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Cold Adaptation in Budding Yeast[D]

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We have determined the transcriptional response of the budding yeast *Saccharomyces cerevisiae* to cold. Yeast cells were exposed to 10°C for different lengths of time, and DNA microarrays were used to characterize the changes in transcript abundance. Two distinct groups of transcriptionally modulated genes were identified and defined as the early cold response and the late cold response. A detailed comparison of the cold response with various environmental stress responses revealed a substantial overlap between environmental stress response genes and late cold response genes. In addition, the accumulation of the carbohydrate reserves trehalose and glycogen is induced during late cold response. These observations suggest that the environmental stress response (ESR) occurs during the late cold response. The transcriptional activators Msn2p and Msn4p are involved in the induction of genes common to many stress responses, and we show that they mediate the stress response pattern observed during the late cold response. In contrast, classical markers of the ESR were absent during the early cold response, and the transcriptional response of the early cold response genes was Msn2p/Msn4p independent. This implies that the cold-specific early response is mediated by a different and as yet uncharacterized regulatory mechanism.

INTRODUCTION

Unicellular organisms are subjected to a variety of drastic changes in their environment such as fluctuations in nutrients, acidity, osmolarity, and temperature, as well as exposure to toxic agents and radiation. Cells have developed programmed responses to stress; these include rapid changes in processes such as protein phosphorylation and degradation, and longer term effects involving transcriptional changes that become manifested in altered cell states.

The molecular basis of the response to many different stresses has been extensively studied in *Saccharoymces cerevisiae*. For instance, yeast cells undergoing heat shock rapidly induce a large group of heat shock proteins (HSPs) mediated by the transcription factor Hsf1p (Estruch, 2000). HSPs act as molecular chaperones to stabilize cellular proteins and reactivate heat-damaged proteins (Craig, 1993; Boy-Marcotte *et al*., 1998; Estruch, 2000). In response to various other stresses, the transcription of a common set of genes is changed; this defines the general stress response (Ruis and Schüller, 1995). Genome-wide transcriptional profiling has shown that ~10% of the genome is induced or repressed in this response, and the genes involved are defined as the environmental stress response, ESR (Gasch *et al*., 2000), or common environmental response (Causton *et al*., 2001). Induced ESR genes are involved in a variety of cellular functions such as protein folding and degradation, transport, and carbohydrate metabolism. Repressed ESR genes generally function in cell growth-related processes, including RNA metabolism, nucleotide biosynthesis, secretion, and ribosomal performance. The regulation of the ESR is determined by the function of the two transcription factors, Msn2p and Msn4p, that bind to stress response elements (STREs) in the promoters of their target genes (Martinez-Pastor *et al*., 1996; Schmitt and McEntee, 1996; Görner *et al*., 1998).

Little is known about the mechanisms responsible for growth and survival at low temperature. Cold causes a variety of changes in the physical and biochemical properties of the cell. For instance, a decrease in membrane fluidity results in slower lateral diffusion of membrane proteins, decreased activity of membrane-associated enzymes, and a major reduction in membrane transport (Vigh *et al*., 1998). In prokaryotes, a direct consequence of cold is the stabilization of mRNA secondary structures, particularly the 5′-untranslated region, that makes the Shine-Dalgarno sequence unavailable for ribosomes and therefore prevents the initiation of protein translation (Ermolenko and Makhatadze, 2002). The ability to adapt to such dramatic changes is determined by different regulatory mechanisms. In bacteria, especially *Escherichia coli*, a group of genes induced upon cold treatment has been identified that encode the cold shock proteins, CSPs (Thieringer *et al*., 1998). These CSPs are involved in transcription and translation processes (Jiang *et al*., 1997; Ermolenko and Makhatadze, 2002). In *S. cerevisiae*, differential hybridization has revealed a small set of genes upregulated in response to reduced temperature that includes *NSR1*, *TIP1*, *TIR1*, and *TIR2*. *NSR1* encodes a nucleolin-like

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protein that is involved in pre-rRNA processing and ribosome biogenesis (Kondo and Inouye, 1992). *TIP1* and its two homologues *TIR1* and *TIR2* encode serine- and alanine-rich cell wall proteins and may be involved in maintaining cell wall integrity during stress (Kondo and Inouye, 1991; Kowalski *et al*., 1995). The fatty acid desaturase gene *OLE1* is also induced upon cold (Nakagawa *et al*., 2002). A cold-dependent induction of fatty acid desaturases has been identified in other eukaryotic organisms such as plants (Uemura *et al*., 1995; Browse and Xin, 2001), dimorphic fungi (Laoteng *et al*., 1999), fish (Tiku *et al*., 1996), and prokaryotes (Sakamoto and Bryant, 1997; Thieringer *et al*., 1998; Aguilar *et al*., 1999), suggesting that membrane fluidity adaptation is a ubiquitous common response to cold.

This article describes the global transcriptional analysis of cold response in *S. cerevisiae* wild-type and Δ*msn2* Δ*msn4* cells. We compare the cold response to the responses to other stress stimuli and measure the hallmarks of the general stress response such as trehalose and glycogen accumulation in cold-stressed *S. cerevisiae* cells.

MATERIALS AND METHODS

Strains

We used strains BY4743 (*MAT***a**/α, wild-type) and BSY25 (BY4743, except homozygous Δ*msn2*::*kanMX* Δ*msn4*::*kanMX met15*), which was derived from a cross of the two single-mutant strains obtained from the American Type Culture Collection (Manassas, VA). For growth curve experiments, W303 (*MAT***a**/α, wild-type) also was used.

Growth Medium and Culture Conditions

Cultures were grown in YPD medium (2% glucose, 2% bactopeptone, and 1% yeast extract). For each experiment, cultures were inoculated from a fresh colony and grown overnight at 30°C in 50 ml of medium in 250-ml Erlenmeyer flasks shaken at 170 rpm. The overnight cultures were then diluted to 0.05 OD_{600} in 500 ml of fresh medium, grown to 0.6 OD_{600} at 30°C in 1500-ml flasks shaken at 170 rpm, and transferred to a 10°C water bath shaker, in which they were incubated for 10, 30, or 120 min at 170 rpm before harvesting. The temperature decreased 4°C per minute. To ensure that cells from all experiments were harvested during early log phase, the doubling time of a culture at 10°C (20.7 h) was determined. Based on this doubling time, the diluted overnight cultures for the 12-h experiments were grown only to 0.4 OD_{600} before they were shifted to 10°C. For the 60-h experiments, the overnight cultures were diluted to 0.05 OD_{600} in 100 ml of fresh medium in 250-ml flasks, grown to 0.4 OD_{600}, and diluted again to 0.05 OD_{600} in 500 ml of fresh medium in 1500-ml flasks. When the culture reached 0.1 OD_{600}, the cells were transferred to 10°C. Thus, each culture reached a final OD_{600} of 0.6–0.8 before cells were harvested by centrifugation at 10 or 30°C (control) for 2 min at 3500 rpm. Cell pellets were snap-frozen in liquid nitrogen and stored at −80°C.

Isolation of RNA

Total RNA was isolated using the hot-phenol method (Kohrer and Domdey, 1991) with the following modifications. The cells from a 500-ml culture were processed in 50-ml tubes by extracting with phenol twice for 10 min apiece. For the 60-h experiment, RNA extraction was found to be inefficient, and it was therefore improved by adding glass beads (425–600 μm; Sigma-Aldrich, St. Louis, MO). mRNA was purified using the Oligotex Spin-Column Protocol (Oligotex mRNA Midi kit; QIAGEN, Valencia, CA).

RNA Labeling and DNA Microarray Hybridization

Three micrograms of mRNA was labeled by directly incorporating Cy3- and Cy5-dCTP through reverse transcription. The resulting cDNA was hybridized onto yeast genomic DNA microarrays (obtained from the University Health Network Microarray facility; http://www.microarrays.ca). Prehybridization was done in 20:1:1 DigEasyHyb solution (Roche Applied Science, Laval, Quebec, Canada), yeast tRNA (1 mg/ml, Baker's yeast; Roche Applied Science), and sonicated salmon sperm DNA (10 mg/ml; Invitrogen, Burlington, Ontario, Canada) for 2 h at 42°C. Microarrays were washed twice in 0.1× SSC buffer for 2 min per wash at 42°C, airstream dried, and immediately hybridized. Detailed protocols are available at http://www.irb-bri.cnrc-nrc.gc.ca/business/microarraylab/products_e.html.

Data Acquisition and Analysis

Microarray slides were scanned using a ScanArray lite scanner (Packard Bioscience, PerkinElmer-Cetus, Wellesley, CA) at a 10-μm resolution, and the resulting 16-bit TIFF files were quantified using the QuantArray software (PerkinElmer-Cetus; version 2.0 and 3.0). Normalization and quality controls of the QuantArray files were performed in Microsoft Excel by using standardized spreadsheets, as described previously (Nantel *et al*., 2002). Each DNA spot had to pass three quality controls to be included in the normalization and subsequent analysis: 1) the signal intensity had to be significantly greater than the local background (the signal intensity minus half the SD had to be greater than the local background plus half the SD); 2) the signal intensity had to be within the dynamic range of the photomultiplier tube; and 3) the raw intensities of the duplicate spots for each gene had to be within 50% of one another. For spots that met these criteria, the ratio of intensities of the two channels was normalized by the median ratio for the entire subarray consisting of 400 spots that had passed the quality control. To correct for variation in local intensities across the surface of the array, we performed subarray normalization by normalizing each subarray individually, which was found to produce more reproducible data (Smyth and Speed, 2003). Finally, the log2 values of the ratio for each duplicate spot were averaged. Statistical analysis and visualization were performed with GeneSpring software (Silicon Genetics, Redwood City, CA). Hierarchical clustering (Eisen *et al*., 1998) was performed in GeneSpring based on the matrix of standard correlation.

Experimental Design

To help ensure that each culture used for the microarray experiments was in the same physiological state, samples were taken before harvesting to determine the budding index of the cells (Supplementary Figure S3) and the glucose content in the medium. The cultures at each time point showed an average of 70% budded cells, and the medium glucose content was ~16 g/l on average. To ensure that no diauxic shift occurred during the continuous growth at 10°C, the transcriptional profiles of the 12- and 60-h experiments were analyzed for diauxic shift-inducible genes (DeRisi *et al*., 1997). Marker genes of the diauxic shift such as *ACO1*, *CIT1*, *FUM1*, *ALD2*, *IDP2*, and *FBP1* did not show transcriptional changes during long-term cold treatment for 12 and 60 h.

The time-course experiments with the wild-type strain were performed with time points of 0, 2, and 12 h (two independent biological repeats) and of 10 min, 30 min, and 60 h (three independent biological repeats). Two independent biological repeats were carried out for each of the experiments with the Δ*msn2* Δ*msn4* strain except for the 12-h time point (three repeats). For each experiment performed, the Cy dyes were swapped for the reference and experimental samples. In addition, control microarrays were carried out to determine the variability of the experimental factor using independently grown cultures at 30°C (3 technical repeats with dye swapping). From these control hybridizations, reliable data were obtained for 5559 genes and only 14 genes (0.25%) showed an average variation >1.5-fold. To ensure significant data quality, we selected genes with at least twofold variation and a Student's *t* test p value of <0.03 for our experimental analysis (634 genes for the wild-type and 120 genes for the Δ*msn2* Δ*msn4* mutant). The expression ratios were averaged. In this study, a total of 43 microarrays were used. The complete data set is available for retrieval from our website (http://cbr-rbc.nrc-cnrc.gc.ca/genetics/cold/).

Comparison with Other S. cerevisiae Stress Data

The list of 830 *S. cerevisiae* ESR genes was obtained from the Web site of Gasch *et al*. (2000), whose comprehensive study of the responses to a variety of stresses was obtained at http://genome-www.standford.edu/yeast_stress/. The expression data for the cold response described by Sahara *et al*. (2002) were obtained at http://staff.aist.go.jp/t-sahara/. Comparisons were performed with GeneSpring using standard correlation.

Biochemical and Analytical Procedures

Determination of glycogen and trehalose levels were performed as described previously (Parrou and François, 1997). For these experiments, the cells were grown and harvested as described for the DNA microarray analysis. Glucose concentrations were determined using the Glucose kit (Sigma-Aldrich).

RESULTS

Cold Response of S. cerevisiae

When subjected to low temperature (10°C), *S. cerevisiae* cells showed a reduced growth rate but a normal growth curve. Exponentially growing cultures with a doubling time of ~90 min were shifted from 30 to 10°C, after which the doubling time was immediately reduced to 20.7 h without a detectable growth arrest (Supplementary Figure S1, A and B). After ~120 h, the cultures reached stationary phase, consistent with the observed reduced glucose concentration in the medium (Supplementary Figure S1, C). The ability to adapt

to decreased temperature is potentially accompanied by changes in gene expression. We have applied global transcriptional profiling by using DNA microarrays to examine such possible changes.

Our results show that *S. cerevisiae* cells do respond to a rapid temperature shift from 30 to 10°C with transient changes in gene expression. The data were organized by two-dimensional hierarchical clustering (Figure 1A; Eisen *et al.*, 1998). There were five main clusters: three with induced genes and two with genes that were repressed in response to cold. Among these cold-responsive genes, a subset was induced particularly during the first 2 h of cold treatment (clusters D and E), whereas another subset was induced or repressed after 12 and 60 h (clusters A–C). These two subsets were defined as early cold response (ECR) and late cold response (LCR). The numbers of genes involved and their relative expression levels were considerably higher during the LCR with a peak at 12 h. A classification of the ECR and LCR genes into functional categories according to MIPS is shown in Figure 1, B and C.

To test whether the cold induction treatment was effective, we followed the transcriptional response of five previously identified cold-responsive genes (*NSR1*, *TIP1*, *TIR1*, *TIR2*, and *OLE1*). All of them were induced more than twofold, with *NSR1* showing increased transcript abundance during almost the entire time course; *OLE1* after 10, 30, and 120 min; *TIP1* after 30 and 120 min; *TIR1* after 2 and 12 h; and *TIR2* after 12 h. These results demonstrate effective cold induction.

Early Cold Response

We defined induced ECR genes as being reproducibly induced ≥2-fold at one or more of the three early time points examined and identified 130 open reading frames (ORFs) that met this criterion (Figure 1A, clusters D and E). These genes are mainly associated with transport, lipid and amino acid metabolism, and transcription, and also include many ORFs of unknown function (Figure 1B).

Studies in prokaryotes have shown the induction of a set of cold shock proteins that include RNA helicases (Jones *et al.*, 1996). We also identified a set of ECR genes involved in transcription, including the RNA helicase genes *DED1* and *DBP2*; the RNA processing genes *NSR1*, *HRP1*, *NRD1*, *STP4*, *NOG2*, and *HUL5*; and the RNA polymerase subunit gene *RPA49*.

Another important factor during cold adaptation is the control of membrane fluidity by alteration of the concentration of unsaturated fatty acids in membrane lipids, and a variety of studies in both prokaryotes and eukaryotes have identified cold-inducible fatty acid desaturases (Wada *et al.*, 1990; Gibson *et al.*, 1994; Tiku *et al.*, 1996; Kodama *et al.*, 1997; Aguilar *et al.*, 1998; Nakagawa *et al.*, 2002). We were interested in the identification of genes involved in lipid metabolism that may contribute to a change in membrane fluidity in yeast. A group of ECR genes included *OLE1*, encoding a Δ9-fatty acid desaturase. *OLE1* is regulated by the two endoplasmic reticulum (ER) membrane-bound transcription factors Spt23p and Mga2p (Zhang *et al.*, 1999), whose activation requires the chaperone-like complex $CDC48^{UFD1/NPL4}$ (Hoppe *et al.*, 2000; Rape *et al.*, 2001). We found that *UFD1* was significantly induced during the ECR. Both *MGA2* and a gene encoding another component of the chaperone complex, *NPL4*, also showed reproducible increases in transcript abundance during the ECR, but the induction never exceeded twofold.

S. cerevisiae cells also responded to a temperature downshift by rapidly decreasing the levels of expression of some genes. The expression of 32 genes was reduced by at least twofold within the first 2 h (Figure 1A, cluster F), including genes encoding heat shock proteins such as the cytosolic and mitochondrial chaperones Hsp104p, Hsp82p, Hsp60p, and Hsp10p, which are required for correct protein folding and play an important role in response to stress (Craig, 1993; Hohfeld and Hartl, 1994).

Late Cold Response

We identified 280 LCR genes that were reproducibly induced twofold or more at 12 and/or 60 h (Figure 1A, cluster C). These genes include ones encoding metabolic enzymes involved in carbohydrate metabolism, particularly in glycolysis (*GLK1*, *HXK1*, *PYK2*, and *GPD1*), glycogen metabolism (*GLC3*, *PGM2*, *GPH1*, *GDB1*, *GYS1*, and *GYS2*), and trehalose metabolism (*TPS1*, *TPS2*, and *TSL1*). In addition, some genes required for the regulation of carbohydrate metabolism, including the transcription factor-encoding genes *HAP5* and *TYE7*, were coordinately induced.

Another set of induced LCR genes (*HSP12*, *HSP26*, *HSP42*, *HSP104*, *YRO2*, and *SSE2*) encodes members of the heat shock protein family that are known to be involved in stress response. These functionally conserved proteins prevent protein aggregation and facilitate protein degradation or refolding (Lindquist, 1992). In addition, genes previously shown to be induced by oxidative stress and implicated in detoxification processes, including *GTT2* (glutathione transferase), *HYR1* and *GPX1* (glutathione peroxidase isoforms), *TTR1* (glutaredoxin), and *PRX1* (thioredoxin peroxidase) (Gasch *et al.*, 2000; Rep *et al.*, 2000), were induced in the LCR.

The long-term cold treatment also triggered repression of a variety of genes, and 256 cold-repressible LCR genes were identified (Figure 1A, clusters A and B). One large subset of these genes (36%) is involved mainly in protein synthesis (ribosomal protein genes; in cluster A), whereas a second set is associated with nucleotide biosynthesis, protein modification, and vesicle transport (in cluster B). These results suggest that the repression of ribosomal genes and other genes involved in protein synthesis contributes to the adaptation to cold.

Cold Response Compared with Other Environmental Stress Responses

Analysis of the LCR genes revealed a set of induced genes common to the ESR, during which they are regulated by the transcription factors Msn2p and/or Msn4p via STREs in their promoter regions (Boy-Marcotte *et al.*, 1998; Moskvina *et al.*, 1998). The increasing global expression profiling data that are available allow comparison of the transcriptional responses to a wide range of stress stimuli. We therefore compared the ECR and LCR transcription profiles to the transcription profiles produced by a variety of environmental stresses, including different cold stresses (Gasch *et al.*, 2000). For this study, we chose the 2-h time point to represent ECR and the 12-h time point to represent LCR, because the maximum changes in transcript abundance were observed at these times.

The comparison of the ECR with the transcriptional pattern produced by a temperature downshift from 37 to 25°C revealed a similar transcriptional response with a time-dependent gradual decrease in similarity (Figure 2). Forty-seven percent of the induced ECR genes also showed a transient increase in expression after a shift from 37 to 25°C (Figure 2, cluster b). This group contains genes involved in transcription and in amino acid and fatty acid metabolism.

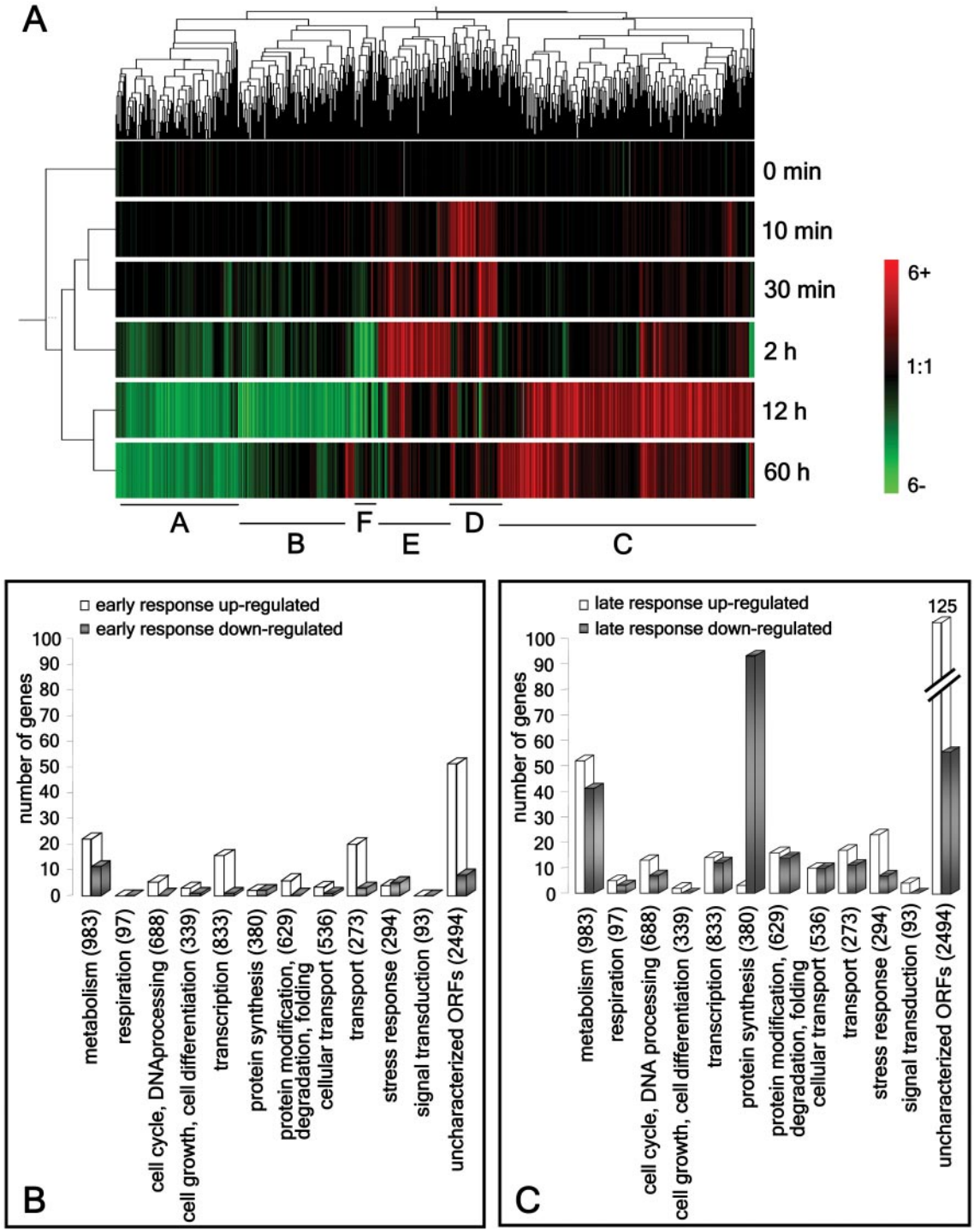

Figure 1. Transcriptional response to cold. (A) Two-dimensional hierarchical cluster analysis of microarray data obtained from a time-course experiment with *S. cerevisiae* wild-type diploid cells (BY4743) incubated at 10°C for the indicated times. The analysis was performed on 634 genes that showed a statistically significant variation of at least twofold in at least one of the experiments (see *Materials and Methods* for details). Ratios of the changes in transcript abundance obtained by dividing the experimental by the reference samples are represented with a green-to-red color scale. Down-regulated genes are green, whereas up-regulated genes are red. Similarities between gene expression patterns are represented by the horizontal dendrogram; the vertical dendrogram represents the similarities between the different times of exposure to cold. Labels D and E represent the ECR genes; labels A, B, and C represent the LCR genes. (B and C) Classification of ECR (B) and LCR (C) genes. The diagrams show the distributions of the most representative functional categories, each of which is subdivided into up- and down-regulated genes. The ORFs were categorized based on MIPS classification and the SGD database. The total numbers of genes classified in each category according to MIPS are represented in parentheses.

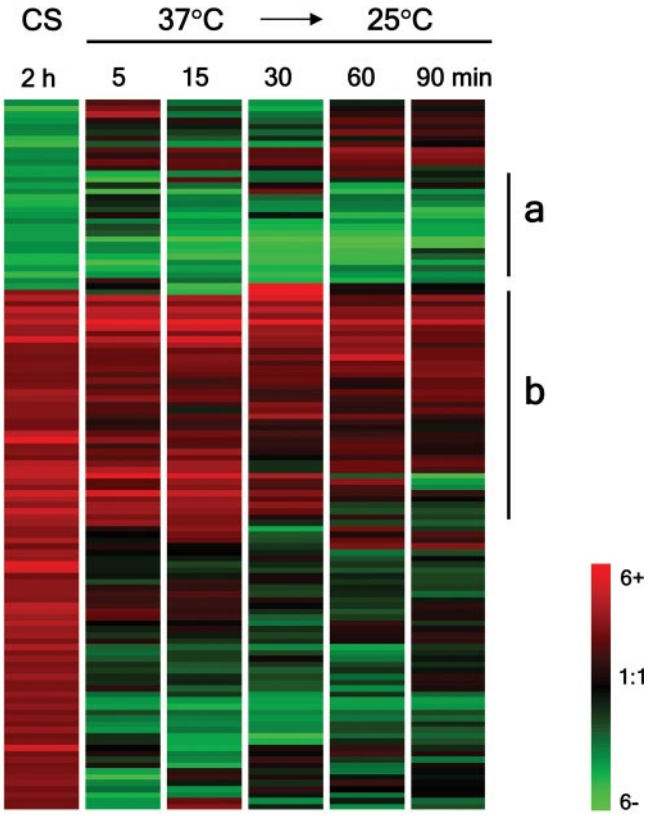

Figure 2. Transcriptional profiles of early cold response during temperature downshifts. The ECR genes, represented by the 2-h time point, were compared with a temperature downshift from 37 to 25°C at the indicated time points (Gasch *et al.*, 2000). Labels a and b represent genes showing a correlation in transcriptional response after shift from 37 to 25°C.

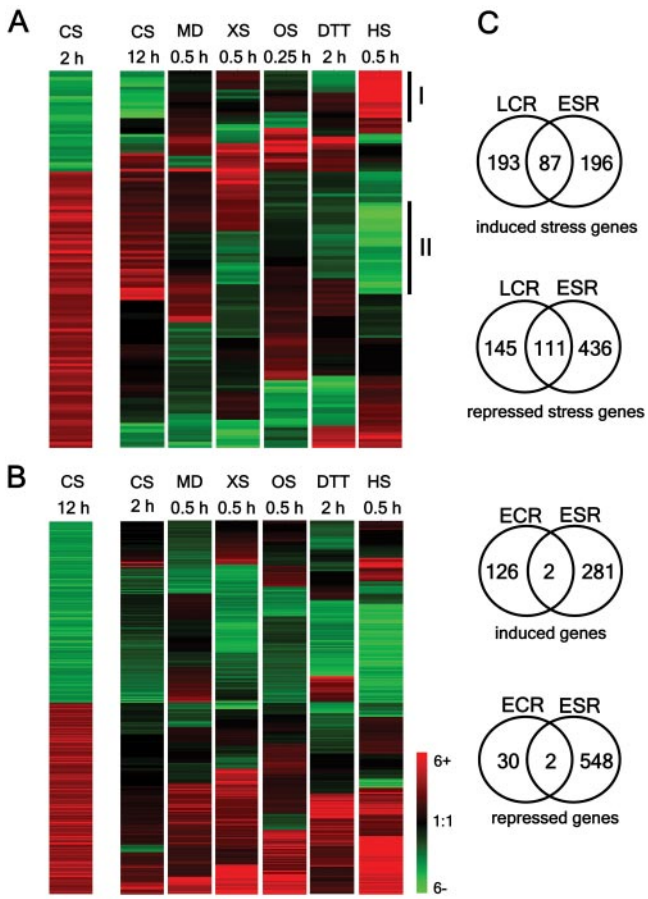

Figure 3. Comparison of the transcriptional responses to cold and other environmental stresses. (A) Transcriptional responses of ECR genes (CS, 2 h) compared with those of LCR genes (CS, 12 h) and to the responses to MD, XS, OS, DTT, and HS at the indicated times (Gasch *et al.*, 2000). ECR genes with a reciprocal transcriptional response during heat shock are labeled with I and II. Each experiment was individually compared with the CS, 2-h data by using GeneSpring (standard correlation). (B) Similarly, the transcriptional responses of the LCR genes were individually compared with those of ECR genes and to the responses to menadione, oxidative stress, osmotic stress, dithiothreitol, and heat shock (Gasch *et al.*, 2000). (C) The LCR (two diagrams from top) and ECR (two diagrams from bottom) were compared with ESR, and the numbers of genes in common are shown in Venn diagrams for both the induced and missing repressed genes in each case. The overlaps with ESR are significant for both induced (p value of 1×10^{-47}) and repressed (p value of 1×10^{-48}) LCR genes, whereas the overlaps with ESR are not significant for induced (p value of 0.98) and repressed (p value of 0.78) ECR genes.

The majority of repressed ECR genes were also repressed during a temperature downshift from 37 to 25°C (Figure 2, cluster a), including the HSP genes. Notably, similar results also were obtained when the transcriptional responses at 10 and 30 min after the shift from 30 to 10°C were compared with those observed after downshift from 37 to 25°C (our unpublished data). In contrast, when the transcription profiles from cultures grown continuously (20 h) at particular low temperatures (15, 17, or 21°C) were compared with the ECR and LCR profiles, only weak correlations were seen (Supplementary Figure S2).

Comparing the ECR expression profiles with those produced by different stress stimuli such as oxidative stress (0.3 mM H_2O_2, XS; or 1 M menadione, a superoxide-generating drug, MD), osmotic stress (1 M sorbitol, OS), a disulfide-reducing agent (2.5 mM dithiothreitol, DTT), and heat shock (25–37°C, HS) revealed unexpected correlations for OS after 15 min, for MD and XS after 0.5 h, and for DTT after 2 h. Many ECR genes showed a reciprocal behavior under the other stress stimuli: induced ECR genes were repressed, whereas repressed ECR genes were induced (Figure 3A). In contrast, only a minor group of ECR genes was found to be coinduced or corepressed compared with the other stress stimuli. The most intriguing correlation was observed between the ECR and heat shock. Almost half of the repressed ECR genes were induced during heat shock, including HSP genes and genes involved in amino acid and carbohydrate metabolism (Figure 3A, cluster I). In addition, 40% of induced ECR genes were repressed after 0.5 h of heat shock. This set includes genes associated with RNA metabolism (RNA helicase, RNA polymerase, and RNA processing) and fatty acid metabolism (Figure 3A, cluster II). Furthermore, ~18% of induced ECR genes showed no heat shock response, and relatively few genes were coexpressed. A similar reciprocal transcription pattern also was observed in a recent study that compared heat shock (25–37°C) and temperature downshift (37–25°C) responses (Gasch *et al.*, 2000).

Strikingly, when the expression profiles of LCR genes were compared with those seen under the other stress conditions, we observed similar transcriptional responses in all cases (Figure 3B). This result is of particular interest because

of the reciprocal response pattern of the ECR, which reverts back to a general stress response during LCR. Based on the remarkable similarities between LCR and other stress responses, we focused our further analysis on a systematic comparison. In the earlier comprehensive study, 283 genes were defined as induced ESR genes (Gasch *et al*., 2000). Comparing these genes with the induced LCR genes revealed a significant overlap of 87 genes (Figure 3C, top). This set of genes includes the classical hallmarks of the ESR such as *HSP12* and *HSP104*, as well as genes involved in carbohydrate metabolism (*GLK1*, *HXK1*, *PGM2*, *GSY2*, *TPS1*, and *TPS2*). Similarly, the repressed LCR genes showed a significant overlap of 111 genes (Figure 3C, second from top), including genes involved in nucleotide biosynthesis and ribosomal genes. In contrast, the comparison of ESR with ECR genes showed no significant overlap. Only two induced and two repressed ECR genes were coexpressed in the ESR (Figure 3C, bottom two diagrams). These observations strongly suggest that the LCR involves the ESR, whereas the ECR indicates a "cold-specific" transcriptional response.

Regulation of the Transcriptional Response to Cold

Many stress genes are regulated by the transcription factors Msn2p and/or Msn4p, for instance *HSP12*, *GLK1*, *PGM2*, *HSP104*, *HXK1*, and *GSY2* (Boy-Marcotte *et al*., 1998; Moskvina *et al*., 1998). We asked whether these transcription factors are involved in the regulation of cold-responsive genes by performing microarray analyses with a strain deleted for *MSN2* and *MSN4*. First, the Δ*msn2* Δ*msn4* strain was characterized during growth at different steady-state temperatures. In comparison with a wild-type strain, a slight reduction in growth at 37°C was observed after 2-d incubation, whereas cold exposure (15 and 10°C) caused no detectable lag in growth compared with the wild-type strain (our unpublished data).

We next tested whether Msn2p/Msn4p are required for induction of the LCR genes and whether they are involved in the regulation of the ECR genes. As noted above, we selected the 2- and 12-h time points for this experiment. Furthermore, we chose to directly compare the wild-type and Δ*msn2* Δ*msn4* strains at 10°C. Thus, cold-induced genes dependent on Msn2p/Msn4p show no activation in the mutant strain, and occur with a decrease in intensity relative to the wild-type strain, whereas cold-repressed Msn2p/Msn4p-dependent genes show an increase in the relative signal intensity. An unaltered relative transcriptional abundance indicates genes that are independent of Msn2p/Msn4p and coexpressed in both strains. The analysis showed that the relative expression of 120 genes was affected ≥2-fold (p value of 0.03) in the Δ*msn2* Δ*msn4* strain exposed to cold (Figure 4). Msn2p/Msn4p were required for the activation of 99 LCR genes, including classical hallmarks of the ESR such as the chaperone genes *HSP12* and *HSP104*, as well as carbohydrate metabolism genes (Figure 4, cluster ESR; Martinez-Pastor *et al*., 1996; Boy-Marcotte *et al*., 1998; Moskvina *et al*., 1998). A few genes also were observed that required Msn2p/Msn4p for expression at 30°C (Figure 4, 0 h).

Seventy-eight percent of the LCR genes were unaffected by the absence of Msn2p/Msn4p, suggesting that additional transcriptional regulators for LCR gene expression are involved. These LCR genes are implicated in amino acid metabolism, transport, ubiquitin-dependent protein degradation, protein synthesis (RNA processing and ribosome synthesis), and transcription.

We also investigated the potential role of Msn2p/Msn4p in regulating the ECR genes. In contrast to the findings

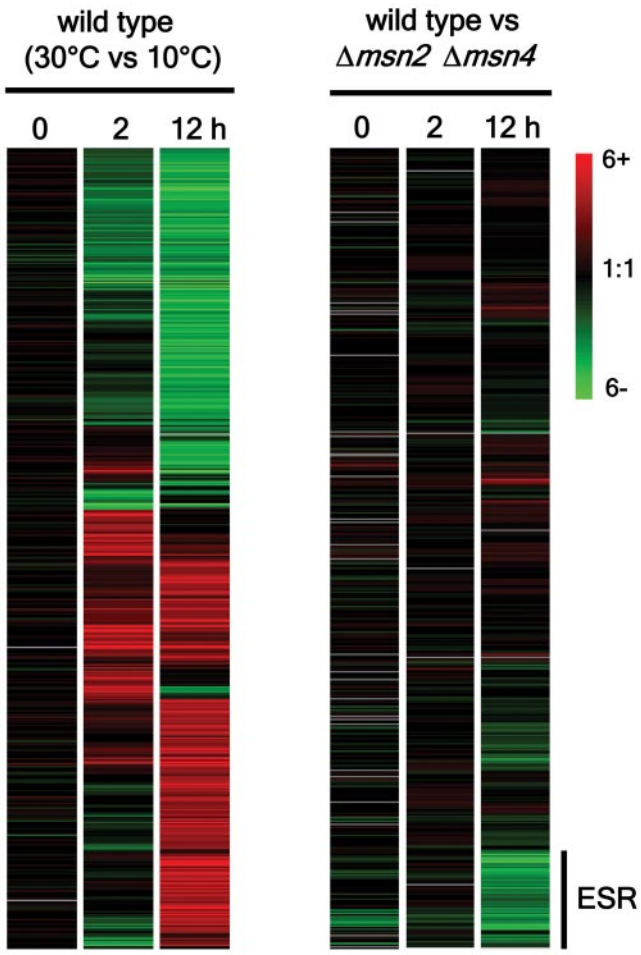

Figure 4. Regulation of gene expression during cold treatment. The 634 cold-responsive genes were clustered based on their expression patterns in wild-type and Δ*msn2* Δ*msn4* strains during ECR (2 h) and LCR (12 h). The expression ratio for each gene in this diagram represents the average from duplicate or triplicate experiments (see *Materials and Methods* for details). The label on the right indicates induced ESR genes.

obtained for the LCR, a 2-h cold treatment revealed no significant differences in transcript abundance between wild-type and Δ*msn2* Δ*msn4* strains, suggesting Msn2p/Msn4p-independent regulation of ECR genes. These results support the comparison of the ECR expression profile to those seen under various stress conditions in indicating a cold-specific response of *S. cerevisiae* during the ECR.

Reserve Carbohydrate Accumulation in Response to Cold

A physiological consequence of the general stress response in *S. cerevisiae* is the accumulation of the two major reserve carbohydrates, glycogen and trehalose. The production of glycogen has been detected upon exposure to stresses like heat and hyperosmotic and oxidative shocks. Glycogen is accumulated up to three times the basal level accompanied by a weak induction of trehalose production in response to heat shock. This low accumulation is due to a turnover phenomenon with induction of genes implicated in the degradation as well as in the production of trehalose (Parrou *et al*., 1997). We have measured the production of the two reserve carbohydrates in response to cold treatment. As

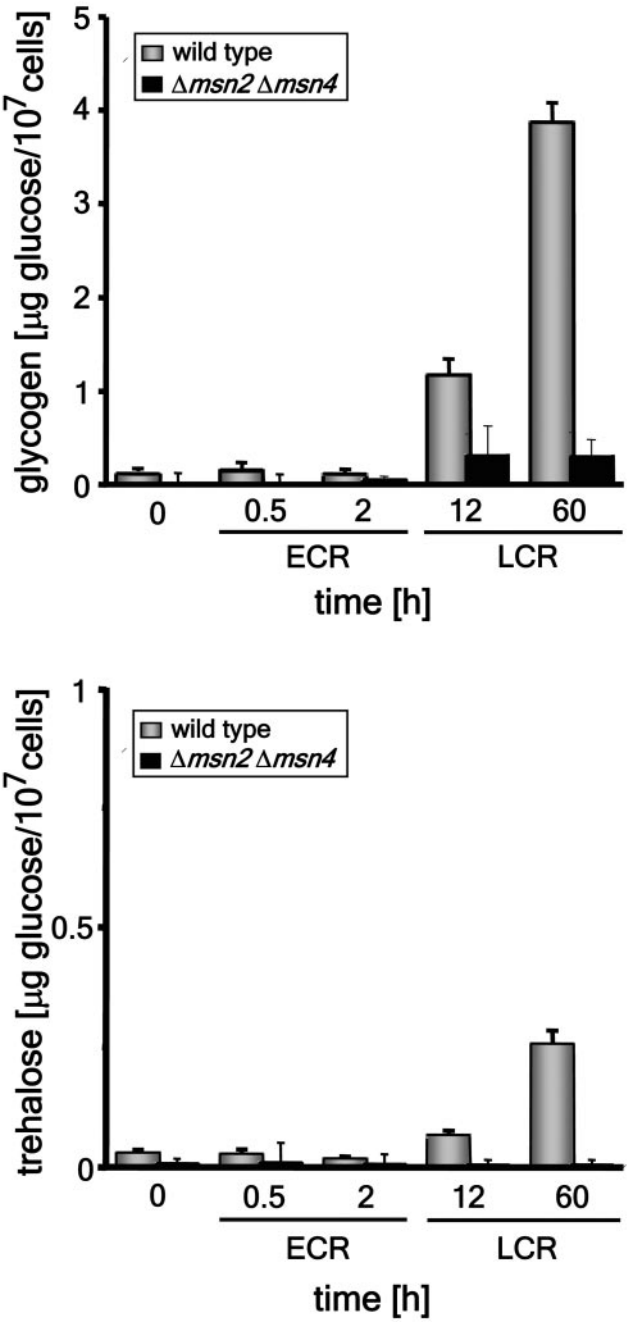

Figure 5. Accumulation of reserve carbohydrates during cold treatment. The effects of cold on glycogen (top) and trehalose (bottom) content in the wild-type and Δ*msn2* Δ*msn4* strains are shown. The levels of glycogen and trehalose were measured at the indicated times after a temperature shift from 30 to 10°C. The results represent the average of three independent experiments.

shown in Figure 5, there is no accumulation in response to cold during the first 2 h, but a reproducible increase in glycogen and trehalose content was observed after 12 h of cold treatment. These results are in agreement with the microarray data: several genes involved in reserve carbohydrate metabolism are induced at this time point. Cells accumulated even higher levels of glycogen and trehalose after 60 h, whereas the induction for most of the genes involved in reserve carbohydrate metabolism dropped to twofold.

The induction of genes involved in reserve carbohydrate metabolism in response to stress depends on the presence of STREs in the promoters of these genes (Ni and LaPorte, 1995; Estruch, 2000; Sunnarborg *et al*., 2001). In the mutant strain lacking both Msn2p and Msn4p, only a small accumulation of glycogen and essentially no accumulation of trehalose occurred in response to cold during the LCR (Figure 5). These data correlate with the loss of induction of these genes during cold treatment in the Δ*msn2* Δ*msn4* strain.

DISCUSSION

The ability to adapt rapidly to changing environmental conditions is essential for all organisms. In this study, the model organism *S. cerevisiae* was used to study the transcriptional response to an abrupt temperature decrease from 30 to 10°C. *S. cerevisiae* initiates different expression programs during the response to cold, and their regulation is gene and time specific (Figure 1). We identified two distinct cold responses defined as the ECR (times ≤2 h) and the LCR (times ≥12 h). Major characteristics of the ECR are the induction of genes implicated in RNA metabolism and lipid metabolism, whereas genes induced during the LCR mainly encode proteins that are involved in protecting the cell against a variety of stresses.

Decreased temperatures are known to affect the stability of RNA secondary structures, leading to a rate-limiting step of translation initiation (Jones and Inouye, 1996; Farewell and Neidhardt, 1998). Thus, in bacteria, ATP-dependent RNA helicases play an essential role during cold adaptation by removing cold-stabilized mRNA secondary structures to allow efficient translation initiation (Jones *et al*., 1996; Thieringer *et al*., 1998; Chamot and Owttrim, 2000). In yeast, we identified cold-induced genes encoding RNA helicases, RNA-binding proteins, and RNA-processing proteins during the ECR. Interestingly, mutations in some of these genes, such as *NSR1* (Kondo and Inouye, 1992), *DED1* (Chuang *et al*., 1997), and *DBP2* (Barta and Iggo, 1995), lead to cold-sensitive phenotypes. For the RNA helicase Ded1p, an active role in translation initiation has been suggested, particularly in melting secondary structures during scanning by the ribosomal subunit (de la Cruz *et al*., 1999; Linder, 2003). Thus, Ded1p may be required for unwinding cold-stabilized mRNA secondary structures, thereby increasing the efficiency of the translation initiation process. The identification of cold-inducible RNA helicases also in plants (Seki *et al*., 2002) demonstrates that RNA helicases are generally important factors during cold adaptation.

Another conserved mechanism in response to cold is the adaptation of membrane fluidity. Reduced temperatures cause a decrease in membrane fluidity. This is counteracted by increasing production of unsaturated fatty acids, which involves fatty acid desaturase activity (Vigh *et al*., 1998). Induction of desaturases by cold has been described in bacteria (Aguilar *et al*., 1999), plants (Kodama *et al*., 1997), fish (Tiku *et al*., 1996), and yeast (Nakagawa *et al*., 2002). We found *OLE1*, a yeast fatty acid desaturase gene, to be induced during the ECR together with components involved in its regulation, including the ER-membrane-bound transcription factor Mga2p (Hoppe *et al*., 2000) and Ufd1p and Npl4p of the ubiquitin/proteasome complex $CDC48^{UFD1/NPL4}$, which is involved in the activation of Mga2p (Hoppe *et al*., 2000; Rape *et al*., 2001; Braun *et al*., 2002). A recent study showed that the cold induction of *OLE1* is indeed accompanied by the accumulation of unsaturated fatty acids (Nakagawa *et al*., 2002).

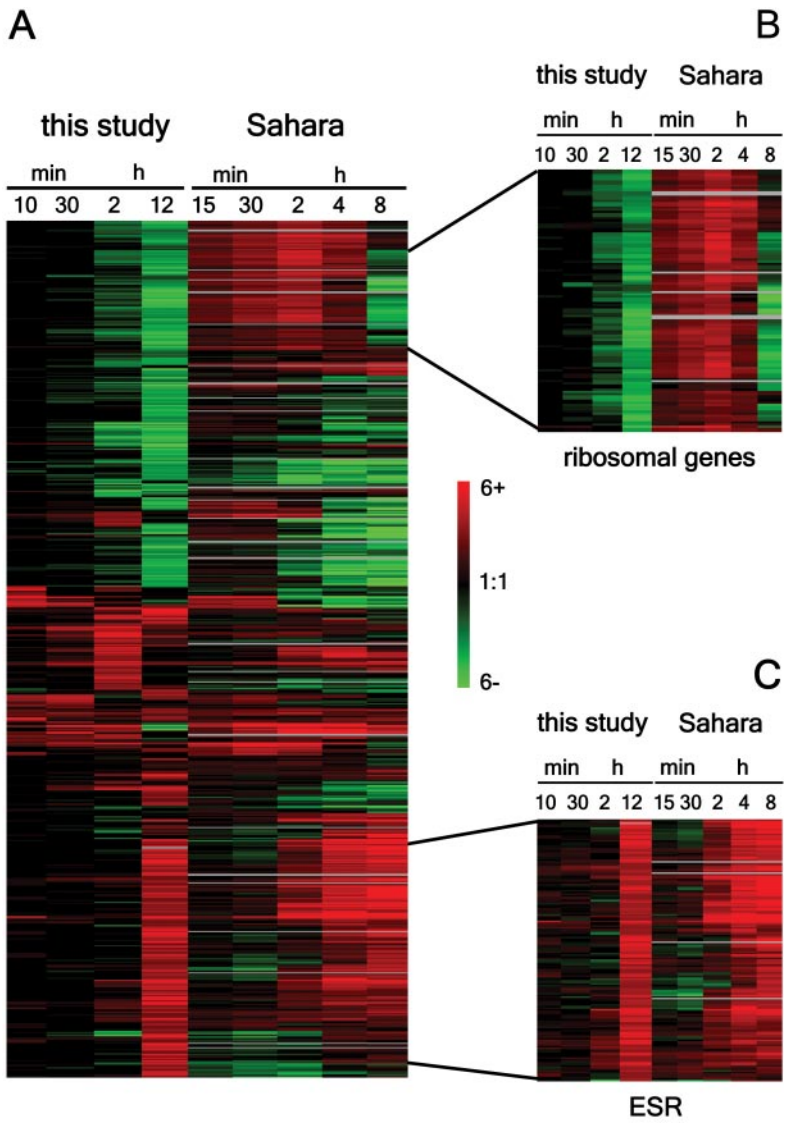

Figure 6. Comparison of the transcriptional response to cold (10°C) observed in this study to that reported by Sahara *et al*. (2002). The transcriptional profiles of the 634 cold-responsive genes identified in this study were clustered according to their transcriptional profiles in both studies using GeneSpring (standard correlations). In such a representation, each gene is represented by a single line and colored according to its change in transcript abundance under the indicated condition. Fold changes in transcript abundance are represented by a color scale as indicated. Gray indicates genes for which there are no data in the Sahara *et al*. (2002) set. (A) The complete data set. (B) Close-up representation of a cluster from (A), enriched in genes encoding ribosomal proteins, and showing substantial differences between the two studies. (C) Close-up representation of a cluster from A, enriched in environmental stress genes that showed increased transcript abundance in response to cold in both studies.

The gene expression program activated during the LCR includes metabolic genes and stress genes, possibly to compensate for cold-related reduction in enzyme activities and to synthesize stress-protective molecules. In yeast, trehalose has been shown to protect cells against autolysis (Attfield, 1997), to increase freezing tolerance (Diniz-Mendes *et al*., 1999), and to stabilize membrane structures (Iwahashi *et al*., 1995), and it facilitates protein folding by Hsp104p (Simola *et al*., 2000). Both trehalose and glycogen accumulate in cells subjected to heat shock, oxidative stress, or osmotic stress (François and Parrou, 2001), and we observed accumulation of both reserve carbohydrates during the LCR, confirming the observed induction of the genes involved in their synthesis. Genes involved in trehalose and glycogen catabolism also were induced, which at first sight seems paradoxical. However, similar observations have been made for other stress conditions, and it was suggested that stress stimulates recycling of glycogen and trehalose (Parrou *et al*., 1997, 1999). It was recently reported that induction of trehalose-synthesizing enzymes is important for the survival of yeast cells at near-freezing and freezing temperatures (Kandror *et al*., 2004), and similar observations were made in *E. coli* (Kandror *et al*., 2002). Together, these data suggest that trehalose is an important component of the cold-adaptation process. However, we could not detect a decrease either in growth rate or in viability when either a Δ*tps1* Δ*tps2* strain (which does not accumulate trehalose; Bell *et al*., 1998) or a Δ*msn2* Δ*msn4* strain (which does not accumulate either trehalose or glycogen; Parrou *et al*., 1997) was incubated at 10°C under our experimental conditions (our unpublished data).

A variety of HSP genes were also found in the set of LCR induced genes, suggesting a requirement for molecular chaperones for protein folding and maintaining protein conformation in the cold. The induction of HSP genes has been described in response to a variety of stress conditions, including near-freezing temperatures and freezing (Gasch *et al*., 2000; Rep *et al*., 2000; Causton *et al*., 2001; Kandror *et al*., 2004). Proteins that cannot be folded properly are targeted for degradation, and genes that are involved in protein degradation (ubiquitination enzymes, proteasome components, and proteases) also are induced during the LCR.

A second set of general stress response genes belongs to the glutathione/glutaredoxin system. Glutaredoxin is involved in protein folding, sulfur metabolism, and repair of oxidatively damaged proteins (Grant, 2001). Various studies in yeast and plants have reported an increase of intracellular

H_2O_2 concentration and the induction of antioxidant genes during exposure to cold (Prasad *et al*., 1994; O'Kane *et al*., 1996; Zhang *et al*., 2003), which also may result in increased levels of toxic metabolites caused by lipid peroxidation (Grant, 2001). Consistent with this possibility, the transcriptional profile of genes of the glutathione/glutaredoxin system suggests activation during the LCR.

We have compared the global stress-transcription profiles of *S. cerevisiae* (Gasch *et al*., 2000) with the gene expression data for the response to cold (Sahara *et al*., 2002; this study). However, comparisons of microarray data obtained from different laboratories have potential problems. For example, there are neither standard applications for global transcriptional profile measurement and data analysis nor standard conditions used for stress induction. Thus, quantitative comparisons must be interpreted cautiously even when the other data sets have been reconstructed to fit our applied data analysis strategy. By comparing the data for the 634 cold-responsive genes identified in our study to the transcriptional cold response described by Sahara *et al*. (2002), we observed a common cluster of genes during the LCR that includes various general stress-response genes (Figure 6, A and C). However, there were major differences between the two data sets during the ECR (Figure 6, A and B). For instance, Sahara *et al*. (2002) described the induction of ribosomal genes during short cold treatments, whereas we observed a decrease in transcript abundance for ribosomal genes. This discrepancy may be due to differences in strain background or in experimental design. For example, we used cells in early log phase in contrast to the mid-log phase cells (OD_{600} of 2–4) examined by Sahara *et al*. (2002). Another recent publication also studied the global transcriptional response of yeast grown at 4, 15, or 35°C (Homma *et al*., 2003). However, direct comparisons of these data with ours or with the data from Gasch *et al*. (2000) or Sahara *et al*. (2002) could not be performed, because the expression data (fold variation) of Homma *et al*. (2003) were not available in a suitable form.

Comparison of our data with those of Gasch *et al*. (2000) has yielded some interesting results. There was a significant overlap between the LCR and ESR genes, indicating that the environmental stress response is activated during the LCR, whereas comparison with the ECR revealed very different expression profiles, suggesting a reciprocal stress response during the first 2 h of cold adaptation. Such a reciprocal expression pattern has been observed previously when yeast cells were subjected to opposite stresses (Gasch *et al*., 2000). In addition, a similar phenomenon (repression of heat shock proteins during cold shock) has been described in bacteria (Jones and Inouye, 1994; Graumann and Marahiel, 1999), suggesting that the responses to cold and heat are generally counteractive.

The general stress induction during the LCR is controlled by the Msn2p/Msn4p transcription factors, as shown by analysis of a Δ*msn2* Δ*msn4* strain, and its activation is supported by the accumulation of glycogen and trehalose in cold-treated cells. About 36% of the LCR induced genes, including classical target genes such as HSP genes and genes encoding enzymes of both glycogen and trehalose synthesis (Boy-Marcotte *et al*., 1998; Moskvina *et al*., 1998), required Msn2p/Msn4p for their induction (Figure 4). However, another substantial portion of the LCR genes was regulated in an Msn2p/Msn4p-independent manner, indicating that other regulators also may govern the LCR. The regulation of a subset of LCR repressed and induced genes has been shown to be dependent on the protein kinase A pathway in response to carbon source and on the protein kinase C pathway in response to a defective secretory pathway (Klein and Struhl, 1994; Neuman-Silberberg *et al*., 1995; Nierras and Warner, 1999; Mutka and Walter, 2001).

Interestingly, the ECR transcriptional pattern was unchanged in a Δ*msn2* Δ*msn4* double mutant. Together with the comparison of the ECR gene expression profile to those observed with other forms of stresses, this observation suggests a cold-specific mechanism for the ECR, which may involve as yet uncharacterized regulatory factors.

In summary, the transcriptional cold response of *S. cerevisiae* is comprised of two distinct expression patterns during the early and late phases. The early phase may produce adjustments of membrane fluidity and destabilization of RNA secondary structures to allow efficient protein translation. The late phase involves the environmental stress response and is presumably a consequence of the altered physiological state of the cell caused by decreased transport, accumulation of misfolded proteins, and reduced enzyme activities. The expression data also indicate the involvement of other regulatory mechanisms. The transcriptional response to cold involves both general stress and cold-specific mechanisms and it is likely that multiple other physiological changes also contribute to survival and growth at low temperatures, including cellular processes regulated at the translational and/or posttranslational level. Further experiments will be needed to elucidate the key regulatory mechanisms that allow cells to survive and grow in the cold.

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