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Global Translational Responses to Oxidative Stress Impact upon Multiple Levels of Protein Synthesis*[S]

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Global inhibition of protein synthesis is a common response to stress conditions. We have analyzed the regulation of protein synthesis in response to oxidative stress induced by exposure to H_2O_2 in the yeast *Saccharomyces cerevisiae.* Our data show that H_2O_2 causes an inhibition of translation initiation dependent on the Gcn2 protein kinase, which phosphorylates the α-subunit of eukaryotic initiation factor-2. Additionally, our data indicate that translation is regulated in a Gcn2-independent manner because protein synthesis was still inhibited in response to H_2O_2 in a *gcn2* mutant. Polysome analysis indicated that H_2O_2 causes a slower rate of ribosomal runoff, consistent with an inhibitory effect on translation elongation or termination. Furthermore, analysis of ribosomal transit times indicated that oxidative stress increases the average mRNA transit time, confirming a post-initiation inhibition of translation. Using microarray analysis of polysome- and monosome-associated mRNA pools, we demonstrate that certain mRNAs, including mRNAs encoding stress protective molecules, increase in association with ribosomes following H_2O_2 stress. For some candidate mRNAs, we show that a low concentration of H_2O_2 results in increased protein production. In contrast, a high concentration of H_2O_2 promotes polyribosome association but does not necessarily lead to increased protein production. We suggest that these mRNAs may represent an mRNA store that could become rapidly activated following relief of the stress condition. In summary, oxidative stress elicits complex translational reprogramming that is fundamental for adaptation to the stress.

Cells must be able to maintain their intracellular homeostasis in the face of changing conditions. Typically, they respond by invoking complex regulatory mechanisms, including global inhibition of translation (1, 2). This reduction in protein synthesis may prevent continued gene expression during potentially error-prone conditions as well as allow for the turnover of existing mRNAs and proteins, whilst gene expression is reprogrammed to deal with the stress.

Four mammalian protein kinases that inhibit translation initiation by phosphorylating eukaryotic initiation factor-2 (eIF2)[2] have been identified. GCN2 (the amino acid control kinase), PKR (the double-stranded protein kinase activated by RNA), HRI (the heme-regulated inhibitor), and PERK/PEK (the PKR-like endoplasmic reticulum eIF2α kinase) are regulated independently in response to various different cellular stresses (2, 3). For example, PERK has been found in all multicellular eukaryotes and is a component of the unfolded protein response. Consistent with its central role in the endoplasmic reticulum (ER) stress response, cells lacking PERK fail to phosphorylate eIF2α and do not down-regulate protein synthesis during ER stress conditions (4, 5). Attenuating protein synthesis may act to reduce the burden of newly synthesized ER client proteins on the ER folding machinery. Additionally, eIF2 phosphorylation induces translation of specific mRNAs, such as that encoding the metazoan activating transcription factor-4 (6, 7). Activating transcription factor-4 mediates the integrated stress response, the targets of which include genes encoding proteins involved in amino acid metabolism and resistance to oxidative stress, ultimately protecting against the deleterious consequences of ER oxidation (8).

In yeast, Gcn2 is the sole eIF2 kinase and phosphorylates eIF2α in response to nutrient starvation and sodium or rapamycin exposure. eIF2 is a guanine nucleotide-binding factor and, in the GTP-bound form, interacts with the initiator methionyl-tRNA (Met-$tRNA_i^{Met}$) to form a ternary complex (eIF2·GTP·Met-$tRNA_i^{Met}$) that is competent for translation initiation. Following each round of initiation, eIF2 is released from the ribosome as a binary complex with GDP. GDP is replaced by GTP in a guanine nucleotide exchange reaction promoted by eIF2B. Phosphorylation of eIF2α by Gcn2 converts eIF2 from a substrate to an inhibitor of the guanine nucleotide exchange factor eIF2B (9). The resulting decrease in eIF2B activity leads to reduced ternary complex levels. Paradoxically, translation of the *GCN4* mRNA is activated in response to low ternary complex levels in a mechanism involving short upstream open reading frames (10). Gcn4 is a transcription factor that activates gene expression of many targets, including amino acid biosynthetic genes (11). Thus, analogous to the mammalian integrated stress response, activation of Gcn4 serves to overcome the imposed starvation, which initially led to the translational control. More recently, we used microarray analysis combined with

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables I–IV.

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[2] The abbreviations used are: eIF2, eukaryotic initiation factor-2; ER, endoplasmic reticulum; ROS, reactive oxygen species; TAP, tandem affinity purification; RT, reverse transcription.

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polysome analysis to demonstrate that lowering ternary complex levels results in widespread translational reprogramming, identifying a fundamental role for translational control in the adaptation to nutrient limitation (12).

All aerobic organisms are exposed to reactive oxygen species (ROS), such as H_2O_2, the superoxide anion, and the hydroxyl radical, during the course of normal aerobic metabolism or following exposure to radical-generating compounds. These ROS cause wide-ranging damage to macromolecules, eventually leading to cell death (13, 14). To protect against oxidant damage, cells contain effective defense mechanisms, including antioxidant enzymes and free radical scavengers (15). Yeast cells can adapt to oxidative stress by altering global transcription, including genes encoding antioxidants and other metabolic enzymes (16, 17). However, we have shown that oxidative stress caused by exposure to H_2O_2 results in a rapid and reversible inhibition of protein synthesis (18). Thus, it is unclear how changes in the gene expression program are translated into the cellular proteome. In this study, we have analyzed the regulation of protein synthesis in response to oxidative stress induced by exposure to H_2O_2. Our data show that H_2O_2 causes a dose-dependent inhibition of protein synthesis mediated in part by Gcn2-dependent phosphorylation of eIF2α. In addition, we provide evidence that protein synthesis is repressed by a Gcn2-independent inhibition of ribosomal transit. We used microarray analysis to demonstrate that certain mRNAs are translationally maintained following oxidative stress, indicating that translational control is a key component of the cellular response to oxidative stress.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—The *Saccharomyces cerevisiae* strains used in this study are isogenic derivatives of CY4 (*MAT***a** *ura3-52 leu2-3 leu2-112 trp1-1 ade2-1 his3-11 can1-100*) (19). Tandem affinity purification (TAP)-tagged strains (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) were purchased from Open Biosystems. Strains were converted to methionine prototrophs by transforming with a PCR-generated *MET15* gene to facilitate radiolabeling with L-[^{35}S]cysteine/methionine. Strains were grown in rich YEPD medium (1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, and 2% (w/v) glucose) or minimal synthetic dextrose medium (0.17% (w/v) yeast nitrogen base without amino acids, 5% (w/v) ammonium sulfate, and 2% (w/v) glucose) supplemented with appropriate amino acids and bases (20) at 30 °C and 180 rpm. Media were solidified by the addition of 2% (w/v) agar.

Assays of GCN4-lacZ Reporter Expression—Standard methods for measuring the β-galactosidase activity for strains bearing *GCN4-lacZ* fusions were used (21). β-Galactosidase levels are expressed as nanomoles of *o*-nitrophenol β-D-galactopyranoside hydrolyzed per min/μg of total protein.

Western Blot Analysis—Protein extracts were electrophoresed under reducing conditions on SDS-polyacrylamide minigels and electroblotted onto polyvinylidene difluoride membrane (Amersham Biosciences). Blots were probed using anti-eIF2α and anti-phospho-eIF2α antibodies as described previously (22). TAP-tagged proteins were detected by Western blot analysis using peroxidase·anti-peroxidase complex (Sigma).

Analysis of Protein Synthesis—The rate of protein synthesis was measured in exponential phase cells treated with H_2O_2 for 15 min and pulse-labeled for the last 5 min with 85 μM L-[^{35}S]cysteine/methionine (18). TAP-tagged proteins were immunoprecipitated using IgG-Sepharose beads as described previously (23).

For the analysis of ribosome distribution on sucrose density gradients, yeast cultures were grown to exponential phase and treated with H_2O_2 as appropriate. Extracts were prepared and analyzed as described previously (24). Monosome and polysome peaks were quantified using NIH Image J software.

For transit time measurements, cells were pulsed-labeled with 85 μM L-[^{35}S]cysteine/methionine, and aliquots were taken at regular intervals. Extracts were prepared in 200 μg/ml cycloheximide, and radioactive incorporation was measured in the total (completed and unfinished proteins) and completed protein fractions by liquid scintillation counting (25). Transit times were determined by comparing the incorporation of radioactive amino acids into total proteins and completed proteins released from ribosomes.

Polysome Analysis and RNA Preparation—Cell extracts were prepared as described previously (12). Briefly, 60 A_{260} units were layered onto 35-ml 15–50% sucrose gradients and sedimented via centrifugation at 16,900 rpm for 13 h in a Beckman ultracentrifuge. Gradients were collected, and RNA quality was assessed using a 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). For comparison of RNA levels, fractions 4–8 and 11–15 from the gradient were pooled together for monosomal and polysomal RNA samples, respectively (see Fig. 4*B*). Thus, we focused on only the most strongly and poorly translated mRNAs, omitting some mRNAs that are located in the dimer region of the gradient. Total RNA samples were prepared according standard protocols (www.cogeme.man.ac.uk/Facilities/TRF%20Protocols.htm). RNA analysis by real-time reverse transcription (RT)-PCR was carried out using the MyiQ single-color real-time PCR detection system and iQ SYBR Green Supermix (Bio-Rad).

Affymetrix GeneChip Expression Microarray Analysis—Microarray experiments were performed using the GeneChip yeast genome S98 oligonucleotide array (Affymetrix) according to the manufacturer's instructions (www.affymetrix.com/support/technical/manuals.affx). Approximately 10 μg of polysomal, monosomal, or total RNA was processed into biotinylated cRNA according to the Affymetrix protocols. 15 μg of biotinylated cRNA targets was fragmented and hybridized to the arrays at 45 °C for 16 h. The arrays were then processed using the Affymetrix EukGE-WS2 fluidics protocol (Version 4_450) and stained with R-phycoerythrin conjugated to streptavidin (Molecular Probes). Microarray images were acquired using GeneChip Scanner 2500 (Affymetrix) and Microarray Suite Version 5.0 software. For consistency with previous studies (12), we used robust multi-array analysis normalization to normalize the array data as well as further analysis using the Affymetrix library of procedures (Affy Version 1.5.8) in Bioconductor (Version 1.5, www.bioconductor.org) within R (Version 2.0.1, www.r-project.com). Robust multi-array analysis nor-

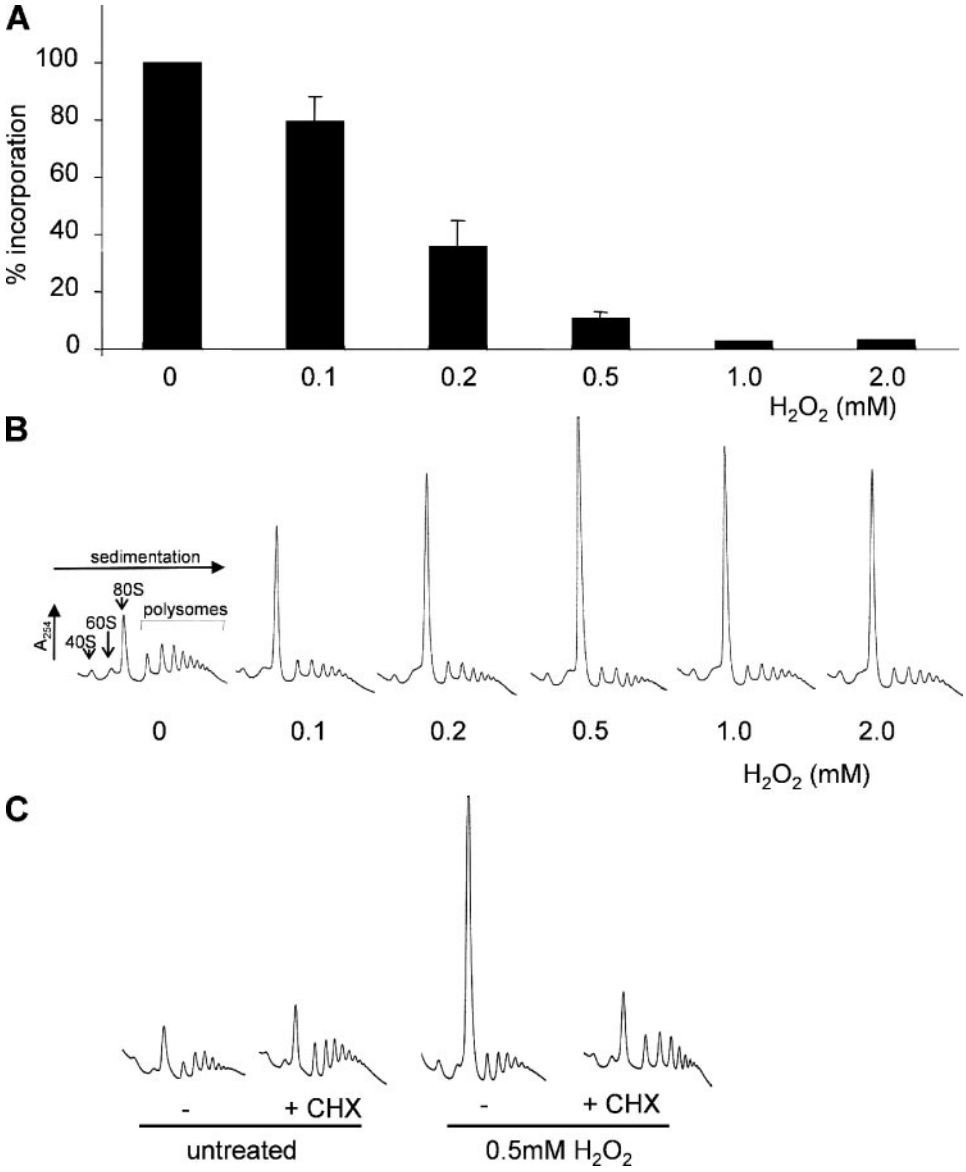

FIGURE 1. **Oxidative stress inhibits protein synthesis.** *A*, wild-type cells were grown to exponential phase in minimal synthetic dextrose medium, and protein synthesis was measured by pulse labeling with [^{35}S]cysteine/methionine for 5 min. Data are shown for untreated cultures (100%) and following treatment with H_2O_2 for 15 min. *B*, H_2O_2 treatment specifically inhibits translation initiation. Polyribosome traces are shown for the wild-type strain treated with the indicated concentrations of H_2O_2 for 15 min. The peaks containing the small ribosomal subunit (40 S), the large ribosomal subunit (60 S), and both subunits (80 S) are indicated by *arrows*. The polysome peaks generated by 2, 3, 4, 5, etc., 80 S ribosomes on a single mRNA are *bracketed*. *C*, peroxide does not inhibit translation by activating mRNA degradation. Polyribosome traces are shown for the wild-type strain treated with 0.5 mM H_2O_2 in the presence or absence of cycloheximide (*CHX*).

malization was observed to produce more robust treatment of replicates as seen from standard MA plots used to compare the biological replicate samples (data not shown). Pooled polysomal and monosomal fractions generated very modest dispersal, demonstrating that the procedure we have developed is highly reproducible and fulfills all of the standard criteria generally applicable to microarray data (26). The data sets are publicly available at ArrayExpress (accession number E-MEXP-526).

RESULTS

Hydrogen Peroxide Inhibits Protein Synthesis—Cells were treated with H_2O_2 for 15 min, and the rate of protein synthesis was measured during the final 5 min by the incorporation of [^{35}S]cysteine/methionine. H_2O_2 caused a dose-dependent inhibition of protein synthesis with maximal inhibition observed at concentrations >1.0 mM (Fig. 1*A*).

The inhibition of protein synthesis caused by H_2O_2 prompted us to analyze translational activity by examining the distribution of polysomes. Polysomes are ribosomes that are actively translating mRNAs. They can be separated on sucrose density gradients and quantified by measuring absorbance at 254 nm. Extracts prepared from the untreated strain exhibited normal profiles, including peaks corresponding to 40 S and 60 S ribosomal subunits, monosomes (80 S ribosomes), and polysomes (Fig. 1*B*). There was a dramatic shift of ribosomes from the polysomal region into the monosome or 80 S peak following treatment with H_2O_2. The accumulation of ribosomes in the 80 S peak of a sucrose gradient is indicative of decreased translation initiation. Polysome profiles from cells treated with cycloheximide during the peroxide treatment were analyzed to ensure that this effect did not arise because of peroxide activating global mRNA degradation in yeast. Incubation with cycloheximide was found to preserve polysomes during peroxide treatment, ruling out any effect on mRNA degradation (Fig. 1*C*).

Oxidative Stress Induces Gcn2-dependent eIF2α Phosphorylation—One of the best characterized translational regulatory pathways in yeast involves phosphorylation at Ser^{51} of the eIF2α subunit by the Gcn2 kinase. Gcn2 is activated in response to amino acid starvation and ultimately brings about an inhibition of translation initiation (10). To test whether the H_2O_2-dependent translation inhibition relies upon this pathway, we examined eIF2α phosphorylation by immunoblot analysis using anti-phosphorylated eIF2α antibody. Phosphorylation of eIF2α was observed in response to all concentrations and was maximal at 0.5 mM H_2O_2 (Fig. 2*A*), corresponding with maximal polysome decrease (Fig. 1*B*).

Deletion of *GCN2* abrogates phosphorylation of eIF2α and results in translational resistance to amino acid starvation (10). Similarly, no inhibition of translation initiation was observed in

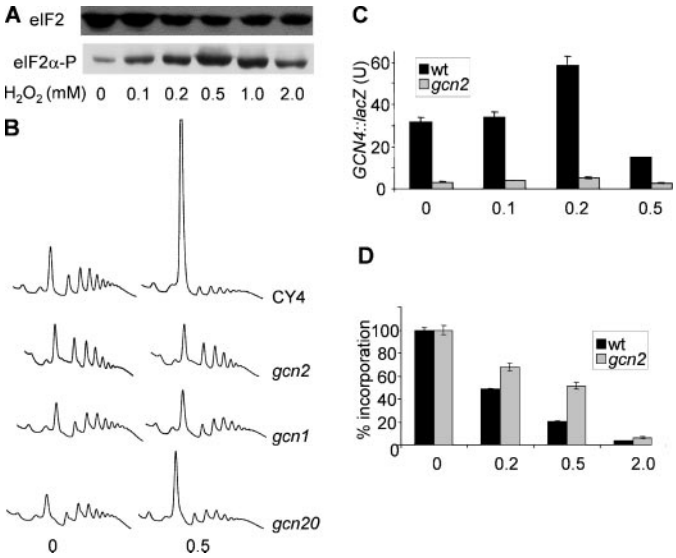

FIGURE 2. **Role of eIF2α phosphorylation in response to oxidative stress.** *A*, shown are the results from Western blot analysis of eIF2α and phospho-eIF2α (*eIF2α-P*). The wild-type strain was grown to exponential phase in minimal synthetic dextrose medium and treated with the indicated concentrations of H_2O_2 for 15 min. *B*, polysome traces are shown for the wild-type, *gcn2*, *gcn1*, and *gcn20* mutant strains following treatment with 0.5 mM H_2O_2 for 15 min. *C*, *GCN4-lacZ* expression was measured in the wild-type (*wt*) and *gcn2* mutant strains following treatment with the indicated concentrations (millimolar) of H_2O_2 for 2 h. The values shown are the means of at least three independent determinations. *D*, wild-type and *gcn2* mutant cells were grown to exponential phase, and protein synthesis was measured as described in the legend to Fig. 1*A*.

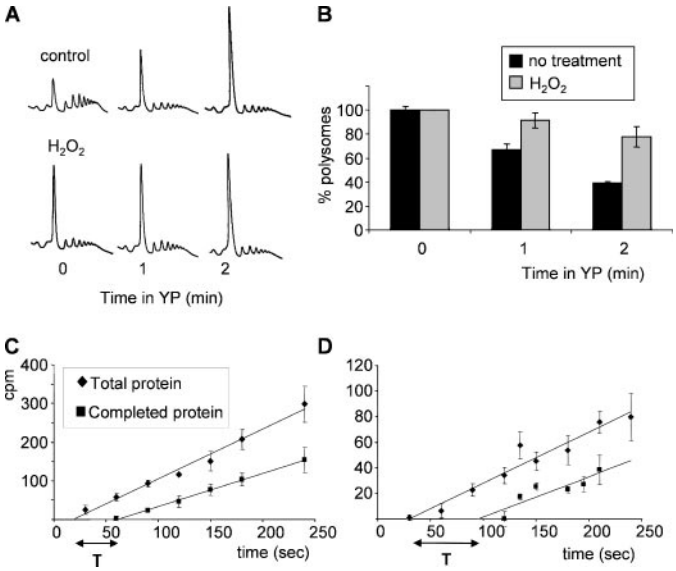

FIGURE 3. **Hydrogen peroxide stress inhibits ribosomal transit.** *A*, yeast cultures were grown to exponential phase in YEPD medium and treated with 2 mM H_2O_2 for 15 min. Cells were washed and resuspended in medium lacking glucose (*YP*) to inhibit translation initiation. Following incubation at 30 °C for 1 or 2 min, polysome extracts were prepared to analyze ribosomal runoff. *B*, the ribosomal runoff shown in *A* was quantified. The values shown are the means of three independent determinations. Polysome maintenance is expressed as the percentage of total ribosomes present as polysomes. *C* and *D*, the average ribosomal transit time was estimated. The incorporation of [^{35}S]cysteine/methionine into total protein and completed protein is shown for unstressed (*C*) and H_2O_2-treated (*D*) *gcn2* mutant cells. Stressed cells were treated with 0.5 mM H_2O_2, and the values shown are the means of at least two to seven determinations. Linear lines were drawn and extrapolated to the *abscissa* to determine the half-average transit time (*T*) as described previously (25).

a *gcn2* mutant in response to oxidative stress because loss of *GCN2* prevented the accumulation of monosomes in response to H_2O_2 (Fig. 2*B*). However, polysomes were maintained in the *gcn2* mutant following H_2O_2 treatment compared with the wild-type strain, indicating an effect at the post-initiation phase of translation (see below). Inhibition of translation initiation was also abrogated in a strain containing a mutant form of eIF2α lacking Ser^{51} that is the target of phosphorylation (data not shown). The Gcn1·Gcn20 complex binds to Gcn2 and is thought to mediate the activation of Gcn2 by uncharged tRNA. The inhibition of translation initiation promoted by H_2O_2 stress was reduced by loss of *GCN1* or *GCN20*, indicating that the activation of Gcn2 by H_2O_2 is liable to operate via a mechanism similar to amino acid starvation (Fig. 2*B*).

Phosphorylation of eIF2α causes a global inhibition of protein synthesis as well as gene-specific translational activation of *GCN4*, which encodes a transcriptional activator protein (27). *GCN4* expression has been widely measured using a *GCN4-lacZ* reporter containing the *GCN4* promoter and 5′-untranslated region driving expression of the *lacZ* gene. This reporter provides a convenient means to assess Gcn2-dependent inhibition of eIF2B activity mediated by eIF2α phosphorylation. When expression from this *GCN4-lacZ* reporter was measured following H_2O_2 treatment for 2 h, a modest 2-fold induction was seen in response to 0.2 mM H_2O_2 (Fig. 2*C*). This induction was dependent on Gcn2 because there was no induction in a *gcn2* mutant. Surprisingly, *GCN4* expression was inhibited at higher concentrations of H_2O_2 (Fig. 2*C*) despite the finding that maximal phosphorylation of eIF2α was at 0.5 mM H_2O_2 (Fig. 2*A*). To investigate the reason for this inhibition, we measured the rate of protein synthesis in a *gcn2* mutant that could not inhibit translation initiation in response to H_2O_2 (Fig. 2*B*) and found that protein synthesis was still inhibited in the *gcn2* mutant, albeit to a lesser extent (Fig. 2*D*). Taken together, these data indicate that H_2O_2 exposure causes a global inhibition of protein synthesis via Gcn2-dependent inhibition of translation initiation as well as via an additional mechanism that does not affect initiation.

Oxidative Stress Inhibits Ribosomal Transit—Polysome analysis showed that H_2O_2 caused an inhibition of translation initiation (Fig. 1*A*). The extracts for these profiles were prepared in the presence of the translation elongation inhibitor cycloheximide to prevent continued elongation and ribosomal runoff. An inhibition of ribosomal transit (elongation plus termination of polypeptide chains) should be seen as a preservation of polysomes when extracts are prepared in the absence of cycloheximide. However, even a diminished rate of ribosomal runoff is extremely rapid and difficult to detect (28). We therefore took the approach of inhibiting translation initiation by shifting cells to medium lacking glucose following the treatment with H_2O_2. Glucose withdrawal from the growth medium results in a rapid (<1 min) inhibition of translation initiation (24), and we reasoned that this would allow us to better detect any effect on ribosomal transit without the added complication of *de novo* translation initiation. Cells were either left untreated or treated with 2 mM H_2O_2 prior to the switch to medium lacking glucose. Glucose withdrawal resulted in a rapid inhibition of translation initiation as expected (Fig. 3*A*), and ribosomal runoff occurred

in control experiments, resulting in a loss of ~60% of the polysomes within 2 min (Fig. 3*B*). In contrast, polysomes were maintained following the H_2O_2 treatment, and ~80% of the polysomes were maintained for 2 min.

Ribsosomal transit times can be measured by comparing the incorporation of [^{35}S]cysteine/methionine into total protein and completed polypeptide chains released from ribosomes (25). Using this technique, we determined that the average mRNA transit time in a *gcn2* mutant is ~86 s (Fig. 3*C*). H_2O_2 treatment (0.5 mM) increased the transit time by ~50% to ~131 s (Fig. 3*D*). Because a *gcn2* mutant could not inhibit translation initiation (Fig. 2), these data confirm that there is an inhibition of translation elongation or termination in response to H_2O_2.

Global Gene Expression Profiling Reveals That Certain mRNAs Are Translationally Maintained following Oxidative Stress—The use of expression profiling techniques such as microarray analyses can be used to analyze protein synthesis (29). We used this technology to identify mRNAs that are translationally regulated in response to oxidative stress conditions. Cell extracts were prepared from yeast cells treated with 0.2 or 2 mM H_2O_2 for 15 min. These concentrations were chosen because they had a similar effect on translation initiation (Fig. 1*B*) but inhibited protein synthesis to different extents (Fig. 1*A*). The 15-min treatment with 0.2 mM H_2O_2 reduced cell viability by ~20% compared with 65% loss of viability upon treatment with 2.0 mM H_2O_2. The 2.0 mM treatment also significantly slowed the growth of cells compared with the 0.2 mM treatment (Fig. 4*A*). Polysomal gradients from the selected stress conditions were separated into fractions (as described below), and in addition, total RNA samples (total stressed or total control) were prepared from stressed and control yeast cells to quantify standard transcript level changes. The resulting RNA samples were processed into cRNA and hybridized to Affymetrix microarrays. The analysis was performed in duplicate, and the data were processed and compared using the bioinformatics analyses described under "Experimental Procedures."

Expression profiling studies generally compare the ratio of mRNAs in polysomal (P) and monosomal (M) fractions between stressed (S) and control (C) yeast cells. For example, when translation is inhibited at the level of initiation, the association of mRNAs with the polysomal *versus* non-polysomal fraction can be used as a marker that represents the translational activity of specific mRNAs (29). We recently defined the ratio PS/MS:PC/MC as the "translational state" of an individual mRNA and used it to identify mRNAs that are translationally maintained in response to the eIF2B-targeting stresses, amino acid starvation and fusel alcohol addition (12). However, when translation is inhibited by attenuating elongating ribosomes, as is the case for H_2O_2, increased polysome association cannot be used as a marker of the translational state of mRNAs. It was therefore necessary to develop a novel strategy to identify candidate mRNAs that are translationally regulated following H_2O_2 stress. Specifically, we compared the mRNAs in monosomal and polysomal fractions during stress conditions (PS+MS) with the mRNAs in monosomal and polysomal fractions during control conditions (PC+MC) (Fig. 4*B*). We reasoned that any mRNAs that can overcome the initiation block would have more ribosomes bound during stress conditions, resulting in an

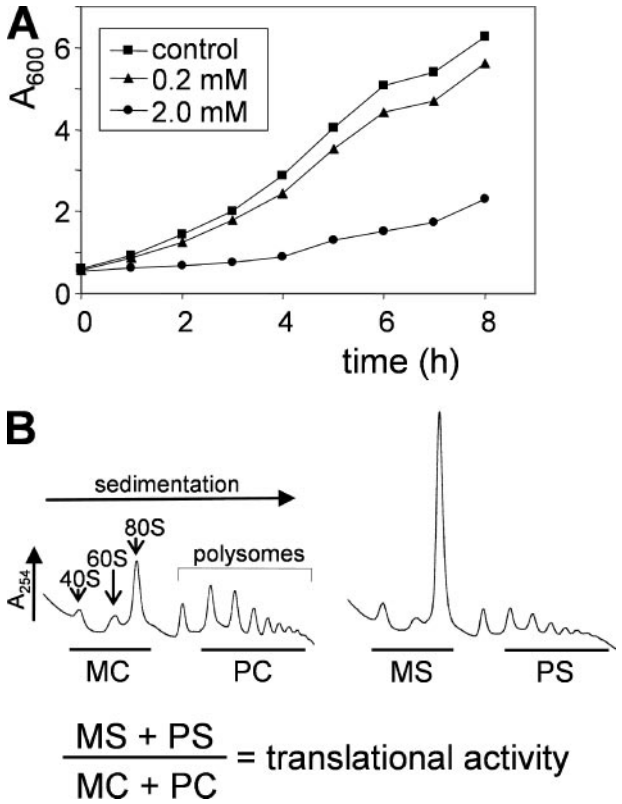

FIGURE 4. **Analysis of the effect of oxidative stress on growth and translation.** *A*, H_2O_2 treatment inhibits growth. Exponential phase wild-type cells were treated with 0.2 or 2.0 mM H_2O_2 for 15 min, washed, and resuspended in fresh synthetic dextrose medium. Growth was monitored by measuring absorbance at 600 nm. *B*, strategy of polysome/microarray analysis. mRNAs from unstressed and stressed cells were fractionated on sucrose density gradients. Pooled microsomal and polysomal fractions containing stressed mRNAs (*MS + PS*) and control mRNAs (*MC + PC*) were used for microarray analysis. Translational activity is defined as the ratio PS+MS:PC+MC.

increase in PS+MS compared with PC+MC. These values were plotted on a scatter plot for stressed (*y* axis) and unstressed (*x* axis) cells (Fig. 5, *A* and *B*), where the intersection generates the change in translational activity for the mRNAs (PS+MS:PC+MC). This analysis facilitates the identification of those mRNAs that can overcome the block in translation initiation because they contain more ribosomes (both monosomal and polysomal) following the stress treatment. Within this population of mRNAs, we reasoned that it is possible to identify those mRNAs that are also somewhat resistant to the ribosomal transit block because they will contain more polysomes bound during stress conditions compared with control conditions, resulting in an increase in the PS:PC ratio (Fig. 5, *A* and *B*, *red*). Thus, we were able to identify those mRNAs that can overcome the inhibition of translation initiation (using the PS+MS:PC+MC ratio) as well as the mRNAs within this population that can overcome the inhibition of ribosomal transit to accumulate an increase in polyribosomes (using the PS:PC ratio).

Interestingly, the two stress conditions gave very different translational profiles. Using a 2-fold cutoff value for the change in translational activity (PS+MS:PC+MC), 231 mRNAs (130 up and 101 down) and 230 mRNAs (98 up and 132 down) were significantly altered following treatment with 0.2 or 2.0 mM H_2O_2, respectively (Fig. 5, *A* and *B*). The majority of these

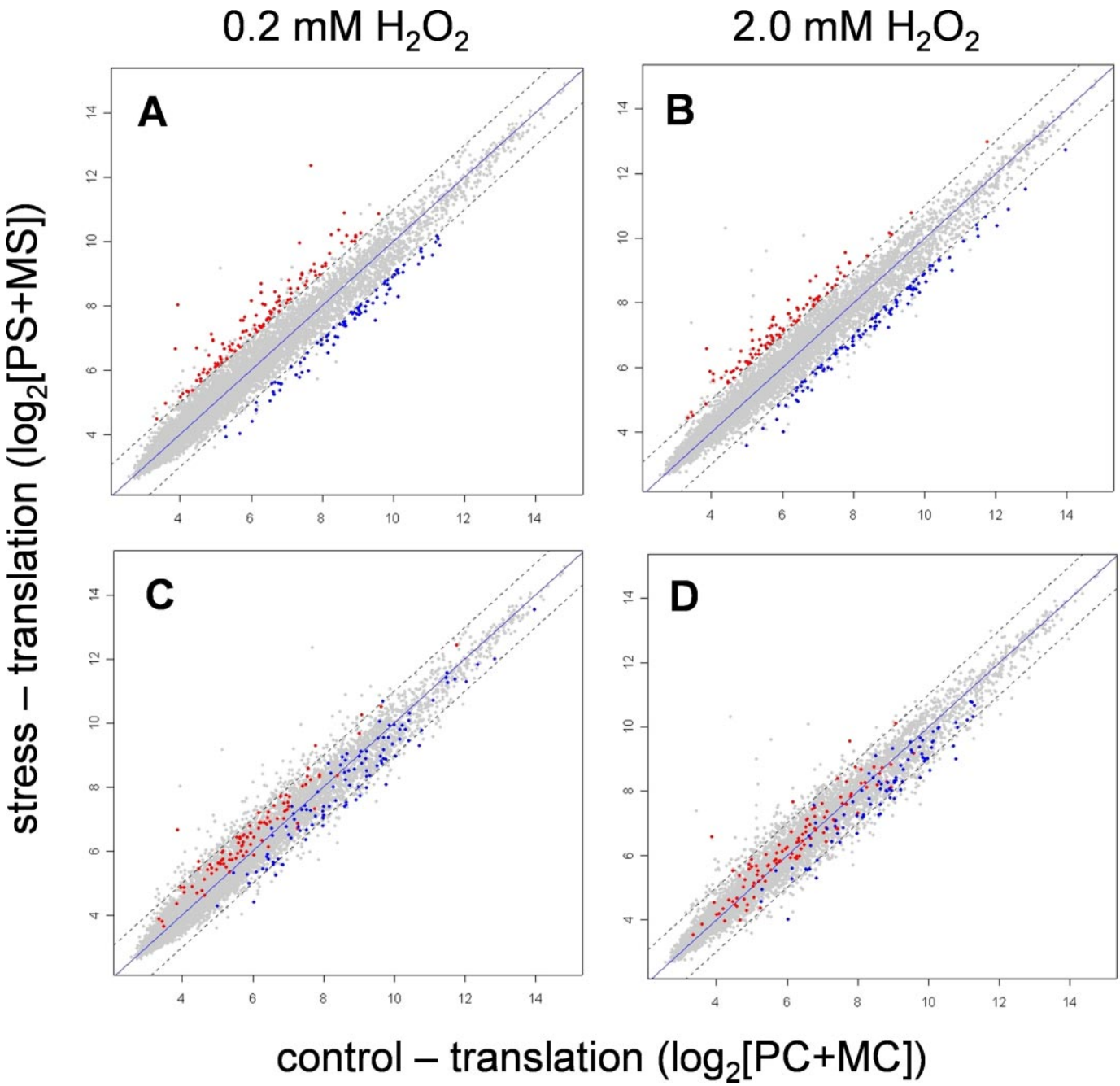

FIGURE 5. **Translational control in response to oxidative stress.** *A* and *B*, graphical representations of the translational microarray data for 0.2 and 2.0 mM H_2O_2, respectively. The mRNAs in monosomal and polysomal fractions during stress conditions (log_2(PS+MS)) have been plotted against the mRNAs in monosomal and polysomal fractions during control conditions (log_2(PC+MC)). Those mRNA data points falling above or below a 2.0-fold cutoff are classified as overcoming the block in translation initiation. mRNAs that were also resistant or sensitive to the ribosomal transit block (log_2(PS:PC)) have been colored *red* and *blue*, respectively. *C* and *D*, the plots are the same as in *A* and *B*, but only the translationally regulated mRNAs (up-regulated in *red* and down-regulated in *blue*) for the 2.0 mM H_2O_2 treatment have been highlighted on the 0.2 mM H_2O_2 plot (*C*) and vice versa (*D*).

mRNAs were also somewhat resistant to the ribosomal transit block because they contained more polysomes bound during stress conditions compared with control conditions (0.2 mM, 199 mRNAs; and 2.0 mM, 198 mRNAs) (Fig. 5, *A* and *B*). To assess whether a similar set of mRNAs are altered following each stress condition, we highlighted those mRNAs that changed following the 2.0 mM treatment on the 0.2 mM plot (Fig. 5*C*), and conversely, we highlighted the 0.2 mM regulated mRNAs on the 2.0 mM plot (Fig. 5*D*). These plots show that the mRNAs up- or down-regulated were different for each stress condition.

To assess whether the response to these stress conditions is coordinated in terms of transcript level and translational activity, we plotted the change in translational activity (PS+MS: PC+MC) against the change in transcript level for each stress condition (Fig. 6). mRNAs that were translationally up-regulated and also changed at the transcript level and mRNAs that were translationally down-regulated and also changed at the

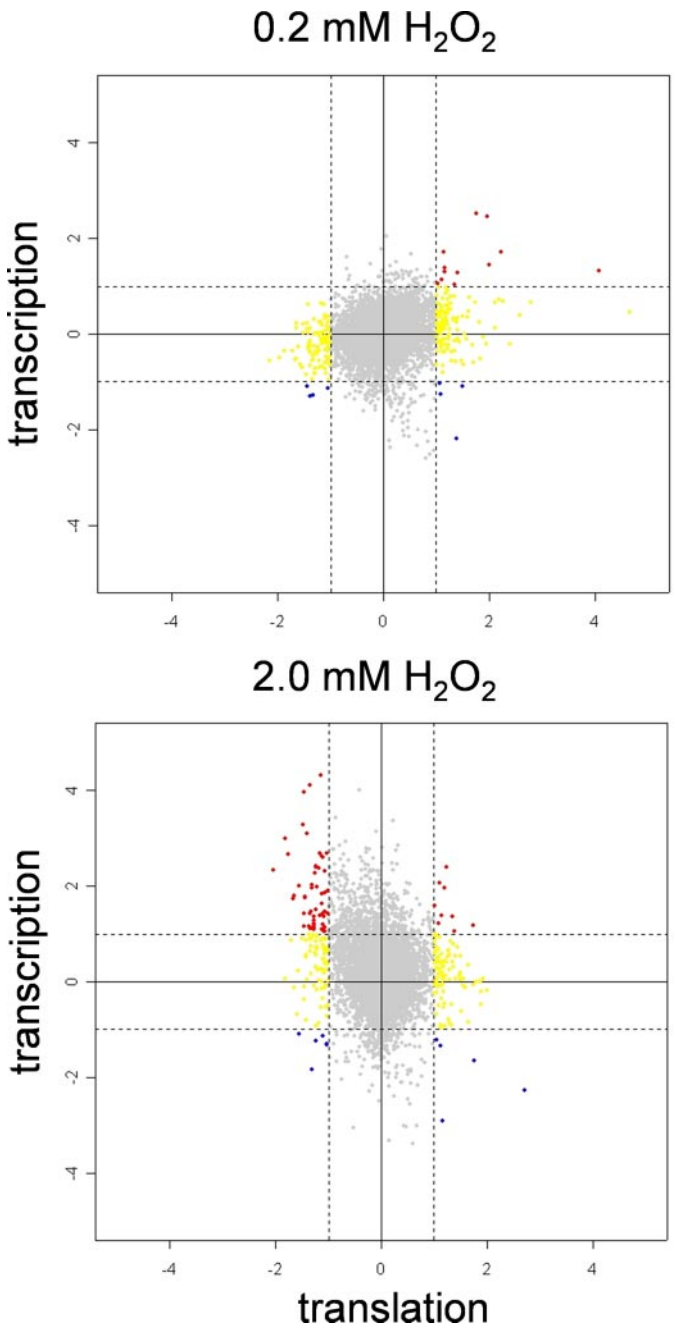

FIGURE 6. **Comparison of alterations in transcript level and translation in response to H_2O_2 stress.** Shown are graphical plots comparing the transcript level ($\log_2$(TS:TC)) with the change in translational state ($\log_2$(PS+MS) − $\log_2$(PC+MC)) after 0.2 mM (*top*) and 2.0 mM (*bottom*) H_2O_2 treatment. Cutoff values of 2.0 for the change in transcript level and translational state are depicted as *dashed lines*. Translationally regulated mRNAs that were not transcriptionally regulated are colored *yellow*. Translationally up-regulated mRNAs that were also regulated at the transcript level are colored *red*, whereas translationally down-regulated mRNAs that were also regulated at the transcript level are colored *blue*.

transcript level are depicted, along with other translationally regulated mRNAs (Fig. 6, *red*, *blue*, and *yellow*, respectively). This analysis did not reveal any strong correlation between changes in transcript level and the translational response to H_2O_2. This may be because of the relatively short exposure times used in these experiments. Of the genes that were translationally up-regulated, just 10% (13 of 130) and 11% (11 of 98) were also induced at the transcriptional level by the 0.2 and 2 mM H_2O_2 treatments, respectively (supplemental Tables I and II). A co-regulation of the transcript level and translation has been noted previously following heat shock, rapamycin treatment, and amino acid starvation of yeast and has been termed "potentiation" (12, 30). In contrast, no potentiation was observed here or for butanol stress, suggesting that co-regulation of the transcript level and translational activity is a stress-specific phenomenon (12).

We confirmed the microarray results using real-time RT-PCR analysis for a range of mRNAs in terms of both the overall abundance of the mRNA and the change in abundance across polysomal gradients (Fig. 7*A*). The Affymetrix system used for the microarray analysis requires that equal cRNA concentrations are used for each RNA hybridization. This effectively normalizes any changes in monosomal or polysomal fractions between stressed and unstressed samples for most mRNAs even though global translation has been inhibited by the stress. This is best illustrated by *ACT1* mRNAs, which were unaffected by the 0.2 or 2.0 mM treatment according to the microarray analysis, whereas the RT-PCR analysis showed that less *ACT1* mRNA was associated with monosomes and polysomes following stress conditions consistent with the global inhibition of translation (Fig. 7*A*). Nevertheless, RT-PCR analysis could be used to confirm the general trends that were detected from the microarray data. For example, RT-PCR analysis confirmed that the *HSP30* mRNA was increased in abundance in monosomal and polysomal fractions following both stress conditions, that *OYE3* and *SRX1* mRNAs were increased in monosomal and polysomal fractions following the 0.2 mM H_2O_2 treatment, and that the *YCF1* mRNA was increased in the polysomal fraction following the 0.2 mM H_2O_2 treatment.

Our data show that mRNAs were differentially associated with polyribosomes in response to oxidative stress conditions. To begin to address whether this results in increased protein production, we analyzed protein synthesis and levels for a selected group of proteins that are available with a TAP tag. To analyze protein synthesis, cells were treated with 0.2 or 2.0 mM H_2O_2 for 15 min, and proteins were pulse-labeled with [^{35}S]cysteine/methionine during the final 5 min of treatment. SDS-PAGE analysis confirmed a global inhibition of protein synthesis (Fig. 7*B*). Decreased protein production was observed following both peroxide treatments but was more pronounced for the 2 mM H_2O_2 treatment, with relatively few proteins detected.

The microarray analysis indicated that the *HSP30* mRNA increased in abundance in monosomal and polysomal fractions following both stress conditions. No Hsp30 was detected by Western blot analysis during normal growth conditions (Fig. 7*C*). In contrast, Hsp30 was dramatically increased following the 0.2 mM treatment. Similarly, microarray analysis indicated an increase in the translational activity of *TRR1* and *SOD2* in response to 0.2 mM H_2O_2. In agreement with this observation, immunoprecipitation of pulse-labeled proteins revealed increased synthesis of both proteins at 0.2 mM H_2O_2. In the case of Sod2, increased protein levels were also detected by Western blot analysis, whereas no changes in the high basal levels of Trr1 were observed over this relatively short treatment period (Fig. 7*C*). From the microarray analysis, *HSP30* and *TRR1* belong to

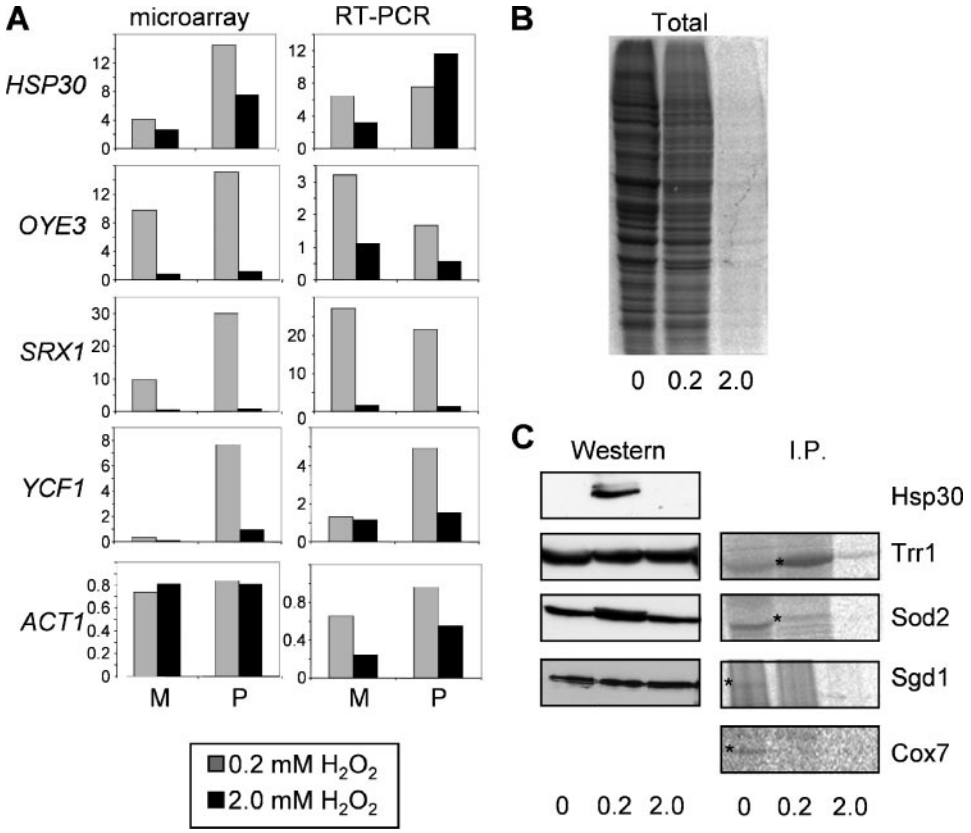

FIGURE 7. **Confirmation of microarray data.** *A*, the microarray data were confirmed by RT-PCR analysis for selected genes. Microarray and RT-PCR data are shown for *HSP30*, *OYE3*, *SRX1*, *YCF1*, and *ACT1* mRNAs across monosomal (*M*) and polysomal (*P*) fractions from 0.2 (*gray bars*) and 2.0 (*black bars*) mM H_2O_2-stressed cells. The values are expressed relative to untreated control cultures and are the means of duplicate (array) and triplicate (RT-PCR) determinations. *B*, shown are the results from the analysis of protein synthesis in cells exposed to 0.2 or 2.0 mM H_2O_2 for 15 min. Cultures were pulse-labeled with [^{35}S]cysteine/methionine during the final 5 min of the H_2O_2 treatment, and proteins were analyzed by SDS-PAGE. *C*, total protein levels were analyzed by Western blot analysis of TAP-tagged Hsp30, Trr1, Sod2, Sgd1, and Cox7 following a 15-min treatment with 0.2 or 2.0 mM H_2O_2. No signal could be detected for the Cox7 protein. Proteins were pulse-labeled with [^{35}S]cysteine/methionine, and TAP-tagged proteins were immunoprecipitated (*I.P.*) using IgG-Sepharose beads. It was not possible to identify Hsp30 because of the presence of a number of nonspecific proteins in the immunoprecipitate.

the class of mRNAs that were somewhat resistant to the block in ribosomal transit (PS:PC ratio >2), whereas the *SOD2* mRNA did not show any increase in polyribosomes following the H_2O_2 treatment. Thus, both classes of mRNA result in increased protein production. Microarray analysis indicated that the translational activity of *HSP30*, *SGD1*, and *COX7* mRNAs was increased in response to 2.0 mM H_2O_2. However, no increase in protein production was observed for these proteins as analyzed by immunoprecipitation of pulse-labeled proteins (Fig. 7*B*). Taken together, these data indicate that low peroxide (0.2 mM) results in increased protein production, whereas high peroxide (2.0 mM) increases polyribosome association with certain mRNAs but does not necessarily result in increased protein production.

Functional Classification of mRNAs Regulated by H_2O_2—The data were analyzed in terms of the precise function of the regulated genes and the likely impact that changes in translational activity would have on cellular physiology. Gene products were grouped into functional categories according to the MIPS Functional Catalogue Database (mips.gsf.de/genre/proj/yeast/index.jsp) and the *Saccharomyces* Genome Database GO Term Mapper (db.yeastgenome.org/cgi-bin/SGD/GO/goTermMapper). Genes that were regulated at the translational level relative to the global trend are included in these categories (Fig. 8 and supplemental Tables I–IV).

The two stress conditions showed distinct patterns of regulation. The low peroxide (0.2 mM) condition induced several antioxidants, as might be expected (supplemental Table I). These included a cytosolic catalase (CTT1) and an atypical 2-Cys peroxiredoxin (GPX2), which can reduce H_2O_2 directly (31–33); thioredoxin reductase (*TRR1*), which provides the reducing power for peroxiredoxins; sulfiredoxin (*SRX1*), which can reduce cysteinesulfinic acid residues that are formed in peroxiredoxins following oxidative stress (34); and a glutathione transferase (*GTT2*) and two GS-X pumps (*YCF1* and *YBT1*), which form part of the glutathione conjugation/removal system of cells that is active against a broad range of toxic substrates (35). The most prominent classes of genes up-regulated by 0.2 mM H_2O_2 included genes encoding various cellular transporters. Strikingly, nine ABC (ATP-binding cassette) transporters from a predicted 31 proteins belonging to the ubiquitous ABC superfamily were identified (36, 37). These included seven "full-size" ABC transporters (from a predicted 16), which can confer considerable multidrug resistance. A number of iron regulon genes were translationally up-regulated, including those involved in cell-surface iron uptake (*FET3*, *FTR1*, *FRE1*, and *FRE2*), iron transport across the vacuole membrane (*FET5* and *FTH1*), and a mitochondrial iron transporter (*MRS4*). Iron is an essential nutrient that is largely thought to be regulated at the level of transcription via the iron-responsive transcriptional activators Aft1 and Aft2 (38, 39). Increased iron uptake during oxidative stress is somewhat surprising given that it can potentially lead to the generation of the hydroxyl radical via the Fenton reaction (40). However, these data may indicate a requirement for restoration of iron homeostasis following oxidative stress. Cellular iron is found largely complexed in cells, *e.g.* in iron-sulfur clusters. Oxidation of these clusters causes release of the iron, resulting in enzyme inactivation (41). A number of metabolic genes were up- or down-regulated following treatment with 0.2 mM H_2O_2 (supplemental Tables I and II). These included genes affecting carbon, amino acid, nitrogen, lipid, and energy metabolism, indicating that significant metabolic reconfiguration is required following oxidative stress.

The high peroxide (2.0 mM) condition resulted in significant up-regulation of genes involved in ribosome biogenesis and

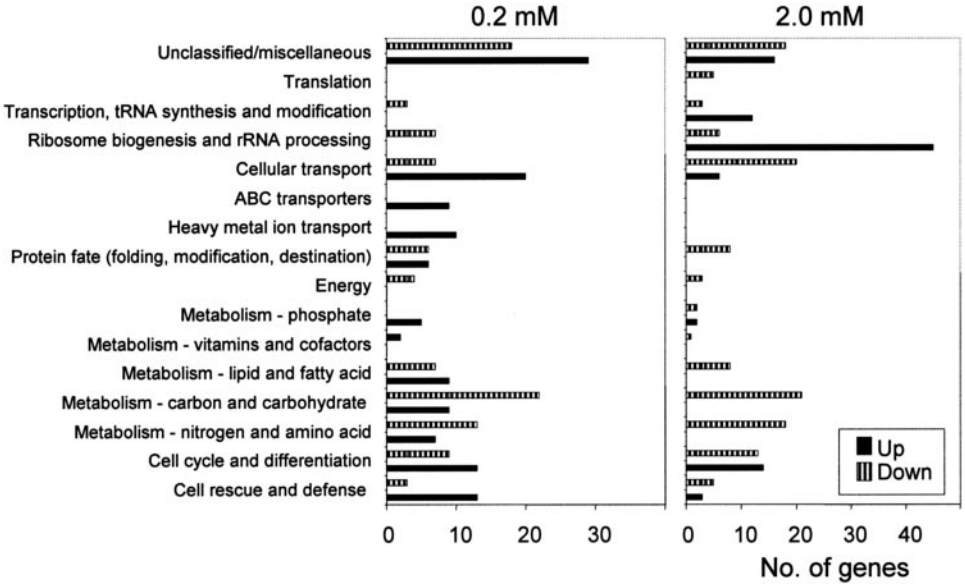

FIGURE 8. **Functional classification of the regulated mRNAs.** Shown is a summary of the functional classification in supplemental Tables I–IV.

rRNA processing (45 of 98 genes induced) (supplemental Table III). This is in contrast to the large number of similar genes that are transcriptionally repressed as part of the environmental stress response (42). Transcriptional co-regulation of genes involved in ribosome biogenesis has been linked to cell proliferation, and ribosome synthesis may serve as a measure for cell cycle progression (43, 44). However, very few of the genes in our data set showed changes in transcript levels following the short H_2O_2 treatment time used in this study (5 of 45 genes) (supplemental Table III). Additionally, a set of 236 genes that are transcriptionally co-regulated by the Spf1 transcription factor has been defined and termed the *Ribi* regulon (44). There is only a partial overlap between the *Ribi* regulon and the mRNAs that were translationally induced by 2.0 mM H_2O_2 (48 genes) (data not shown). Rather than indicating a link between ribosome production and cell proliferation, these data may indicate that there is a requirement to replace ribosomal proteins and rRNA that become damaged by oxidative stress. In contrast to the low peroxide treatment, there was no significant up-regulation of metabolic genes. However, many genes affecting several aspects of metabolism were down-regulated in response to 2.0 mM H_2O_2, in agreement with the idea that metabolic reconfiguration is required in response to oxidative stress (supplemental Table IV). Surprisingly, a significant number of the genes that were translationally down-regulated in response to 2.0 mM H_2O_2 were increased at the transcriptional level (59 of 132 mRNAs) (Fig. 6 and supplemental Table IV). These data indicate that certain genes are increased at the transcriptional level in response to H_2O_2 but remain poorly translated. These mRNAs could represent an mRNA store that would become rapidly activated following relief of the stress condition.

DISCUSSION

Global inhibition of protein synthesis is widely recognized as a response of biological systems to stress conditions. However, it is becoming increasingly recognized that not all translation is inhibited and that translational control of specific mRNAs is required for survival during growth under stress conditions (12, 30). The complement of proteins expressed in individual cells (or the proteome) is fundamental to their nature and diversity because proteins catalyze most of the reactions in cells and also serve numerous structural and regulatory roles. Control of protein levels via translational regulation offers a significant advantage to the cell because of the immediacy of the regulatory effect. Regulation of protein levels is therefore one of the key aspects in determining the fate of cells during stress conditions.

Eukaryotic translation initiation is a complex highly regulated process involving >30 polypeptide factors interacting with ribosomal subunits, Met-$tRNA_i^{Met}$, and mRNAs (45). The initiation phase of protein synthesis is rate-limiting and is a target of extensive regulation. In mammalian cells, eIF2α is phosphorylated by PERK in response to oxidative stress as part of the integrated stress response (8). Yeast does not contain PERK, and hence, H_2O_2 stress has not previously been shown to inhibit protein synthesis.

Yeast Gcn2 phosphorylates eIF2α in response to nutrient starvation and sodium or rapamycin exposure. Depletion of amino acids leads to an accumulation of uncharged tRNA, which activates the Gcn2 protein kinase through its histidyl-tRNA synthase-related domain. The signals activating Gcn2 in response to rapamycin or NaCl are not well understood. Rapamycin appears to work by blocking Tor-mediated phosphorylation of Gcn2 at Ser^{577} (46). However, activation of Gcn2 by rapamycin and NaCl still requires the histidyl-tRNA synthase-related domain of Gcn2 as well as Gcn1 and Gcn20, which are thought to mediate the activation of Gcn2 by uncharged tRNA (47). Similarly, the inhibition of translation initiation in response to H_2O_2 requires Gcn1 and Gcn20. Oxidative stress may conceivably cause an accumulation of uncharged tRNA through a variety of mechanisms. Free amino acids and amino acids in proteins are highly susceptible to oxidation by ROS (48). Oxidized amino acids can be detected in yeast cells, and for example, oxidized phenylalanine (*m*- and *o*-Tyr) is elevated following exposure to concentrations of H_2O_2 (0.2–2 mM) (49) similar to those used in this study. Although the levels of oxidized amino acids detected are relatively low, representing about one modification/10^3 phenylalanine residues, the total oxidative load on the amino acid pool may trigger an amino acid starvation response. Alternatively, the proteins and nucleic acids that are required for tRNA aminoacylation may be susceptible to oxidation, resulting in an accumulation of uncharged tRNA and activation of Gcn2. For example, oxidative damage to RNAs, including tRNAs, has been implicated in the pathogenesis of Alzheimer disease (50, 51) and ROS can

affect the stability and activity of aminoacyl-tRNA synthetases (52).

We have previously determined the nature and extent of mRNAs that are translationally regulated in response to two stresses that lower eIF2B activity (12). Amino acid starvation leads to an accumulation of non-aminoacylated tRNAs and subsequent activation of Gcn2, whereas exposure to the fusel alcohol butanol inhibits eIF2B activity. Surprisingly, even though the stresses impact upon the same translation initiation factor (eIF2B), they have quite different outcomes in terms of the specific mRNAs that are translationally controlled. This creates a highly specific stress response that facilitates adaptation to the particular stress condition (12). We have suggested that, although these stresses both act via eIF2B, there must be other stress-specific modulatory inputs on the translational pathway that ultimately change which mRNAs are translationally selected following stress. The impact of oxidative stress on ribosomal initiation and transit is therefore particularly interesting because it indicates that control of the translational machinery at a stage subsequent to initiation can be used to modulate a proteomic output.

In contrast to amino acid starvation, protein synthesis is still inhibited in response to H_2O_2 in the absence of Gcn2. Decreased ribosomal runoff is observed following H_2O_2 stress, consistent with an inhibition of translation elongation or termination. Similarly, the average mRNA transit time is increased by ~50% in a *gcn2* mutant, confirming that H_2O_2 causes a post-initiation inhibition of protein synthesis. Regulation of mRNA expression levels by modulating translation elongation or termination is relatively poorly understood. It is known that cells can alter the bulk rate of protein synthesis in response to different growth conditions or hormones by changing the overall rates of elongation and/or termination (reviewed in Ref. 53). Similarly, oxidative stress in mammalian cells elicits a marked increase in eIF2 phosphorylation and oxidative modification, which is thought to contribute to an inhibition of translation (54, 55). Attenuating elongating ribosomes in response to stress conditions, as opposed to ribosomal initiation, offers the advantage that ribosomes remain bound to mRNAs and can rapidly resume protein synthesis once the stress is removed or detoxified. For an oxidative stress condition, it would also prevent continued protein synthesis during potentially error-prone conditions. There are very few characterized examples in which the expression of individual mRNAs is regulated via alteration of ribosomal transit times (reviewed in Ref. 56). One good example is provided by the tyrosine aminotransferase mRNA, in which the rate of ribosomal transit is increased 5-fold in response to dibutyryl cAMP (57). Our data indicate that the majority of mRNAs that are translationally up-regulated following H_2O_2 stress contain more ribosomes in polysomes following the stress condition. An obvious focus for future experimentation will be an investigation of how these mRNAs overcome this translation block.

The environmental stress response cluster encompasses ~900 genes that are transcriptionally activated or repressed by a large number of stress conditions, including ROS such as H_2O_2 (16). The genes that are transcriptionally induced as part of the environmental stress response encode products that are thought to protect against and/or detoxify the stress agent as well as repair the resulting cellular damage. However, our data indicate that relatively few (~15%) of the mRNAs that are translationally up-regulated in response to H_2O_2 show concomitant increases in transcript levels. Increased transcript levels in the absence of active translation may therefore provide a source of mRNAs that can become rapidly translated once the stress is removed. In this view, the mRNAs that are actively translated during exposure to H_2O_2 may provide the crucial functions that are required to detoxify and remove H_2O_2 and its metabolic products. It is therefore particularly interesting that the low peroxide treatment results in increased production of stress protective proteins, whereas the high peroxide treatment increases the number of ribosomes associated with certain mRNAs but does not result in increased protein production. The low peroxide treatment used in this study has previously been shown to promote an adaptive response whereby cells become resistant to a subsequent higher and more lethal treatment with H_2O_2 (58). Our data indicate that this may be explained by increased production of stress protective proteins, including antioxidants, at the low H_2O_2 (0.2 mM) treatment. In contrast, the high H_2O_2 (2.0 mM) treatment increases ribosome association on key mRNAs, which can then become rapidly translated once the oxidative stress is removed. In summary, our data indicate that the response to oxidative stress is complicated, requiring both translational and transcriptional reprogramming.

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