MATERIALS AND METHODS

Strains

Strains used in this study are shown in Table 1. The *yap1* deletion strain was constructed by homologous recombination using G418 resistance as a selectable marker. The *KanMX4* gene (Winzeler *et al*., 1999) was amplified by PCR introducing to each end of the PCR product 45 bp homology to the genomic sequence immediately flanking the *YAP1* open reading frame. Diploid cells transformed with the resulting PCR product and resistant to 0.5 mg/mL G418 were selected. Correct integration was verified by PCR using one primer internal to the integrated *KanMX* gene and one primer upstream of the *YAP1* start site. Diploids were sporulated, and G418 resistance segregated 2:2 in the dissected tetrads.

A DBY7286 derivative in which *MSN2* and *MSN4* had been deleted was received from J. DeRisi (*Mat**a** msn2::KanMX msn4::URA3 GAL2*).

Media and growth conditions

YP medium (Sherman, 1991) was used in most experiments, supplemented with 2% weight-to-volume glucose in most cases (YPD media). Cultures were grown at 30^{o}C unless otherwise noted and shaken at 250-300 rpm. The culture volume did not exceed 25% of the flask capacity.

Harvesting cells from liquid culture

In most cases, cells were grown to early log phase (OD_{600} 0.2 to 0.4), and an aliquot of cells was collected to serve as the time=0 reference. Cells were collected by centrifugation at 3,000 g for 3 to 7 minutes at room temperature. Each 50 mL cell pellet was resuspended in 3 to 10 mL of Lysis Buffer (10 mM Tris-Cl pH 7.4, 10 mM EDTA, 0.5% SDS), frozen in liquid nitrogen, and stored at -80^{o}C until RNA preparation.

Cell lysis and total RNA isolation

To each cell suspension, an equal volume of water-saturated phenol was added, and the emulsion was incubated at 65^{o}C for approximately 30 minutes with periodic

vortexing. Following extraction of the cell lysate, total RNA was precipitated from the aqueous phase by addition of 0.3 M sodium acetate pH 5.3 and either one volume isopropanol or 2.5 volume ethanol. RNA was recovered by centrifugation and resuspended in TE buffer at a final concentration of approximately 1 mg/mL.

mRNA isolation

Where noted, poly-adenylated RNA was purified using oligo-dT cellulose (Ambion). mRNA was precipitated with 0.3 M sodium acetate pH 5.3 and one volume isopropanol, recovered by centrifugation, and resuspended in TE at a final concentration of approximately 0.5 - 1 ?g/?L.

Probe preparation

Probes for the microarrays were prepared by labeling either total RNA or purified mRNA, as indicated for each experiment. Either 15 ?g of total RNA or 2 ?g mRNA was combined with 5 ?g oligo dT in a final volume of 15 ?L and incubated at 70°C for ten minutes. Following chilling on ice, the solution was mixed with 3 ?L of 2.5 mM Cy3- or Cy5-conjugated dUTP (Amersham), 3 ?l 0.1 M DTT, 6 ?l first strand buffer (Life Technologies), 0.5 ?l dNTPs (25 mM each of dATP, dCTP, dGTP, and 15 mM dTTP), and 2 ?l Superscript II (Life Technologies). The reaction was incubated at 42°C for two hours, and then diluted with 500 ?L TE buffer. The labeled cDNA was purified from unincorporated nucleotide by retention on a centricon-30 column (Ambion) until the volume was reduced to 3 to 10 ?L. Unless otherwise noted, in each experiment RNA isolated from the unstressed, asynchronous culture was labeled with Cy3-dUTP, and RNA from stressed samples were labeled with Cy5-dUTP.

Microarray hybridization, data acquisition, and image analysis

Microarrays were hybridized as previously described (DeRisi *et al*., 1997) and were scanned using a commercially-available scanning laser microscope (GenePix 4000) from Axon Instruments (Foster City, CA). Full details on using the GenePix 4000 can be obtained from Axon. All arrays were analyzed using the program ScanAlyze (available from http://rana.stanford.edu/) as described in the manual.

Heat shock from 25°C to 37°C

Two heat shock time series were performed and are referred to as hs-1 and hs-2. Cells were grown at 25°C to early-log phase. YPD heated to 49°C was added until the cell culture reached 37°C, and the cells were grown at 37°C. Samples were removed at 5, 10, 15, 20, 30, 40, 50, and 60 minutes, and total RNA was harvested for array analysis.

For the hs-2 series, an aliquot of cells was collected for the time=0 reference, and the remaining culture was resuspended in an equal volume of media that had been preheated to 37°C and returned to 37°C for growth . Samples were removed at 5, 15, 30, 45, and 60 minutes after cell resuspension. For array analysis, total RNA from each of the samples, including the unshocked sample, was labeled with Cy5-dUTP. A reference pool was prepared by combining an equal mass of total RNA from each sample, and this reference was labeled with Cy3-dUTP. For array analysis, each Cy5-labeled sample was compared to the Cy3-labeled reference pool. Following data acquisition and clustering analysis, the data were mathematically "zero transformed" for visualization by dividing the expression ratios for each gene measured on a given array by the corresponding ratios measured for the unshocked, time-zero cells. Therefore, in all figures the ratios represent the expression level at each time point relative to the expression level in the unshocked, time-zero sample.

Heat shock from various temperatures to 37°C and steady-state temperature growth

Six cultures were inoculated with over night cultures grown at the respective 17°, 21°, 25°, 29°, 33°, or 37°C, and each culture was returned to its respective temperature for approximately 6 hours. Half of the culture was collected to serve as the unstressed reference, and the remainder of each culture was collected by centrifugation and immediately resuspended in YPD heated to 37°. After 20 minutes at 37° the cells were harvested, and total RNA was isolated.

Two separate steady-state temperature experiments were performed and are referred to as ct-1 and ct-2. Each of the samples grown continuously at 17°, 21°, 25°, 29°, 33°, or 37°C in the previous experiment were compared to cells grown at 33°C to observe steady-state gene expression at different temperatures. A second experiment was

performed nearly-identically, with an additional sample grown at 15°C**,** except that for array analysis each sample was compared to a reference pool of all of the samples. The data were mathematically transformed by dividing the expression ratios for each gene measured on a given array by the corresponding ratios measured for the cells grown at 33°C.

Temperature shift from 37°C to 25°C

YPD media at 37°C was inoculated with an overnight culture of DBY7286 growing at 37°C, and the culture was grown to OD_{600} ~ 0.3. Cells were collected by centrifugation for 2 minutes; an aliquot was frozen for use as the reference sample, and the remainder of the culture was resuspended in two volumes of YPD media at 25°C. Samples were collected at 5, 15, 30, 45, 60, and 90 minutes after shock, and total RNA was collected for array analysis. To monitor expression at steady-state temperature levels, cells growing continuously at 37°C were compared to cells growing at 25°C. In addition, a mock temperature shock was performed by collecting cells grown at 37°C and resuspending them in 37°C media.

Mild heat shock at variable osmolarity

To compare the effects of mild heat shock at different osmolarities, three experiments were performed. In the first, a YPD culture of DBY7286 was grown at 29°C to OD_{600} 0.3. Cell were collected by centrifugation, the culture was resuspended in media at 33°C, and samples were collected at 5, 15, 30 minutes. A second time series was performed identically, except that cells were grown in YPD supplemented with 1 M sorbitol through the experiment. In the third experiment, cells growing continuously in YPD with 1 M sorbitol at 29°C were collected and resuspended in YPD without sorbitol at 33°C, and time points were collected. Total RNA was isolated for array analysis.

Response of mutant cells to heat shock

Wild type and mutant strains were exposed to heat shock in triplicate experiments. Wild type, *yap1*, or *msn2msn4* cultures grown at 30°C were collected and resuspended in an equal volume of media preheated to 37°C. The culture was shaken in

a water bath at 37°C for 20 minutes, cells were collected, and total RNA was isolated.

Hydrogen peroxide treatment

Because H_2O_2 is rapidly removed from the culture media by the cells, the concentration of H_2O_2 in the culture was maintained throughout the experiment. A 500 mL YPD culture was grown to OD_{600} 0.4. Following the removal of 100 mL as a reference, 100 mL of prewarmed YPD supplemented with H_2O_2 (Sigma) was added to give a final concentration of 0.30 mM H_2O_2. Samples were collected at 10, 20, 30, 40, 50, 60, 80, 100, and 120 minutes. Following sample removal, an equal volume of prewarmed YPD supplemented with 0.3 mM H_2O_2 was added to the culture to maintain the culture volume. In addition, YPD prewarmed to 30°C and supplemented with H_2O_2 was dripped into the culture throughout the experiment. The concentration of H_2O_2 was monitored every three minutes using a horseradish-peroxidase based assay (Green and Hill, 1984) which showed that the concentration of H_2O_2 was maintained at 0.32 +/- 0.03mM H_2O_2 over the course of the experiment (data not shown). For array analysis, mRNA was isolated from each sample.

Response of mutant cells to H_2O_2 exposure

Wild type and mutant strains were exposed to 0.3 mM H_2O_2 in triplicate experiments. 100 mL YPD cultures of either wild type, *yap1*, or *msn2msn4* were grown to OD_{600} 0.5, and 50 mL was removed as a reference. A single dose of H_2O_2 was added to 0.3 mM, and after 20 minutes the remaining culture was collected, and mRNA was isolated.

Menadione exposure

Menadione bisulfite (Sigma) was suspended immediately before use in water at a concentration of 1 M and filter sterilized through a 0.45 ? m filter. Menadione bisulfite was added to a concentration of 1 mM, samples were removed from the culture at 10, 20, 30, 40, 50, 60, 80, 105, and 120 minutes, and mRNA was isolated. A control experiment characterizing the effects of 1 mM Sodium bisulfite (Sigma) and revealed that there were no significant changes in gene expression in response to bisulfite (data not shown).

Diamide treatment

1.5 mM diamide (Sigma) was added to the culture, and samples were recovered at 5, 10, 20, 30, 40, 60, 90 minutes. Poly-adenylated RNA was isolated for array analysis.

DTT exposure

Two separate experiments (designated dtt-1 and dtt-2) were performed in which cells were exposed to 2.5 mM dithiothreitol. In the first experiment, cells were grown at 25^{o}C and DTT (Boeringer Manheim) was added for a final concentration of 2.5 mM. Samples were removed at 5, 15, 30, 45, 60, 90, 120, 180, and total RNA was isolated.

The dtt-2 experiment was performed similar to dtt-1, only cells were collected at 15, 30, 60, 120, 240, 480 minutes. The cells were periodically diluted with YPD supplemented with 2.5 mM DTT to maintain the cell density below OD_{600} 0.5. Total RNA recovered from each time point as well as the unstressed sample was labeled with Cy5-dUTP and compared to a reference pool, consisting of equal mass of total RNA from each sample, that was labeled with Cy3-dUTP. The array data were "zero-transformed" subsequent to clustering analysis.

Hyper-osmotic shock

A YPD culture was inoculated and grown to OD_{600} 0.6. 250 mL of preheated YPD supplemented with 2 M sorbitol was added to the culture, samples were collected at 5, 15, 30, 45, 60, 90, and 120 minutes, and mRNA was isolated.

Hypo-osmotic shock

YPD supplemented with 1 M sorbitol was inoculated with an overnight culture of DBY7286 grown in YPD plus 1 M sorbitol. The culture was grown to OD_{600} 0.15 at which point the culture was resuspended in YPD without sorbitol. Samples were collected at 5, 15, 30, 60 minutes, and total RNA was isolated for array analysis. To control for gene expression changes that were due to cell handling, cells grown in 1 M sorbitol were also collected and resuspended in identical media, containing 1 M sorbitol.

Amino acid starvation

Cells were grown in complete minimal media (SCD). Cells were collected by centrifugation and resuspended in an equal volume of minimal media lacking amino acids (YNB-AA, 2% glucose, 20 mg/L uracil) and allowed to grow. Samples were then harvested after 0.5 h, 1 h, 2 h, 4 h, and 6 h, and total RNA was collected.

Nitrogen source depletion

Cells were grown in complete minimal media (SCD), collected by centrifugation, and resuspended in an equal volume of minimal media without amino acids or adenine and with limiting concentrations of ammonium sulfate (YNB-AA-AS, 2% glucose, 20 mg/L uracil, 0.025% ammonium sulfate), and returned to the 30^{o}C shaker. Samples were subsequently harvested after 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 1 d, 2 d, 3 d, and 5 d, of culture incubation, and mRNA was isolated.

Stationary phase

Two nearly-identical experiments were performed, one at 25^{o}C (ypd-1) and one at 30^{o}C (ypd-2). YPD cultures were grown to OD_{600} 0.3, at which point cell culture was collected to serve as the time = 0 reference. Samples were recovered at 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 1 d, 2 d, 3 d, and 5 d, of culture incubation. Total RNA was isolated for array analysis.

Steady-state growth on alternative carbon sources

Two identical experiments (identified as car-1 and car-2) were performed in which cells were grown in YP media supplemented with 2% weight to volume of either glucose, galactose, raffinose, fructose, sucrose, or ethanol as a carbon source. For both experiments, the cultures were grown to an OD_{600} of between 0.22 and 0.37 and samples were collected. Total RNA harvested from each of the samples was labeled with Cy5-dUTP. For both experiments, a reference pool was created by combining an equal mass of RNA from each sample, and the reference pool was labeled with Cy3-dUTP. The data were transformed relative to the YP glucose experiment subsequent to clustering.

Steady-state growth at constant temperatures

Two separate steady-state temperature experiments were performed and are referred to as ct-1 and ct-2. The first experiment compared samples isolated as part of the previous series of variable temperature shocks. As described above, cells were grown continuously at 17°C, 21°C, 25°C, 29°C, 33°C, and 37°C. Total RNA recovered from the 33°C sample was used as a reference and labeled in Cy3-dUTP while all other samples were labeled in Cy5-dUTP.

The second series was performed identically to the first experiment only an additional 15°C was inoculated from a 17°C culture. For the second series, a reference pool was created by mixing an equal mass of total RNA from each sample, and this pool was labeled with Cy3-dUTP. Total RNA from each culture was labeled in Cy5-dUTP and compared to the labeled reference pool. After data acquisition and clustering analysis, data were transformed relative to the 33°C array data.

Overexpression studies

Overexpression constructs pRS-*MSN2* and pRS-*MSN4* as well as the parent vector pRS416 were received from Tae Bum Shin. DBY7286 was transformed with one of the three plasmids, and the three resulting clones were grown overnight in SC-Ura with 2% glucose. The cells were then washed three times with an equal volume of SC-Ura with 2% galactose and used to inoculate a fresh culture in SC-Ura supplemented with 2% galactose. The cells were grown approximately 6 hours to OD_{600} 0.3 and harvested. Total RNA from the cells harboring either pRS-*MSN2* or pRS-*MSN4* was labeled with Cy5-dUTP and compared to total RNA from control cells harboring pRS416.

Hierarchical clustering

Hierarchical clustering of the data was performed similar to that previously described (Eisen *et al*., 1998) using the program Cluster (available at http://rana.stanford.edu). 142 arrays observing wild type samples were clustered, including previously-published data (DeRisi *et al*., 1997), and the analysis was performed before mathematical transformation of the data from experiments in which a

reference pool was used. The data from each array experiment were weighted by the program Cluster (available at http://rana.stanford.edu/software/) according to the overall similarity of each array to others in the data set, which served to under-weight arrays that were highly similar. The resulting cluster was visualized using the program TreeView (available at http://rana.stanford.edu/software/). Genes sharing greater than 80% identity at the genomic level, and genes whose open reading frames overlap, are likely to cross-hybridize on the microarrays. In all figures, genes suspected of cross-hybridizing on the microarrays are marked by an asterisk in the gene annotation.

Promoter analysis

For co-regulated genes, either 600 bp or 1000 bp, as indicated, upstream of each gene start site was recovered using Yeast Tools (http://copan.cifn.unam.mx/Computational_Biology/yeast-tools/tool-list.html). Sequence motifs common to the upstream sequences were identified by the MEME algorithm (http://www.sdsc.edu/MEME/meme/website/meme.html (Bailey and Elkan, 1994)). Upstream sequences were searched for specific sequence motifs using Yeast Tools