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Comprehensive Expression Analysis of Time-dependent Genetic Responses in Yeast Cells to Low Temperature*

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We performed genome-wide expression analysis to determine genetic responses in *Saccharomyces cerevisiae* to a low temperature environment using a cDNA microarray. Approximately 25% of the genes in the yeast genome were found to be involved in the response of yeast to low temperature. This finding of a large number of genes being involved in the response to low temperature enabled us to give a functional interpretation to the genetic responses to the stimulus. Functional and clustering analyses of temporal changes in gene expression revealed that global states of the expressions of up-regulated genes could be characterized as having three phases (the early, middle, and late phases). In each phase, genes related to rRNA synthesis, ribosomal proteins, or several stress responses are time-dependently up-regulated, respectively. Through these phases, yeast cells may improve reduced efficiency of translation and enhance cell protection mechanisms to survive under a low temperature condition. Furthermore, these time-dependent regulations of these genes would be controlled by the cAMP-protein kinase A pathway. The results of our study provide a global description of transcriptional response for adaptation to low temperature in yeast cells.

Low temperatures are known to have several effects on biochemical and physiological properties in various cells (*e.g.* low efficiency of protein translation, low fluidity of cellular membrane, stabilization of double helix or secondary structure of DNA or RNA molecule, slow folding of protein, and decrease of enzymatic activities) (1–3). Most organisms would have developed adaptive mechanisms to cope with these phenomena. The mechanisms underlying low temperature-dependent gene expression and responses to low temperature have been studied in few organisms (4–6).

In prokaryotes, especially *Escherichia coli*, when cells grown at 37 °C are exposed to a low temperature, such as 15 °C, a set of proteins called cold shock proteins are transiently induced (7). CspA has been identified as a major cold shock protein (8) and has been suggested to act as an RNA chaperone to increase efficiency of translation under a low temperature condition (9). It has been reported that *Bacillus* and *Synechococcus* species increase synthesis and stability of desaturases, which catalyze unsaturation of fatty acids in the membrane phopholipids under a low temperature condition (4, 10). Induction of desaturases by low temperature has also been found in eukaryotic species, such as plants (5, 6), protozoan (12), dimorphic fungus (13), fish (14), and yeast (15). These findings suggest that cellular responses to low temperature, such as improvement of reduced translation and decreased membrane fluidity, and their mechanisms are common in various organisms.

In yeast, *Saccharomyces cerevisiae*, several cold-inducible genes have been identified. *NSR1*, one of the cold-inducible genes, encodes a nucleolin-like protein related to rRNA processing and ribosomal biosynthesis (16–18). *TIP1* (temperature-inducible protein) and the other members of its family (*TIR1* and *TIR2*) are also induced by cold shock (19, 20). These genes encode a serine- and alanine-rich protein and are also induced by an anaerobic growth condition (21, 22). The *OLE1* gene, encoding sole Δ9 fatty acid desaturase, has been shown to be induced by hypoxia (23–25) and low temperature (15) through ubiquitin/proteasome-dependent processing of membrane-bound transcription factors (15, 26, 27). However, the entire mechanisms of low temperature response and low temperature-dependent gene expression are still unclear in any of organisms.

In this study, we analyzed global gene expression in low temperature-exposed yeast cells using a yeast cDNA microarray to obtain fundamental information on low temperature response and low temperature-dependent gene expression in yeast cells. Several sets of cooperatively regulated genes were identified by clustering of time-dependent gene expression profiles and functional analyses. Our findings suggest that several steps of global expression changes play an important role in adaptation to a low temperature environment.

EXPERIMENTAL PROCEDURES

Materials—An *S. cerevisiae* cDNA microarray was purchased from DNA Chip Research Inc. (Kanagawa, Japan). All other reagents were of the highest grade available.

Strains and Culture Conditions—*S. cerevisiae* YPH500 (*MATα*, *ura3–52*, *lys2–801*, *ade2–101*, *trp1-Δ63*, *his3-Δ200*, *leu2-Δ1*) (28) was used for all analyses. Unless otherwise noted, yeast cells were cultured aerobically in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30 °C and shaken at 100 rpm.

Sample Collection and RNA Isolation—Yeast cells were grown to a midlog phase (A_{600} = 2.0), and then 50 ml of the yeast culture was collected for a time 0 reference. The cells were harvested by centrifugation at 3,000 × *g* for 5 min at 30 °C. The harvested cells were flash-frozen in liquid nitrogen and stored at −80 °C until RNA preparation. The remaining cultured cells were cold-shocked at 10 °C in a precooled water bath and were then aerobically cultured at 10 °C at 100 rpm. Cells were collected at 0.25, 0.5, 2, 4, and 8 h after the cold shock by centrifugation at 3,000 × *g* for 5 min at 10 °C. The harvested cells were also flash-frozen and stored as described above.

Total RNA was prepared by an acidic phenol method (29) and further purified by using an RNeasy Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

Probe Preparation and Microarray Hybridization—Total RNA (15 μg) and 5 μg of oligo(dT) (Amersham Biosciences) were used to prepare fluorophore-labeled cDNA probes for array hybridization. In all experiments, Cy3-dUTP (Amersham Biosciences) and Cy5-dUTP (Amersham Biosciences) were used to label the time 0 reference and experimental samples (cold-shocked samples), respectively. Microarray hybridiza-

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TABLE I
Global view of changes in expression of yeast genes after exposure to low temperature

	Total genes[a]	No. of genes				
		15 min	30 min	2.0 h	4.0 h	8.0 h
≥2-Fold up-regulated genes						
Cell growth, cell division and DNA synthesis	(836)	3	12	24	32	51
Cell rescue, defense, cell death and aging	(369)	3	4	16	38	46
Cellular communication, signal transduction	(135)	1	2	3	7	10
Cellular transport and transport mechanisms	(498)	1	7	13	24	36
Energy	(252)	1	4	9	29	46
Ionic homeostasis	(124)	2	3	5	8	13
Metabolism	(1065)	9	24	39	78	121
Protein destination	(589)	1	5	14	28	51
Protein synthesis	(359)	1	28	104	30	13
Transcription	(793)	10	42	43	37	48
Transport facilitation	(311)	3	8	16	20	27
Unclassified proteins	(2420)	16	73	90	190	209
Total number of genes		41	188	323	438	536
≤2-Fold down-regulated genes						
Cell growth, cell division and DNA synthesis	(836)	0	1	26	54	61
Cell rescue, defense, cell death and aging	(369)	1	5	16	16	24
Cellular communication, signal transduction	(135)	0	0	7	8	9
Cellular transport and transport mechanisms	(498)	0	1	9	18	24
Energy	(252)	1	3	9	9	14
Ionic homeostasis	(124)	1	1	6	9	7
Metabolism	(1065)	1	2	35	94	117
Protein destination	(589)	1	2	11	23	23
Protein synthesis	(359)	0	0	2	6	41
Transcription	(793)	0	2	19	35	38
Transport facilitation	(311)	0	1	3	16	18
Unclassified proteins	(2420)	0	8	125	121	175
Total number of genes		4	27	246	361	488

[a] Total number of genes classified in each category according to the MIPS functional database (mips.gsf.de/proj/yeast/CYGD/db/index.html) is indicated in parentheses. The number of genes up- or down-regulated by ≥2-fold at each time point is represented.

tions were carried out according to the manufacturer's manual for the *S. cerevisiae* cDNA microarray. After the hybridization, microarrays were sequentially washed at room temperature in the following solutions: 2× SSC for 20 min; 2× SSC, 0.1% SDS for 20 min; 0.2× SSC, 0.1% SDS for 20 min; 0.2× SSC for 5 min; and 0.05× SSC for 5 min. All experiments were carried out in duplicate.

Data Acquisition and Analysis—Microarrays were scanned by a scanning laser microscope, GenePix 4000A (Axon Instruments, Foster City, CA). Images obtained by the scannings were analyzed by a computer program, GenePix Pro 3.0 (Axon Instruments). Quantified fluorescence intensities for all spots were exported to a microarray data analysis software, GeneSpring 4.2 (Silicon Genetics) and then normalized by the algorithm of a "per chip normalization" method in the analysis software. Expression ratio values from two independent experiments were averaged by the analysis software. The analysis software was also used for further analyses (functional analyses and clustering analyses).

In this study, we used the clustering algorithm "Pearson correlation around zero" in the data analysis software with adequate parameters: separation ratio = 0.5, minimum distance = 0.001. After the clustering analysis, we referred to the Munich Information Center for Protein Sequences (MIPS)[1] functional data base (available on the World Wide Web at mips.gsf.de/proj/yeast/CYGD/db/index.html) and other databases to determine functional relationships among genes in each cluster using the analysis software. As a result, some gene clusters in which members serve identical or similar cellular functions were found.

RESULTS AND DISCUSSION

Global Expression Analysis of Low Temperature Response in Yeast Cells Using a cDNA Microarray—Changes in global expression of genes in yeast cells after exposure to low temperature were analyzed using a microarray containing cDNAs of 5,803 genes in a yeast genome. RNA samples were prepared from yeast cells collected at 0, 0.25, 0.5, 2, 4, and 8 h after a temperature downshift from 30 to 10 °C. During a period of 8 h of exposure to low temperature, yeast cells have continuously grown at a midlogarithmic phase (*e.g.* A_{600} = 3.7 at 8 h). Since A_{600} at a stationary phase was over 10, the diauxic shift should not occur in the cells we used. Indeed, mRNA levels of some diauxic shift-inducible genes (*ACO1*, *CIT1*, and *FUM1*) (30) were down-regulated at 10 °C during 8 h of the low temperature exposure (see Fig. 5, cluster 5F).

We first labeled the time 0 reference sample with Cy3 or Cy5 for microarray analysis to confirm identical labeling efficiency of transcripts between these dyes. In this experiment, the ratio of fluorescent intensities for these dyes was within 2-fold for 98.5% of all cDNA spots on the microarray (data not shown). From this result, we defined significant gene expression change as ≥2-fold change in signal intensity.

Low temperature affected expression levels of ~25% of the yeast genes on the microarray. During the period of exposure to low temperature, the number of genes that were up-regulated by ≥2-fold increased from 41 (at 15 min) to 536 (at 8 h) (Table I, the line of "Total number of genes" in ≥2 fold up-regulated genes). On the other hand, the number of genes down-regulated by ≤2-fold also increased from 4 (at 15 min) to 488 (at 8 h) (Table I, the line of "Total number of genes" in ≤2-fold down-regulated genes). In total, 934 and 756 genes were ≥2-fold up-regulated and ≤2-fold down-regulated during the 8-h period of exposure to low temperature, respectively (data are available on the World Wide Web at staff.aist.go.jp/t-sahara/). We classified significantly up- or down-regulated genes into some functional categories according to the MIPS functional data base. The number of ≥2-fold up- or ≤2-fold down-regulated genes during the period of exposure to low temperature increased in almost all categories (Table I). This result suggests that drastic changes in gene expression programs at a genome-wide level are elicited by a low temperature stimulus to allow yeast cells to adapt to the low temperature environment, as seen in cells exposed to other environmental stresses such as heat, salinity,

[1] The abbreviations used are: MIPS, Munich Information Center for Protein Sequences; RP, ribosomal protein; HSP, heat shock protein; PKA, protein kinase A.

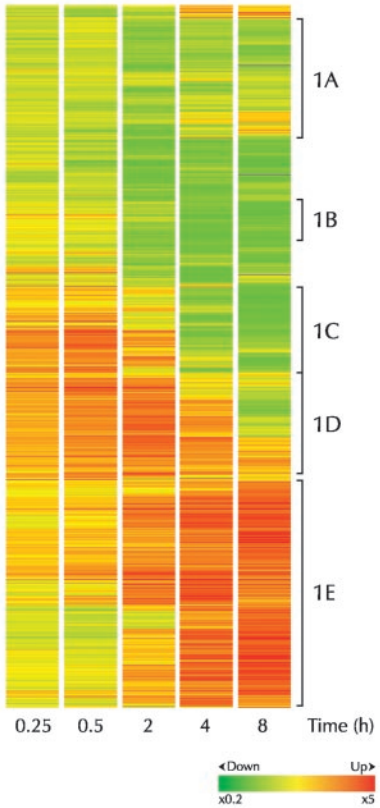

FIG. 1. **Global view of ≥2-fold expression change by hierarchical clustering analysis.** Significantly up- or down-regulated genes were analyzed by a clustering method as described under "Experimental Procedures." *Yellow* denotes no significant difference between the amounts of transcripts in the time 0 reference and a low temperature-exposed sample; *red* and *green* denote transcripts that are more and less abundant in the low temperature-exposed cells, respectively. The intensity of the *colors* is proportional to $\log_{10}$ of the -fold increase or decrease, with maximal intensity corresponding to a 5-fold increase or decrease as represented in the *color scale* (*bottom*). Annotations for clusters given by the microarray data analysis software are shown on the *right*: cluster 1A, unclassified proteins; cluster 1B, amino acid biosynthesis and metabolism; cluster 1C, RNA polymerase I and RNA processing; cluster 1D, ribosomal proteins; cluster 1E, no annotation.

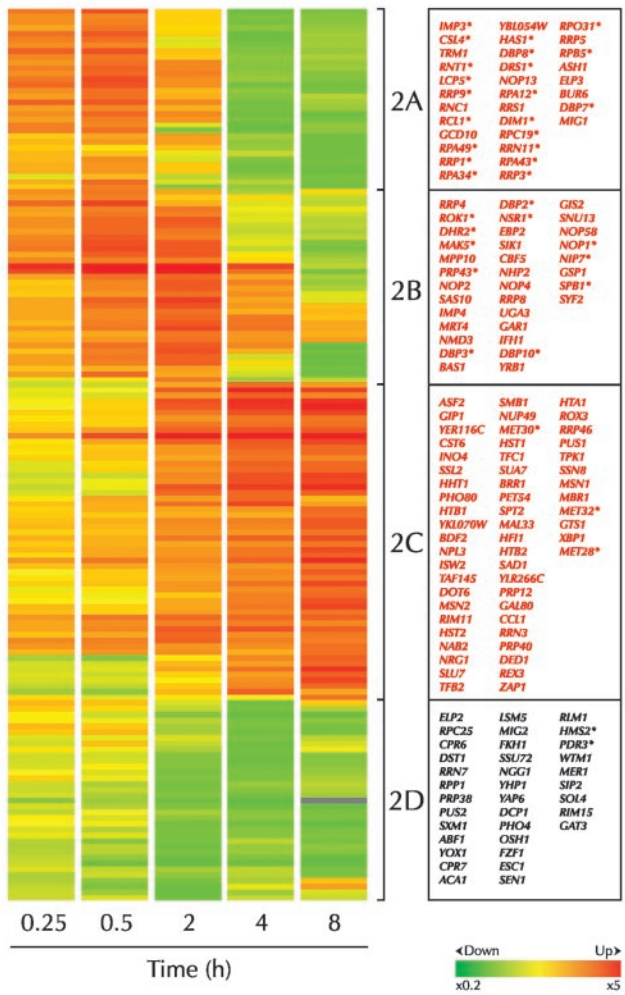

FIG. 2. **Expression profiles of genes classified in the category "transcription."** Significantly up- or down-regulated genes (157 genes) classified in the category of transcription in the MIPS functional data base were analyzed by the clustering method. The results obtained from the analysis are shown in the same manner as the results shown in Fig. 1. Annotations for clusters are as follows: cluster 2A, RNA polymerase I and RNA processing; cluster 2B, rRNA processing; clusters 2C and 2D, mRNA transcription. Genes classified into these clusters are listed in the *columns* on the *right*. Genes printed in *red* are those that were up-regulated by ≥2-fold at any time point. Genes described in this paper are indicated with *asterisks*.

hydrogen peroxide, and osmotic stresses (31–34).

Clustering Analysis of Global Expression Data—Interestingly, ≥2-fold up-regulated genes were divided into three clusters according to their expression profiles (Fig. 1, clusters 1C, 1D, and 1E). In cluster 1C, almost all genes were up-regulated within 30 min (early phase) after exposure to low temperature. Genes in clusters 1D and 1E harbored expression profiles of high up-regulation at 2 h (middle phase) and at 4–8 h (late phase) after exposure to low temperature, respectively. These results suggest that the clustering algorithm successfully identifies cooperatively regulated genes according to the expression profiles of genes. Thus, we performed further clustering analysis of expression profiles for functionally categorized genes to investigate the relevance of the expression profiles to their functions. Significantly up- or down-regulated genes in each functional category of the MIPS functional data base (see Table I) were analyzed by the same clustering method. As we will discuss below, the clustering analyses provided novel insights for understanding cellular functions and the transcriptional regulatory mechanisms for several genes in low temperature stress responses of yeast cells.

Genes Related to Transcription—The "RNA polymerase I and RNA processing" cluster (Fig. 1, cluster 1C) contains genes up-regulated at the early phase. A notable feature in this cluster was cooperative regulation of genes for transcriptional machinery, especially RNA polymerase I. In yeast, RNA polymerase I synthesizes large rRNAs. These rRNAs are first transcribed as a precursor 35 S rRNA and then processed into the mature 18, 5.8, and 25 S rRNAs found in ribosomes. Several components of the RNA polymerase I core unit (*RPA12*, *RPA34*, *RPA43*, *RPA49*, *RPB5*, *RPC19*, and *RPO31*) and a component of the core factor (*RRN11*) were similarly co-regulated (Fig. 2, cluster 2A). In addition, other RNA polymerase I core components (*RPA14*, *RPA135*, and *RPA190*) and a core factor (*RRN7*) were also increased by ≥1.5-fold with similar expression profiles (data not shown). The genes for RNA polymerase I components were up-regulated in the early phase of low temperature exposure, and this up-regulation was followed by abrupt down-regulation (Fig. 2, cluster 2A). Similar expression profiles were also observed for genes related to rRNA processing such as RNA helicases (*DBP2*, *DBP3*, *DBP7*, *DBP8*, *DBP10*, *DHR2*, *DRS1*, *HAS1*, *MAK5*, *ROK1*, *RRP3*, and *PRP43*), ribonucleases (*RNT1*, *RRP1*, and *RRP9*), and other rRNA processing components (*CSL4*, *DIM1*, *IMP3*, *LCP5*, *NIP7*, *NOP1*, *NSR1*, *RCL1*,

and *SPB1*) (Fig. 2, clusters 2A and 2B). Thirty-four of the 41 genes related to transcription in cluster 1C (Fig. 1) participate directly in rRNA synthesis.

On the other hand, as shown in Fig. 2, genes involved in mRNA transcription were clearly divided into two clusters (clusters 2C and 2D). Cluster 2D contained genes that were down-regulated by exposure to low temperature, whereas genes in cluster 2C were up-regulated in the late phase. Genes involved in various transcription control factors were found in these clusters. For instance, the *HMS2* gene, which encodes a heat shock transcription factor homolog (35), and the *PDR3* gene, which encodes a transcription factor for multiple drug resistance genes (36), were down-regulated (Fig. 2, cluster 2D), whereas genes encoding regulatory proteins for biosynthesis of sulfur amino acids (*MET28*, *MET30*, and *MET32*) (37) were cooperatively up-regulated in the late phase (Fig. 2, cluster 2C). The down- or up-regulation of various transcription factors seems to cause various changes in expression states in a large number of their target genes in a later phase.

In summary, our findings suggest that the transcriptional machinery and processing machinery for rRNAs are up-regulated in concert in the early phase of low temperature exposure (discussed below), whereas genes for mRNA synthesis and transcriptional regulation made diverse responses in the late phase.

Ribosomal Protein Genes—Genes for ribosomal proteins (RPs) accounted for the majority of up-regulated genes at 2 h (94 of 323 genes up-regulated by ≥2-fold in Fig. 1, cluster 1D), and these genes were afterward down-regulated in the late phase (Fig. 3, clusters 3A and 3B). The transcripts that had increased at 2 h included 40 proteins of a small subunit and 54 proteins of a large subunit comprising cytosolic ribosomes. A comparison of the expression profiles of the 94 up-regulated RPs revealed that almost all of the RPs had very similar expression profiles, suggesting that they are cooperatively regulated by a low temperature stimulus.

It has been known that translational ability is greatly reduced at low temperature (38). Dysfunction of several genes (*DRS1*, *MPP10*, *NSR1*, *RPS11A*, and *RPS11B*) encoding RPs and proteins involved in rRNA processing has been reported to cause cold sensitivity (39). As described above, transcripts of genes involved in rRNA synthesis and processing were clearly increased in the early phase followed by the cooperative increase of transcripts from genes encoding cytosolic ribosomal proteins in the middle phase. These results suggest that yeast cells recruit transcriptional machinery mainly for cooperative up-regulation of RP genes along with a large set of genes involved in RNA metabolism and protein synthesis in the early to middle phases. We conclude that the primary and important response to low temperature in yeast is to increase ribosomal complex to compensate for the reduced translational ability at low temperature.

Cell Rescue, Defense, Cell Death, and Aging—Among genes in the category "cell rescue, defense, cell death, and aging," expressions of genes encoding heat shock proteins (HSPs), which are up-regulated by a variety of stresses such as oxidative stress, methyl methanesulfonate treatment, and heat shock (40–42), were notably down-regulated during the period of exposure to low temperature (Fig. 4, cluster 4D). Most HSPs (*CIS3*, *HMS2*, *HSC82*, *HSP30*, *HSP60*, *HSP78*, *HSP82*, *HSP150*, *SSA1*, *SSA2*, *STI1*, and *YDJ1*) were down-regulated at all time points, whereas only *HSP12* and *HSP26* were up-regulated in the late phase (Fig. 4, cluster 4C). These results indicate that transcriptions of *HSP12* and *HSP26* are regulated in a different manner from that of other HSPs during exposure to low temperature. It has been reported that transcriptional regulations of these HSPs (*HSP12* and *HSP26*) are negatively controlled by the cAMP-PKA pathway under various stress conditions, including heat, oxidative, and osmotic shocks, and nutrient limitation (43, 44). The transcriptional regulation of these genes controlled by the cAMP-PKA pathway is discussed under "Signal Transduction Components."

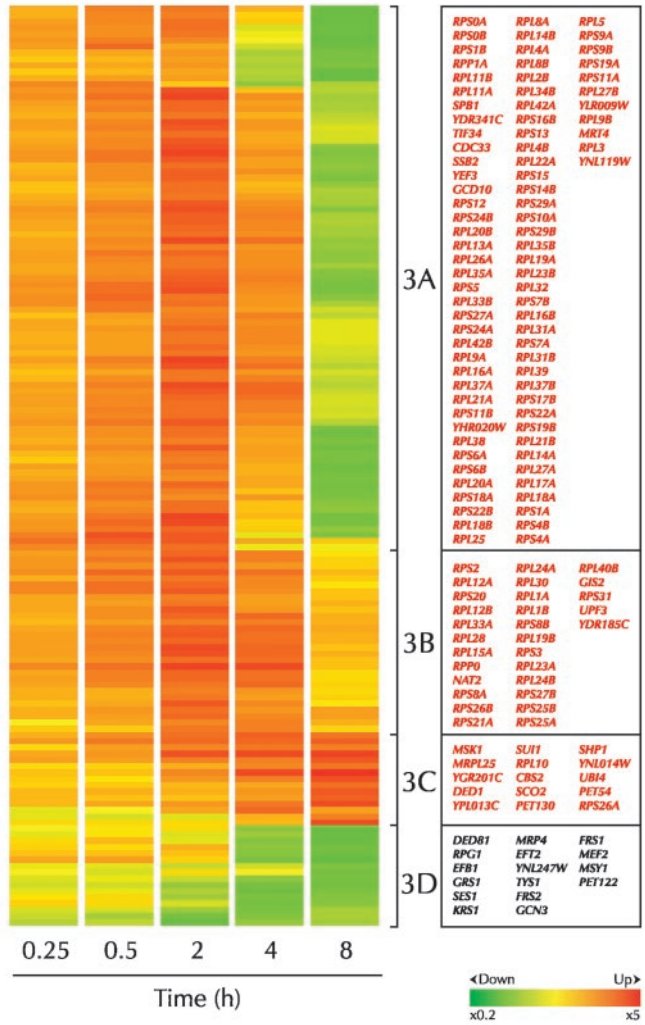

FIG. 3. **Expression profiles of genes classified in the category "protein synthesis."** Significantly up- or down-regulated genes (147 genes) classified in the category of protein synthesis in the MIPS functional data base were analyzed by the clustering method. The results obtained from the analysis are shown in the same manner as the results shown in Fig. 2. Annotations for clusters are as follows: clusters 3A and 3B, cytosolic ribosome; cluster 3C, translational control factors; cluster 3D, tRNA synthetases. Genes classified into clusters are in the *columns* on the *right*.

A process of protein folding in maturation of proteins is affected not only by heat shock but also by low temperature (1–3). In prokaryotic cells, it has been reported that peptidyl-prolyl *cis/trans*-isomerases are induced by low temperature and are thought to play an important role in the protein folding process at low temperature (2). Interestingly, it has been reported that Hsp12p associates with Cpr1p, a peptidyl-prolyl *cis/trans*-isomerase, in yeast cells (45). In addition, the amino acid sequence of Hsp12p has weak similarity with that of another peptidyl-prolyl *cis/trans*-isomerase, Fpr3p, in yeast. Taken together, although the function of Hsp12p is still unclear, Hsp12p may play a role in protein folding at low temperature.

Metabolism and Energy Production—Cooperative regulation of glycogen and trehalose biosynthesis genes was the most

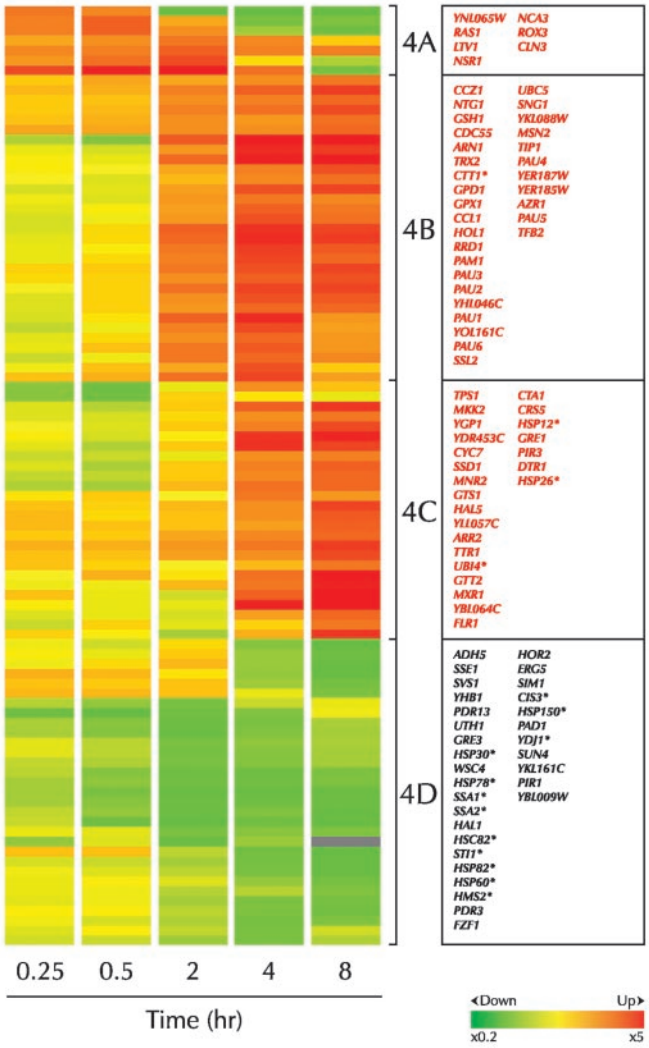

FIG. 4. **Expression profiles of genes classified in the category "cell rescue, defense, cell death, and aging."** Significantly up- or down-regulated genes (95 genes) classified in the category of cell rescue, defense, cell death, and aging in the MIPS functional data base were analyzed by the clustering method. The results obtained from the analysis are shown in the same manner as the results shown in Fig. 2. Annotations for clusters are as follows: cluster 4A, not annotated; clusters 4B and 4C, stress response; cluster 4D, stress response and chaperon. Genes classified into clusters are in the *columns* on the *right*. Genes described in this paper are indicated with *asterisks*.

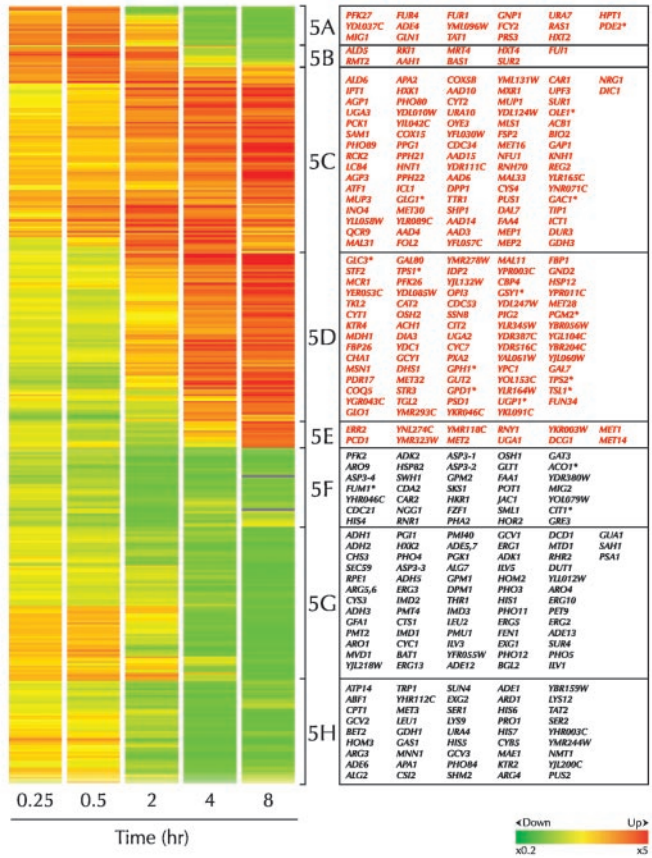

FIG. 5. **Expression profiles of genes classified in the category "energy and metabolism."** Significantly up- or down-regulated genes (343 genes) classified in the category of energy and metabolism in the MIPS functional data base were analyzed by the clustering method. The results obtained from the analysis are shown in the same manner as that of results shown in Fig. 2. Annotations for clusters are as follows: cluster 5A, nucleotide metabolism; clusters 5B and 5E, not annotated; clusters 5C and 5D, C-compound and carbohydrate metabolism; clusters 5F and 5H, amino acid metabolism; cluster 5G, C-compound and carbohydrate utilization. Genes classified into clusters are in the columns on the *right*. Genes described in this paper are indicated with *asterisks*.

notable feature in the clustering analysis of the category "metabolism and energy production." Glycogen and trehalose are two major reserve carbohydrates in yeast cells and are accumulated a level of up to 25% of dry cell mass, depending on environmental conditions (46).

Parrou *et al.* (47) reported accumulation of glycogen and cooperative regulation of the following genes involved in glycogen synthesis in cells exposed to heat, osmotic and oxidative stresses: *GLG1* (a glycogen synthesis initiator), *GSY1* (glycogen synthase), *GLC3* (1,4-glucan-6-(1,4-glucano)-transferase), *GAC1* (a regulatory subunit for phosphoprotein phosphatase type 1, also known as Glc7p, which regulates glycogen synthase-2), and *GPD1* (glycerol-3-phosphate dehydrogenase). We found that these genes were up-regulated in the middle to late phases (Fig. 5, clusters 5C and 5D). However, we also found that *GPH1* (glycogen phosphorylase), which is involved in glycogen degradation, was up-regulated similarly to genes involved in glycogen synthesis (Fig. 5, cluster 5D). These paradoxical simultaneous up-regulations of genes involved in synthesis and degradation of glycogen have already been discussed for cells exposed to heat shock (47, 48). Those studies demonstrated that glycogen was not abundantly accumulated and was futilely recycled despite significant increases in transcripts for genes involved in synthesis of glycogen by heat, hydroxyperoxide, or salt stress. However, the roles of the recycling of glycogen under some stress conditions have not yet been clarified.

It has been reported that trehalose synthesis is stimulated by heat shock and osmotic stress (49–51) and that its accumulation correlates with thermotolerance of yeast cells (52–54). In our analysis, trehalose synthesis seemed to be stimulated by low temperature through cooperative regulation of a trehalose-synthesis multicomplex: *PGM2* (phosphoglucomutase), *UGP1* (UDP-glucose phrophosphorylase), *TPS1* and *TPS2* (trehalose-6-phosphate synthases), and *TSL1* (a 123-kDa regulatory subunit of trehalose-6-phosphate synthase-phosphatase complex) (Fig. 5, cluster 5D). Most genes of the components of this multicomplex were down-regulated in the early to middle phases and then up-regulated in the late phase of low temperature exposure. Furthermore, *NTH1*, which encodes a cytosolic neutral trehalase that mainly catalyzes hydrolysis of trehalose in yeast cells (55–57), was down-regulated at all time points by slightly less than 2-fold (data not shown). These results suggest

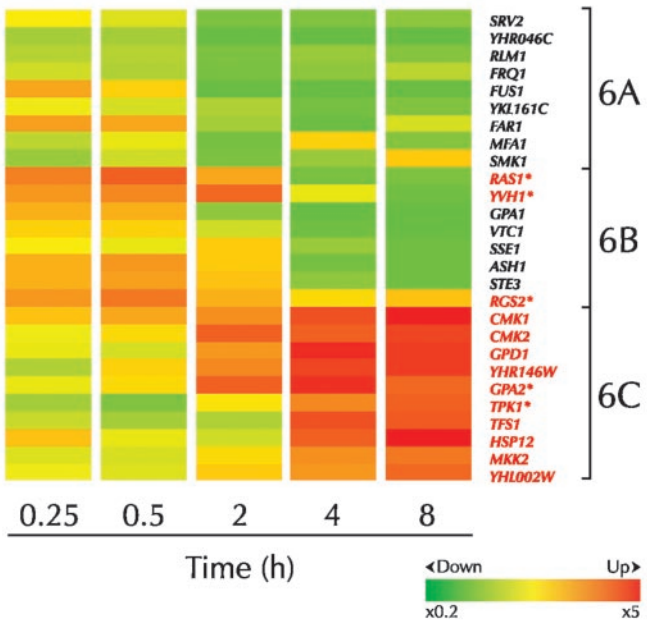

FIG. 6. **Expression profiles of genes classified in the category "signal transduction."** Significantly up- or down-regulated genes (27 genes) classified in the category of signal transduction in the MIPS functional data base were analyzed by the clustering method. The results obtained from the analysis are shown in the same manner as the results as shown in Fig. 2. No annotations were given by the microarray analysis software to the clusters. The gene name for each expression profile is shown in the *column* on the *right*. Genes described in this paper are indicated with *asterisks*.

that trehalose synthesis is induced to accumulate trehalose in yeast cells by low temperature stimulus as well as other stresses.

As a result, trehalose seems to be accumulated at the late phase, when yeast cells are exposed to low temperature. Recently, it has been reported that trehalose biosynthesis is induced in low temperature-exposed *E. coli* cells, and trehalose is essential for the viability of the cells at low temperature (58). In addition, exogenous trehalose restored viability of yeast cells during freezing by possible protection of cellular membrane (59). At high temperature, trehalose can protect cells by acting as a "chemical chaperone" (60), which reduces heat-induced denaturation and aggregation of proteins in yeast cells (61, 62). Considering these results, trehalose may retain viability of yeast cells at low temperature by similar mechanisms.

Signal Transduction Components—Expression levels of 27 of the 135 genes related to signal transduction were changed by ≥2-fold during the period of exposure to low temperature (Fig. 6). In these genes, several components in the cAMP-PKA pathway (43, 63, 64) were notably up-regulated (Fig. 6, clusters 6B and 6C). For instance, *TPK1* (a cAMP-dependent protein kinase; *i.e.* one of the positive effectors) (Fig. 6, cluster 6C) and *PDE2* (a high affinity cAMP phosphodiesterase) (Fig. 5, cluster 5A) were up-regulated. Additionally, *TPK2* (another positive effector of cAMP-dependent protein kinase) was also up-regulated by ≥1.5-fold (data not shown). Furthermore, we found that several genes, *GPA2* (a nucleotide-binding regulatory protein), *RGS2* (a GTPase-activating protein), *RAS1* (an RAS small monomeric GTPase), and *YVH1* (protein-tyrosine phosphatase) (Fig. 6, clusters 6B and 6C), all of which are upstream regulatory components of PKA, were also up-regulated. Induction of PKA-signaling components (especially *TPK1* and *TPK2*) has been reported as one of the notable features of responses to the environmental stresses, such as heat shock, nitrogen depletion, and diauxic shift, and is controlled by Msn2p/4p transcription factors (32).

The cAMP-PKA pathway plays a major role in the control of metabolism, stress resistance, and cell proliferation (64). Downstream targets of activated PKA include many housekeeping gene products and enzymes as well as proteins that are important for stress resistance and cell cycle control. Low activity of PKA causes expression of stationary phase characteristics (*e.g.* high trehalose and glycogen concentrations, high stress resistance, and derepression of STRE-controlled genes) during exponential growth in a glucose medium. Recently, Causton *et al.* (33) demonstrated that Msn2p/4p regulate expressions of 136 genes cooperatively under several stress conditions. We found that 68 of the Msn2p/4p-regulated genes were up-regulated by ≥2-fold in the late phase of low temperature exposure. These 68 genes included genes involved in glycogen synthesis (*GAC1*, *GLC3*, and *GPD1*) (Fig. 5, clusters 5C and 5D), trehalose synthesis (*TPS1* and *TPS2*) (Fig. 5, cluster 5D), and stress resistance (*UBI4*, *CTT1*, *HSP12*, and *HSP26*) (Fig. 4, clusters 4B and 4C). It has also been reported that several yeast heat shock gene promoters (*e.g.* the HSP70 gene *SSA3*, *UBI4*, *CTT1*, *HSP12*, and *HSP26*) are suppressed by cAMP through Msn2p/4p transcription factors (64). These results suggest that genes involved in stress response and in glycogen and trehalose biosynthesis are cooperatively regulated via Msn2p/4p and cAMP-PKA pathway in the late phase of the low temperature exposure. It has also been reported that genes involved in protein synthesis are positively controlled by cAMP (65, 66). As we discussed above (under "Genes Related to Transcription" and "Ribosomal Protein Genes"), genes involved in transcription and protein synthesis were cooperatively up-regulated in the early to middle phases of the low temperature exposure. In addition, it has been reported that PKAs from other organisms, such as insects, frogs, and a bat, were activated in an early phase after low temperature exposure (11, 67–69). In summary, the cAMP-PKA pathway may play an important role in up- and down-regulation of a large number of gene expressions in yeast cells under a low temperature condition.

CONCLUSIONS

We comprehensively analyzed expression states of genes in yeast cells exposed to low temperature using a microarray and demonstrated that a large number of genes for various cellular functions are diversely up- or down-regulated under a low temperature condition. Interestingly, using a clustering and functional analysis, we found that ≥2-fold up-regulated genes are categorized into three phases according to their expression profiles: (i) in the early phase, genes involved in RNA polymerase I and rRNA processing are up-regulated; (ii) in the middle phase, genes involved in cytosolic ribosomal proteins are up-regulated; (iii) in the late phase, genes involved in general stress response are up-regulated (Fig. 1). The result suggests that adaptation mechanisms for low temperature in yeast cells are composed of three sequential molecular events.

It is known that translational efficiency is dramatically reduced by low temperature, because of the formation of secondary structure in RNA molecules and the increase of inactivated ribosome (38). Yeast cells exposed to low temperature first recruited machinery to synthesize rRNAs by the up-regulation of genes encoding components of RNA polymerase I and proteins involved in rRNA processing in the early phase (Fig. 1, cluster 1C). Continuously, genes encoding RPs are induced in the middle phase (Fig. 1, cluster 1D). Through these steps, ribosome seems to be synthesized *de novo* to compensate for inefficient translation suffered from a low temperature condition. The restoration of translational activity will be the most urgent response in yeast to low temperature to support *de novo* synthesis of proteins needed for the restoration of various bio-

chemical and physiological properties.

After the up-regulation of genes involved in translation, about 600 genes are up-regulated in the late phase (Fig. 1, cluster 1E). Some of these genes (*e.g. CTT1*, *HSP12*, *HSP26*, and genes involved in trehalose and glycogen biosynthesis) have been known to be induced by several stress conditions (see "Cell Rescue, Defense, Cell Death, and Aging" and "Metabolism and Energy Production"). These findings suggest that proteins encoded in these genes or metabolites from these enzymes also play important roles in adaptation or tolerance of yeast cells to a low temperature condition as well as other stress conditions.

As one of possibilities, our data suggest that the cAMP-PKA pathway may play an important role for the control of gene expressions under low temperature conditions. The pathway would control positively expressions of genes encoding translational machinery in the early to middle phases followed by controlling negatively expressions of genes related to general stress response in the late phase (see "Signal Transduction Components"). At present, it is unclear why this control change by cAMP-PKA pathway happens between the middle and the late phases. One possibility is that fluidity change of cellular membrane may relate to this control change. The *OLE1* gene, which encodes the sole Δ9 desaturase in yeast, is strongly up-regulated in the middle phase (Fig. 5, cluster 5C) and increases the fluidity of cellular membrane at low temperatures. This improvement of membrane fluidity at low temperatures may affect the phosphorylation state of membrane-bound components of cAMP-PKA pathway. This change of activation state in the pathway finally may achieve global expression changes of genes governed by the pathway in the late phase. However, further investigations are required to clarify this phenomenon.

Detailed characterization of global expression profiles triggered by low-temperature stress is the first step toward elucidation of the role of each gene and each physiological system in cellular adaptation to a low temperature environment. The results have demonstrated a global view of changes in gene expressions under a low temperature condition and have suggested hypotheses for the mechanisms of their regulation. As shown in this study, genetic responses to low temperature in yeast are very diverse. Some genes respond transiently to low temperature in the early phase, whereas other genes are gradually up- or down-regulated. Therefore, a slice of gene expression profiles for a short time gives insufficient information, which may lead to a misunderstanding of the roles of genes in the response to low temperature. Further detailed analysis for mapping of the regulatory pathways that govern the low temperature stress response should provide a clear picture of the mechanisms involved in sensing of and adaptation to a low temperature environment in yeast.

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Comprehensive Expression Analysis of Time-dependent Genetic Responses in Yeast Cells to Low Temperature

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