which one of these responses follows from the other. For example, oxidative stress could cause an arrested state in which glucose derepression is silenced. In any event, both phenotypes are similar in that they represent an inhibition of growth and proliferation. What is clear from the data here is that adaptation to oxidative stress by yeast involves growth limitation including the suppression of gene expression that normally promotes cell and culture growth.

This difference in response to glucose between mating factor-arrested cells and cells arrested by oxidative stress is similar to that noted with respect to RNA synthesis (see Ref. 37 and above), i.e. the stationary phase-like arrest caused by l-ethionine, for example (37), correlated with a strong inhibition of rRNA synthesis. Since rRNA and ribosomal protein synthesis correlates with cell growth (8), this pattern indicates that l-ethionine-treated cells are growth-arrested. In contrast, while mating factor-treated cells are arrested, they appear poised to continue growth since rRNA synthesis is not inhibited (37). This comparison suggests that oxidatively stressed cells are metabolically more like stationary phase cells than mating factor-arrested cells, although, as noted, the rate of their growth recovery indicated that they were not in a true stationary state. Nonetheless, anti-oxidant genes that are induced in stationary phase, e.g. SOD2 and CTT1, were activated in this state indicating that they may be genes transcriptionally activated early in the post-diauxic shift to stationary phase (6).

This work has provided basic information about the physiologic state of a cell under the oxidative stress associated with an acute hyperoxia and a deficiency of SOD1, a stress that will eventually lead to cell death. Expression of activities that are associated with or promote growth (rRNA, UBI1–UBI3, and SUC2) or proliferation (CLN1 and CLN2) is repressed while expression of activities that have the potential of defending against the stress is activated (SOD2, CTT1, and UBI4). The expression of or activation of other genes is unaffected (CUP1 and FUS1), as are mRNA and protein synthesis and overall RNA turnover. The fact that mRNA turnover is not altered in oxidative stress suggests but does not prove that the changes in transcript abundance observed in this work were due solely to changes in message synthesis. A change in the stability of any specific mRNA species in oxidative stress cannot be ruled out. That the biosynthetic capacity of the cell is retained is critical since the cell needs to assemble de novo its defense against the stress. The cell appears to be in a physiologic state that is similar to but not, in fact, stationary phase, although we provide no specific evidence for this inference. At least some of the signaling pathways in the cell are functional, as indicated by copper induction of CUP1 and mating factor induction of the FUS1 promoter in addition to the signaling of the oxidative stress itself. Although our work does not provide explicit information about the mechanism(s) that underlies this transcriptional pattern, the inhibition of glucose derepression in this state is suggestive. Expression of SUC2 requires the relaxation of chromatin structure through the action of the Snp1 and Swi6 gene products (52). The inhibition of glucose derepression seen in acute oxidative stress could indicate that this chromatin structural change is blocked. Maintaining a more condensed state of the chromatin in oxidative stress would appear to have some survival advantage, since it is known that in this state DNA is less susceptible to chemical modification and damage (53). This speculation awaits experimental validation.

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REFERENCES

1. Mager, W. H., and De Kruijff, A. J. J. (1995) Microbiol. Rev. 59, 506–531
2. Mager, W. H., and Ferreira, P. M. (1993) Biochem. J. 290, 1–13
3. Thevelein, J. M. (1991) Mol. Microbiol. 5, 1301–1307
4. Belaiz, T., Wagner, A., Weiser, R., Shanaz, M., Adam, G., Hartig, A., and Ruis, H. (1991) EMBO J. 10, 585–592
5. Finley, D., Ockaynak, K., and Varshavsky, A. (1987) Cell 48, 1035–1046
6. Werner-Washburne, M., Braun, E., Johnston, G. C., and Singer, R. A. (1993) Microbiol. Rev. 57, 383–401
7. Veinot-Drebot, L. M., Singer, R. A., and Johnston, G. C. (1989) J. Biol. Chem. 264, 19473–19474
8. Kraakman, L. S., Grifflon, G., Zerp, S., Groeneveld, P., Thevelein, J. M., Mager, W. H., and Planta, R. J. (1993) Mol. Gen. Genet. 239, 196–204
9. Jamison, D. J. (1992) J. Bacteriol. 174, 6678–6681
10. Collinson, L. P., and Dawes, I. W. (1992) J. Gen. Microbiol. 138, 329–335
11. Flattery-Öbrien, J., Collinson, L. P., and Dawes, I. W. (1993) J. Gen. Microbiol. 139, 501–507
Transcriptional Response to Dioxygen Stress in Yeast

12. Davies, J. M. S., Lowry, C. V., and Davies, K. J. A. (1995) Arch. Biochem. Biophys. 317, 1–6
13. Stephen, D. W. S., Rivers, S. L., and Jamieson, D. J. (1995) Mol. Microbiol. 16, 415–423
14. Jamieson, D. J., Rivers, S. L., and Stephen, D. W. S. (1994) Microbiol. 140, 3277–3283
15. Lindquist, S., and Craig, E. A. (1988) Annu. Rev. Genet. 55, 1151–1191
16. Marchler, G., Schüller, C. Adam, G., and Ruis, H. (1993) EMBO J. 12, 1997–2003
17. Galiazzo, F., and Labbe-Bois, R. (1993) FEBS Lett. 315, 197–200
18. Cadenas, E. (1989) Annu. Rev. Biochem. 58, 79–110
19. Chang, E. C., Crawford, B. F., Hong, Z., Bilinski, T., and Kosman, D. J. (1991) J. Biol. Chem. 266, 4417–4424
20. Chang, E. C., and Kosman, D. J. (1990) J. Bacteriol. 172, 1840–1845
21. Gralla, E. B., and Valentine, J. S. (1991) J. Bacteriol. 173, 5918–5920
22. Gralla, E. B., and Kosman, D. J. (1992) Adv. Genet. 30, 251–319
23. Gardner, P. R., and Fridovich, I. (1991) J. Biol. Chem. 266, 1478–1483
24. Cross, F. R. (1988) Mol. Cell. Biol. 8, 4675–4684
25. Cross, F. R. (1988) Mol. Cell. Biol. 8, 4675–4684
26. Trueheart, J., Boeke, J. D., and Fink, G. R. (1987) Mol. Cell. Biol. 7, 2316–2328
27. Rowley, A., Johnston, G. C., Butler, B., Werner-Washburne, M., and Singer, R. (1993) Mol. Cell. Biol. 13, 1034–1041
28. Cross, F. R. (1990) Mol. Cell. Biol. 10, 6482–6490
29. Rose, M. D., Winston, F., and Heiter, P. (1990) Methods in Yeast Genetics, pp. 179–180, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 7.43–7.48, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Tipper, D. J. (1973) J. Bacteriol. 116, 245–256
32. Herrick, D., Parker, R., and Jacobson, A. (1990) Mol. Cell. Biol. 10, 2269–2284
33. Werner, D., Chenla, Y., and Herzberg, M. (1984) Anal. Biochem. 141, 329–336
34. Miller, J. (1972) Experiments in Molecular Genetics, p. 466, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
35. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
36. Veinot-Drebot, L. M., Singer, R. A., and Johnston, G. C. (1989) J. Biol. Chem. 264, 19552–19554
37. Cross, F. R., and Mason, S. W. (1993) Science 261, 1543–1544
38. Chang, E. C., Crawford, B. F., Hong, Z., Bilinski, T., and Kosman, D. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 90, 8013–8017
39. Trueheart, J., Boeke, J. D., and Fink, G. R. (1987) Mol. Cell. Biol. 7, 2316–2328
40. Rowley, A., Johnston, G. C., Butler, B., Werner-Washburne, M., and Singer, R. (1993) Mol. Cell. Biol. 13, 1034–1041
41. Rowley, A., Johnston, G. C., Butler, B., Werner-Washburne, M., and Singer, R. (1993) Mol. Cell. Biol. 13, 1034–1041
42. Rose, M. D., Winston, F., and Heiter, P. (1990) Methods in Yeast Genetics, pp. 179–180, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
43. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 7.43–7.48, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
44. Cross, F. R. (1988) Mol. Cell. Biol. 8, 4675–4684
45. Cross, F. R. (1988) Mol. Cell. Biol. 8, 4675–4684
46. Trueheart, J., Boeke, J. D., and Fink, G. R. (1987) Mol. Cell. Biol. 7, 2316–2328
47. Rowley, A., Johnston, G. C., Butler, B., Werner-Washburne, M., and Singer, R. (1993) Mol. Cell. Biol. 13, 1034–1041
48. Rose, M. D., Winston, F., and Heiter, P. (1990) Methods in Yeast Genetics, pp. 179–180, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
49. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 7.43–7.48, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
50. Barnes, C. A., Johnstone, G. C., and Singer, R. A. (1990) J. Bacteriol. 172, 317–325
51. Barnes, C. A., Johnstone, G. C., and Singer, R. A. (1990) J. Bacteriol. 172, 317–325
52. Winstein, F., and Carlsson, M. (1992) Trends Genet. 8, 387–391
53. Enright, H. U., Miller, W. J., and Hebbel, R. P. (1992) Nucleic Acids Res. 20, 3341–3346