J. Biochem. **134**, 373–384 (2003)
DOI: 10.1093/jb/mvg155

Response of Genes Associated with Mitochondrial Function to Mild Heat Stress in Yeast *Saccharomyces cerevisiae*

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Received May 12, 2003; accepted June 16, 2003

The genome-wide expression pattern of budding yeast *Saccharomyces. cerevisiae* in response to mild heat treatment in a non-fermentable carbon source was analyzed using DNA microarrays. Of 5,870 open reading frames (nuclear genome transcripts) examined, 104 genes were upregulated and 287 genes were downregulated upon shifting of the cells from 25°C to 37°C. Forty upregulated genes and 235 downregulated genes encoded localization-assigned proteins. Of 113 heat-repressible genes (excluding 122 heat-repressible ribosomal genes), 36 were mitochondria-related genes, whereas only 2 of 40 heat-inducible genes were mitochondria-related. In particular, 9 genes involved in the mitochondrial respiratory chain and 7 genes involved in mitochondrial protein translocation were significantly repressed, suggesting that mitochondrial respiratory function and biogenesis were downregulated. Consistent with these findings, the growth of yeast cells in a non-fermentable carbon source was repressed at 37°C and the mitochondria isolated from heat-stressed cells exhibited compromised preprotein-import activity compared with those from unstressed cells. In contrast, many genes involved in glycolysis and the metabolic pathway to produce glutamate via the tricarboxylic acid cycle, which is essential for biosynthetic reactions, were upregulated. Yeast cells might downregulate mitochondrial function to circumvent heat-induced oxidative stress, upregulate stress-related genes, and remodel genes for metabolic pathways in response to mild heat stress: an adaptive response at the expense of cell growth.

Key words: DNA microarrays, heat stress, mitochondria, stress response, yeast genome.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; COB, cytochrome *b* gene; COX, cytochrome c oxidase; HSP, heat shock protein; MPP, mitochondrial processing peptidase; TCA cycle, tricarboxylic acid cycle; OAA, oxaloacetic acid; ROS, reactive oxygen species; RTG-signaling, retrograde signaling; TIM, translocase of inner membrane; TOM, translocase of outer membrane.

Organisms change their gene expression pattern and regulate cellular functions to adapt to various environmental changes or stress conditions such as heat stress, osmotic stress, or oxidative stress (*1*). Mitochondria are essential organelles involved not only in oxidative ATP synthesis, but in metabolic and biosynthetic reactions such as heme or Fe-S cluster synthesis, the tricarboxylic acid (TCA) cycle, and amino acid synthesis. Most cellular reactive oxygen species (ROS) are toxic by-products of mitochondrial oxidative phosphorylation; approximately 0.4 to 4% of the molecular oxygen consumed by mitochondria is reduced by uncoupled electrons to form ROS (*2*).

In budding yeast *Saccharomyces cerevisiae*, heat treatment induces oxidative stress through ROS that are produced mainly by the heat-damaged mitochondrial electron transport system. Heat-driven oxidative stress is considered to be tightly correlated with cell viability under heat-exposed conditions in yeast. In support of this notion, (i) rho0 petite mutants with decreased amounts of mitochondrial DNA are more resistant to heat stress than wild-type cells (*3*), (ii) cells grown under anaerobic conditions are more resistant to heat stress than those grown in aerobic conditions (*4*), and (iii) deletion of NADH dehydrogenases NDE1 and NDE2, which feed electrons from NADH into the electron transport chain of the mitochondrial inner membrane, attenuates heat-mediated oxidative stress (*5*).

Although mitochondrial respiratory function thus affects the stress sensitivity of cells, it is unclear whether mitochondrial functions *per se* are modified for adaptation of the cells to heat stress. In the present study, we analyzed the genome-wide transcription profile of yeast cells under non-lethal mild heat-stress in a non-fermentable carbon source. DNA microarray analyses of nuclear genome transcripts revealed an intriguing response of genes associated with mitochondrial function, as follows: (i) A set of genes associated with the mitochondrial respiratory chain was downregulated, (ii) a set of genes involved in mitochondrial protein translocation was also downregulated, and (iii) genes encoding cytoplasmic and mitochondrial ribosomal proteins were significantly repressed. This repression was reflected in growth repression of the cells, and a reduction in mitochondrial protein translocation efficiency. In contrast to

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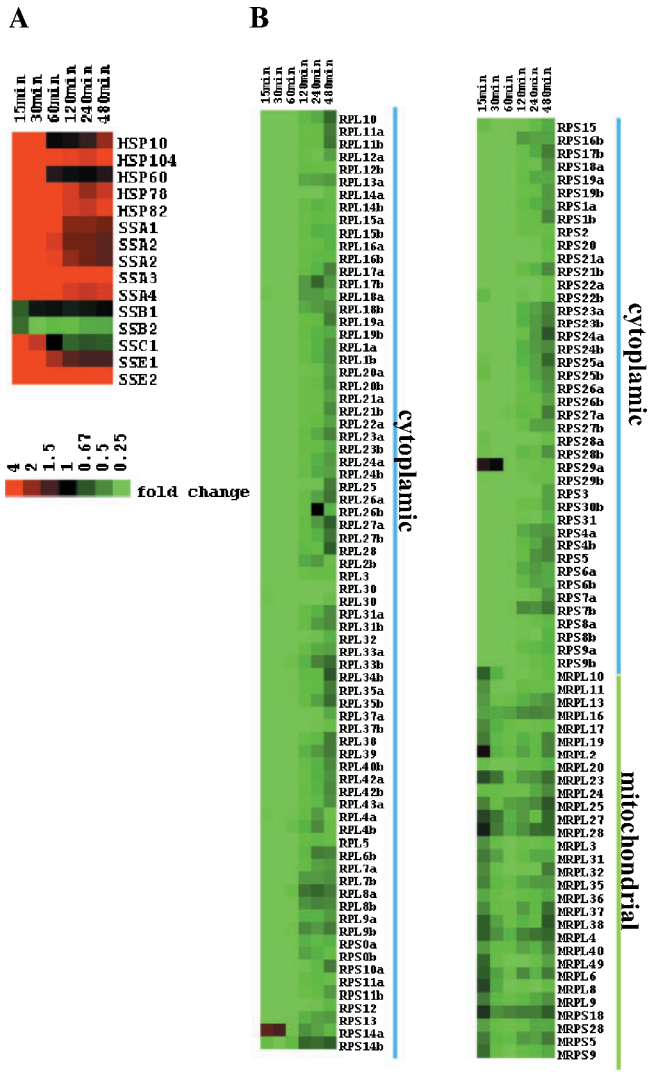

Fig. 1. **Expression profiles of heat shock response genes as visualized by "Horizontal Strip" (7).** (A) Response of heat-shock genes. (B) Response of heat-repressible ribosomal protein genes. Red and blue denote transcripts that are more or less induced, or repressed in cells after exposure to mild heat stress, respectively. The intensity of the colors is proportional to the -fold increase, with the maximum intensity corresponding to a 4-fold increase or decrease as represented by the color scale. The average values of two independent microarray analyses are visualized.

this repressive response, (iv) various stress-related genes, including heat shock protein (HSP) and anti-oxidant genes and (v) a set of genes involved in the glycolytic pathways, were upregulated. Furthermore, (vi) genes involved in the production of oxaloacetic acid (OAA) and acetyl-coenzyme A (CoA) were induced, leading to increased synthesis of α-ketoglutaric acid through the TCA cycle to maintain a sufficient glutamate level, thus maintaining a biosynthetic pathway via glutamate.

Together, these results suggest that yeast cells adapt to mild heat stress at the cost of cell growth by downregulating mitochondrial function to repress ROS production, inducing various cell-stress genes, and remodeling metabolic pathways. Because the heat-response of the mitochondria-related genes described above was mostly unaffected by the ROS scavenger ascorbic acid, factor(s) other than ROS might function as upstream effectors of the above mentioned genes under mild heat-induced stress.

MATERIALS AND METHODS

Yeast Strain and Growth Conditions—All experiments were performed with wild-type strain JK9-3d (leu2-3112; ura3-52; rme1; trp1; his4; GAL+; HMLa) provided by S. Rospert (Max-Planck Research Unit Enzymology of Protein Folding). The growth experiment (Fig. 2) was also performed with SEY6210 (MAT-alp; leu2-3,112; ura3-52; his3Δ 200; trp1Δ 901; lys2Δ 801; suc2Δ 9) and FY1679 (MAT-a; ura3-52; trp1Δ63; leu2Δ 1; his3Δ 200) provided by M. Sakaguchi (Kyushu University) and T. Ito (Kanazawa University), respectively. For microarray and Northern blot analyses, the JK9-3d strain was cultured in YPEG medium (1% yeast extract, 2% peptone, 4% ethanol, and 3% glycerol) at 25°C and grown to 1.0 OD_{600}. The cells were then shifted to 37°C and incubated further. The cells (50 OD600) were collected at 0, 0.25, 0.5, 1, 2, 4, and 8 h to isolate poly(A)-RNAs. In Fig. 4A "AA-120," the cells were grown to 1.0 OD_{600} at 25°C and culture was continued for 2 h at 25°C in the presence of 1 μg/ml antimycin A. For the growth experiment, the JK9-3d strain was cultured in either YPEG or YPD (1% yeast extract, 2% peptone, 2% glucose) at 25°C and grown to 0.1 OD600. The cells were then shifted to 37°C and incubation was continued. Cells were diluted with culture medium so that the OD600 of the cultures ranged between 0.1 and 1.0.

RNA Isolation for Microarray and Northern Blot Analyses—Extraction of yeast total RNA was performed essentially as described by Kohrer *et al.* (*6*). Frozen yeast cells (50 OD600) were suspended in 700 μl each of warmed TES solution (10 mM Tris-HCl [pH 7.5], 10 mM EDTA, and 0.5% SDS) and water-saturated phenol, and then agitated for 1 h at 65°C. After centrifugation, the aqueous phase was collected and mixed with an equal volume of chloroform. The aqueous phase collected by centrifugation was subjected to poly(A)-RNA isolation using polyATract™ system 1000 (Promega) according to the manufacturer's instructions. The obtained mRNA was dissolved in 15 μl diethylpyrocarbonate-treated water.

DNA Microarray—DNA microarrays consisting of 5,870 yeast open reading frames (ORFs; does not include mitochondrial genome-transcripts) were prepared essentially as described by DeRisi *et al.* (*7*). The ORF fragments were prepared by standard polymerase chain reaction methods using plasmids harboring each ORF as templates and, after purification by isopropanol precipitation, were spotted onto poly-L-lysine coated glass slides (Matsunami Glass) by SPBIO (Hitachi Software).

Synthesis of Cy-Labeled cDNA Probe—Fluorescently-labeled cDNA from mRNA was prepared by direct incorporation of a fluorescent nucleotide. The reaction mixture (20 μl), consisting of 2.0 μg of poly(A)-RNA, 1 pmole oligo-dT, 0.5 mM each of dATP, dCTP, and dGTP, 0.3 mM dTTP, 2 nmol either Cy3- or Cy5-dUTP (Amersham Pharmacia Biotech), and 1 U PowerScript™ Reverse Transcriptase (Clontech) in the reaction buffer provided by the manu-

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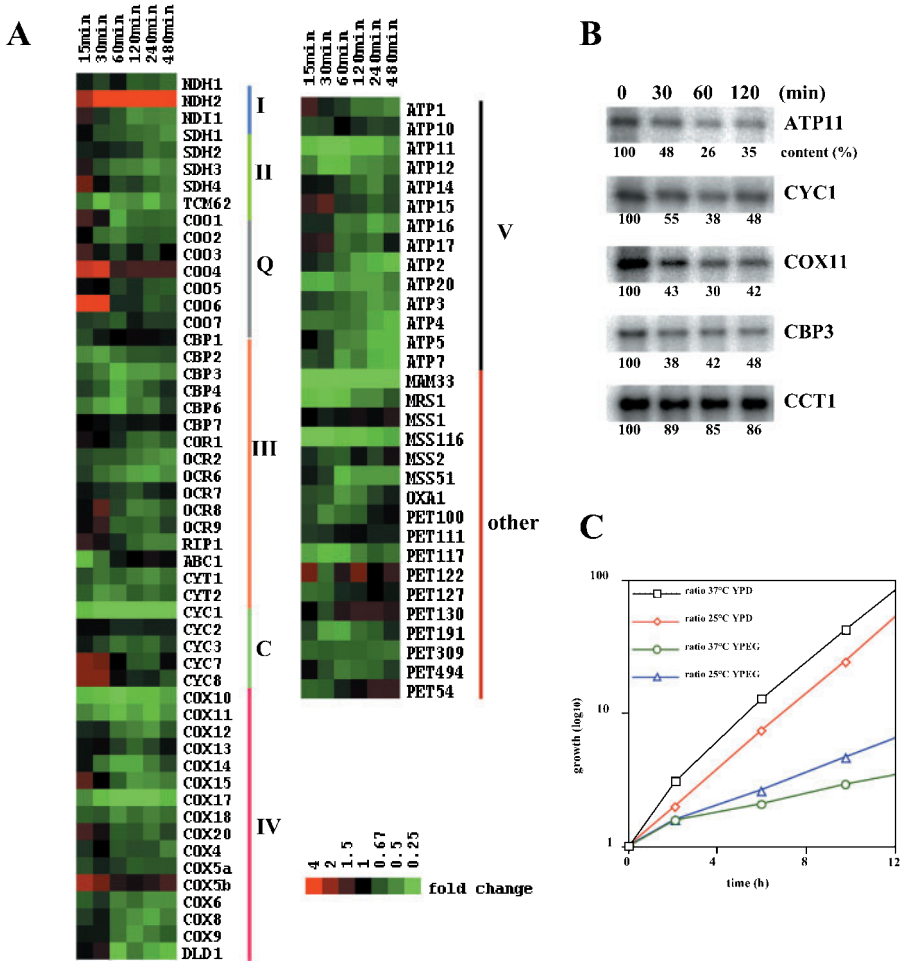

Fig. 2. **Repression of mitochondrial oxidative phosphorylation-related genes after mild heat stress.** (A) Expression profiles of genes related to the mitochondrial respiratory chain. [I], NADPH-dehydrogenase; [II], succinate-dehydrogenase complex; [Q], ubiquinone biosynthesis genes; [III], cytochrome bc1 complex; [C], cytochrome c and its regulator; [IV], cytochrome c oxidase (COX); [V], F_0F_1-ATPase. (B) Northern blot analysis of several respiratory genes. CCT1 was included as a control for non-responding genes. Band intensity was calculated by setting the band intensity at time 0 to 100%. (C) Comparison of the growth rate of yeast cells cultured at 25°C and 37°C in non-fermentable (YPEG) or fermentable (YPD) carbon sources. Yeast cells were grown at 25°C in YPEG or YPD to 0.1 OD_{600} and then the temperature was shifted to 37°C or maintained at 25°C, and culture was continued. Cells were diluted at appropriate time points so that OD_{600} was maintained between 0.1 to 1.0, and culture was continued. Cell numbers are shown as the ratio of OD_{600} at various time points to OD_{600} at time 0.

facturer, was incubated at 42°C for 90 min, after which the mRNA was degraded by the addition of EDTA and NaOH and heating at 65°C for 30 min. The fluorescently-labeled cDNA was purified with a Microcon 30 (Millipore). The cDNA probes derived from non-stressed cells were labeled with Cy5-dUTP and those from stressed cells were labeled with Cy3-dUTP.

Microarray Hybridization and Data Analysis—Hybridization was performed essentially as described by DeRisi *et al*. (*7*). Equal volumes of Cy3- and Cy5-labeled cDNA probe were mixed and hybridized to each microarray in 5× SSC and 0.5% SDS at 60°C for 10 h. After hybridization, the microarrays were washed three times each with 2× SSC/0.2% SDS at room temperature, 0.2× SSC/0.2% SDS at 60°C, and 0.2× SSC/0.2% SDS at room temperature with gentle agitation. The image of the microarray pattern was analyzed by FLA8000 (Fuji Film). Quantification of the fluorescent intensity of each spot was performed by Array Vision 5.0 (Imaging Research). To normalize the baseline lag among the microarray data, data from each array were centered using "CLUSTER" software (*8*). Full-text data of our microarray analysis is available on the Web site (http://www.med.kyushu-u.ac.jp/cell/Ishihara/mild_heat_array_data.xls).

Northern Blot Analysis—A quarter volume of loading buffer [1× MOPS (120 mM MOPS-KOH (pH 7.0), 5 mM Na acetate and 0.5 mM EDTA), 50% formamide, 2.2 M formaldehyde, 0.02% BPB, and 0.75% ficol] was added to each aliquot of mRNA solution and the samples were heated at 65°C and chilled on ice. mRNAs (0.5 μg per lane) were loaded on a denaturing gel (1.2% agarose, 1× MOPS, and 0.41% formaldehyde) that was pre-run for 15 min at 4 V/cm in 1× MOPS running buffer, and electrophoresed at 3 V/cm. The separated RNAs were transferred to a Hybond-XL nylon membrane (Amersham Pharmacia Biotech) with a Turboblotter system (Schleicher & Schuell) in 10× SSC. RNAs were cross-linked to the membrane by UV irradiation. RNA quality was checked by methylene blue staining of the membrane. The DNA probe was labeled with [α-^{32}P]dCTP using a Redivue™ random labeling system (Amersham). Hybridization was performed essentially as described by Maniatis *et al*. (*9*). The membrane was exposed to an imaging plate for several h and the blot images were analyzed by BAS2000 (Fuji Film).

Protein Extraction and Western Blotting—Total protein extracts were prepared basically as described by Yaffe (*10*). Briefly, 1-ml aliquots of cultured yeast cells were treated with 150 μl alkaline solution (75 mg/ml NaOH and 7.5% 2-mercaptoethanol) for 20 min. The solutions were treated with 150 μl trichloroacetic acid on ice for 20 min, briefly centrifuged, and the precipitates were solubilized in SDS-PAGE loading buffer. The solution was clarified by centrifugation and the supernatant corresponding to the extract from 0.1 OD_{600} cells was analyzed by SDS-PAGE and subsequent immunoblotting

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Table 1. **Summary of heat-induced genes (104 genes).**

Response to stress (18genes)	AHA1* GRE1 GRE3* HCH1* HSC82* HSP26* HSP78* HSP82* HSP104* SSA3* SSA4* NTH1* NTH2* TSL1* GPX1
	(response to oxidative stress) ATH1* GAD1*
	(salinity stress) GCY1
Protein folding (12genes)	AHA1* CPR6 HCH1* HSC82* HSP26* HSP78* HSP82* HSP104* SSA3* SSA4* SSE2 STI1
Carbohydrate metabolism (12genes)	(trehalose catabolism/biosynthesis) ATH1* NTH1* NTH2* TSL1*
	(glycolysis / regulation of glycolysis) ENO1 ENO2 GPM1 PFK26
	(others) GND1 TKL2 INO1 GRE3*
Amino acid metabolism (4genes)	ARG1 CHA1 GAD1* STR3
Cell wall organization and biogenesis (4genes)	ECM8 PIR3 SPR28 SSD1
Others (19genes)	BTN2 HXT5 GPG1 MCH2 NCA3 NDH2 PES4 PCL5 POT1 PTP2 RNY1 SGA1 SSU1 TFS1 YPS4 YSP3 VID24 YCL073C YPR078C
Biological process unknown (50genes)	ERR1 ERR2 FUN19 ICY2 IKS1 IML2 OYE3 PHM7 RTN2 SPG1 SPI1 YAL018C, YBL048W YBL049W YBR116C YBR241C YCR013C YDL124W YDL199C YDR070C YDR533C YGR043C YGR146C YGR161C YJL015C YJL016W YJL017W YJL037W YJR008W YKL107W YKL151C YKL153W YLR217W YLR327C YML002W YMR103C YMR107W YMR118C YMR279C YMR322C YMR323W YNL195C YNR014W YNR064C YNR069C YOR338W YOR391C YOR392W YPL277C YPL280W

Gene Ontology (GO) term on Saccharomyces Genome Database (SGD) were refered to for the description of the function of each gene. Among 104 heat-induced genes, 15(*) are categorized into multiple functional groups.

using antibodies against Tom70p, Tom22p, Tom20p, Tom40p, or Hsp60p. Protein bands were visualized with horseradish peroxidase-labeled anti-rabbit IgG (BIOSOURCE) and ECL reagent (Amersham Bioscience) followed by image analysis by a LAS1000*plus* (Fuji Film, Tokyo).

Mitochondrial Import of preSU9-DHFR In Vitro—Yeast mitochondria were isolated from cells grown at 25°C or 35°C in YPEG medium as described previously (*11*). Mitochondrial import of reticulocyte lysate-synthesized ^{35}S-preSU9-DHFR was performed essentially as described previously (*12*), except that 5 mM methionine, 5 mM creatine phosphate, and 100 μg/ml creatine kinase were added to the assay mixture. After import, the mitochondria were subjected to SDS-PAGE and subsequent fluoroimage analysis.

RESULTS

Global gene response under mild heat stress as evaluated by microarray analyses—The genome-wide gene expression profile under mild heat stress was determined by DNA microarray analysis of 5,870 amplified yeast nuclear genome-encoded ORFs. Yeast cells were grown at 25°C to 1.0 OD_{600} in non-fermentable medium, YPEG, then shifted to 37°C and cultured further. The expression pattern after the temperature shift was compared with that of cells grown at 25°C. Responsive genes were defined as genes that were repressed or induced more than 2-fold at 3 or more of 4 time points (1, 2, 4, and 8 h) in both of two independent experiments. There were 104 heat-inducible genes (1.8%) and 287 heat-repressible genes (4.9%). The extracted heat-responsive genes were classified according to the functional category of Gene Ontology (GO) terms on the *Saccharomyces* Genome Database (SGD) and are summarized in Tables 1 and 2. As expected, 12 genes for molecular chaperones and co-chaperones were induced by heat-treatment (categorized as "Protein folding" in Table 1; nine are also categorized as "response to stress" and asterisked). The course of gene expression of the protein folding-related genes, including several of the above described genes, is shown in the "Horizontal Strip" (*7*) in Fig. 1A. In contrast to the positive response of these chaperone genes, SSB1 and SSB2 genes were repressed under these conditions, confirming previous findings (*13*). On the other hand, HSP10, HSP60, and SSC1, which are involved in mitochondrial protein import and protein folding in the mitochondrial matrix, were upregulated shortly after the temperature shift and then down-regulated (Fig. 1A). The expressions of other mitochondrial protein import-related genes, such as a set of the Tom and Tim components, were repressed simultaneously to a significant extent (A sub-category "Mitochondrial translocation" in "Mitochondrial organization and biogenesis" in Table 2), suggesting that mitochondrial biosynthesis is significantly repressed by heat stress (see below). Furthermore, genes involved in mitochondrial energy generation (see below), metabolite transport between cytosol and matrix (DIC1, MIR1, OAC1, YHM2, AAC3, PET9, and YMC1), and heme biosynthesis (HEM1 and HEM12) were significantly repressed (Table 2).

Eighteen genes related to "response to stress" were also induced by heat-treatment (Table 1; nine of them asterisked are also included in "protein folding" category). This class contains four genes (ATH1, NTH1, NTH2, and TSL1) involved in trehalose biosynthesis and metabolism. The concentration of cytoplasmic trehalose increases under heat-stress conditions, which promotes protein folding (*14*). TSL1 is a 123-kDa regulatory subunit of the trehalose synthetase complex (*15*). ATH1, NTH1, and NTH2 encode cytoplasmic and vacuolar trehalase, which are required for recovery of cells from heat stress (*16*). Therefore, an accumulation of trehalose should occur in mild heat stress, which promotes correct folding of the cytoplasmic proteins.

Ribosomal protein genes are transiently down-regulated by heat stress through the repressor activity of Rap1p (*17*). Actually, 122 genes encoding cytoplasmic and mitochondrial ribosomal proteins were repressed ("protein biosynthesis" in Table 2, and Fig. 1B). Furthermore, 14 genes (CAF20, CDC33, MEF1, MTO, PRT1, TEF4,

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Table 2. **Summary of heat-reduced genes (287 genes).**

Protein biosynthesis (145 genes)	RPL11a RPL11b RPL12a RPL12b RPL14a RPL14b RPL15a RPL15b RPL16a RPL16b RPL17a RPL17b RPL18a RPL19a RPL19b RPL20a RPL20b RPL21a RPL21b RPL22a RPL23b RPL24a RPL24b RPL25 RPL26b RPL3 RPL30 RPL31a RPL32 RPL33a RPL34b RPL35a RPL37a RPL37b RPL40b RPL42a RPL42b RPL4a RPL4b RPL5 RPL7a RPL9a RPP0 RPP1a RPP1b RPP2a RPP2b RPS0a RPS0b RPS10a RPS11a RPS11b RPS12 RPS13 RPS14a RPS15 RPS16b RPS18a RPS19a RPS19b RPS1a RPS1b RPS2 RPS20 RPS21a RPS21b RPS22a RPS22b RPS26a RPS26b RPS28a RPS29a RPS29b RPS3 RPS30b RPS31 RPS4b RPS6b RPS7a RPS8a RPS8b RPS9a RPS9b IMG1 MRP1 MRP13 MRP2 MRP21 MRP4 MRP51 MRPL1* MRPL10 MRPL11 MRPL13 MRPL17 MRPL2 MRPL20* MRPL22* MRPL24 MRPL3 MRPL31 MRPL32 MRPL35 MRPL36 MRPL38 MRPL40 MRPL49 MRPL51* MRPL8* MRPL9 MRPS17* MRPS28 MRPS35* MRPS9 NAM9 RML2 RSM10 RSM18 RSM23 RSM24* RSM25 RSM26
	(translation initiation/elongation) CAF20 CDC33 MEF1 MTO1 PRT1 TEF4 TIF1 TIF11 TIF2 TIF3 TIF35 TUF1 YEF3 YGR054W
	(others) CTR1* FAA3* GOG5* GRS1 KRS1 MSK1 MSS51* SSB2 THS1
Small molecule transport# (23 genes)	(ion transport) ATP11* COX17* CTR1* CTR2 DIC1 FET3 FRE1 FRE2 FTR1 MIR1 OAC1 PHO84 PHO89 YHM2
	(nucleobase, nucleotide transport) AAC3 PET9 FCY2
	(siderochrome transport) ARN1 FIT2 SIT1
	(others) GOG5* STL1 YMC1
Mitochondrial respiration# (16 genes)	(aerobic respiration)COX11 DLD1 MAM33 MRPL1* MRPL22* MRPL51* MRPS17* MRPS35* RSM24*
	(protein complex assembly) ATP11* COX17* TCM62
	(RNA processing) MSS51* MSS116
	(ATP synthesized coupled proton transport) ATP7
	(electron transport) CYC1
Ribosome biogenesis (excluding ribosomal proteins 12 genes)	RRB1
	(rRNA modification) GAR1 NOP1 NOP5 NSR1 SIK1
	(ribosomal large subunit assembly and maintenance) BRX1 NIP7 NMD3
	(ribosomal large subunit biogenesis) ARX1 NOP7 RLP7
amino acid metabolism (12 genes)	(branched amino acid metabolism) ARO10 BAT1 ILV3 LEU1 LEU2 LEU4
	(methionine metabolism) HOM2 SAH1 SAM1
	(others) ARO4 GDH1 TRP4
Mitochondrion organization and biogenesis (11 genes)	(mitochondrial translocation) MAS1 MGE1 TIM17 TIM23 TOM6 TOM20 TOM70
	(mitochondrial genome maintenance) MRPL8* MRPL20* MDV1 YHM1
Chromatin assembly/disassembly (6 genes)	HHF1 HHF2 HHT1 HTA1 HTB1 HTB2
Conjugation with cellular fusion (5 genes)	SCW4 CCW12
	(signal transduction during conjugation cell fusion) CDC42 FAR1 STE3
Lipid metabolism (3 genes)	FAA3* ERG13 PIS1
Others (24 genes)	ADH6 ALD6 APT1 ARF1 ARL1 BUD28 CHS1 GAS1 GIS2 GUA1 HEM2 HEM12 HO HXK2 LSC1 MAK3 MATALPHA2 MPT4 PYC1 RKI1 SDA1 SPE3 URA7 VTC1
Biological process unknown (44 genes)	EMI5 FRE7 FYV4 FYV13 HAS1 PRY1 PRY2 QRI5 RMD9 RSM28 SYP1 TOS1 TOS6 YCLX10C YDR134C YDR417C YER156C YFR011C YGL068W YGL069C YGL102C YGR021W YGR219W YIL121W YJR070C YKL177W YLR042C YLR076C YLR179C YLR190W YLR339C YLR349W YML087C YMR157C YNL081C YNL217W YOR248W YOR271C YOR315W YPL142C YPR004C YPR044C YPR099C YPR123C

GO term were refered to for a desicription of the function of each gene product. Among 287 heat-repressed genes, 14(*) are categorized into multiple functional groups., #, arbitrarily set functional categories.

TIF1, TIF11, TIF2, TIF3, TIF35, TUF1, YEF3, and YGR054W) encoding initiation or elongation factors of protein synthesis, and 12 genes (ARX1, BRX1, GAR1, NIP7, NMD3, NOP1, NOP5, NOP7, NSR1, RLP7, RRB1, and SIK1) encoding ribosome biogenesis-related proteins were also repressed ("ribosome biogenesis & protein biosynthesis" in Table 2). In this context, SSB2, which was repressed by mild heat stress, is involved in the passage of the nascent polypeptide chain through the ribosome (*18*). We thus speculated that the cells repressed bulk protein synthesis for the fidelity of protein folding, which might be reflected as the growth defect.

Analysis of our data using the Gene Ontology (GO) term finder at SGD revealed that the enrichments of genes associated with "protein folding" (p = 3.43E-12) and "response to stress" (p = 1.22E-08) in the heat-repressible category, and genes for "protein biosynthesis" (p = 3.05E-62) in the heat-repressible gene category were statistically significant. Similarly, genes associated with copper uptake by the cells (p = 6.44E-05) and branched chain amino acid metabolism (p = 6.51E-05) were classified as down-regulated genes with high probability. Considering that copper ion is essential for the function of mitochondrial cytochrome *c* oxidase (COX) and that

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Table 3. **Subcellular localization of the products of heat-repressed genes.**

Localization	Number of genes	Percentage (%)
Mitochondria	36	31.6
Nucleus	27	23.9
Cytosol / cytopasm	17	15.0
Plasma membrane	12	10.6
Cell wall	6	5.3
Endoplasmic reticulum	3	2.7
Other	16	14.2

SGD was refered to for a description of the localization of each gene products. The localizations of 113 non-ribosomal heat-repressed genes are known. The total percentage is greater than 100%, because 4 of the gene products localize to multiple cellular compartments.

Table 4. **Subcellular localization of the products of heat-induced genes.**

Localization	Number of genes	Percentage (%)
Cytosol	17	42.5
Nucleus	4	10.0
Plasma membrane	3	7.5
Cell wall	2	5.0
Mitochondria	2	5.0
Other	12	30.0

SGD was refered to for a description of the localization of each gene products. Among 104 heat-induced gene products, the localizations of 40 are known.

Cox17p, which delivers copper ion from the cytosol to COX, is down-regulated (see below), the repression of genes for copper transport will compromise mitochondrial respiratory function. The functional significance of the down-regulation of genes associated with branched chain amino acid metabolism is not known.

Repression of Genes Involved in Mitochondrial Respiration—When all transcripts of responsive genes were classified according to their intracellular localization, there were 36 genes encoding mitochondrial proteins among the heat-repressed genes, which account for 31.6% of the total localization-assigned proteins excluding ribosomal proteins (Table 3). In contrast, the heat-inducible genes included only two mitochondrial biogenesis-related genes, which encode 5.0% of the total localization-assigned proteins (Table 4). Analysis using the GO term finder with respect to the intracellular localization also revealed that enrichment of the mitochondria-related genes in the heat-repressible gene category was statistically most significant (p = 5.63E-09).

The mitochondrial protein-encoded genes repressed by heat stress were then classified according to their function. Thirty-nine genes for mitochondrial ribosomal proteins were repressed (see Table 2). Nine of the repressed genes encode key regulatory components of the mitochondrial respiratory chain or its assembly ("mitochondrial respiration" in Table 5). Tcm62p is a chaperone required for the assembly of the succinate dehydrogenase complex (Complex II) (*20*). Although not categorized as "mitochondrial respiration, " Mss51p (see "protein biosynthesis" in Table 2) is required for the translation of the mtDNA-encoded COX1 and cytochrome *b* (COB) mRNAs (*21*). Mss116p is a mitochondrial RNA helicase of the DEAD-box family required for splicing the COX1 and COB group II introns of COX1 and COB (*22*). Cox17p delivers copper ions to the cytochrome c oxidase (COX) complex and this step is crucial for the regulation of COX activity (*23*). Cox11p is the assembly protein of the COX complex (*24*). CYC1 encodes cytochrome c isoform 1, which is required for electron transfer from Complex III to the COX complex under aerobic conditions (*25*). Atp11p is involved in the assembly of mitochondrial F1-ATPase (Complex V) (*26*). Inspection of the "Horizontal Strip" for finer gene response revealed that most of the respiration-related genes, including CBP3, which is required for the assembly of Complex III, and NDH1 and NDI1, which are components of Complex I (*27*, *28*), were down-regulated (Fig. 2A). Down-regulation of ATP11, CYC1, COX11, TCM62 and CBP3 was also confirmed by Northern blot analysis in comparison with a non-responsive gene, CCT1 (Fig. 2B and data not shown). Taken together, the genes involved in the synthesis or assembly of Complexes

Table 5. **heat-repressed genes encoding proteins localized in mitochondria.**

Mitochondrial respiration[#] (9 genes)	(aerobic resipiration) COX11 DLD1 MAM33
	(protein complex assembly) ATP11 COX17* TCM62
	(RNA processing) MSS116
	(ATP synthesized coupled proton transport) ATP7*
	(electron transport) CYC1
Mitochondrion organization and biogenesis (9 genes)	(mitochondrial translocation) MAS1 MGE1 TIM17 TIM23 TOM6 TOM20 TOM70
	(mitochondrial genome maintenance) MDV1 YHM1
Small molecule transport[#] (9 genes)	(ion transport) DIC1 MIR1 OAC1 ATP7* COX17* YHM2
	(nucleobase, nucleotide transport) AAC3 PET9
	(others) YMC1
Protein biosynthesis (5 genes; excluding 39 mitochondrial ribosomal proteins)	(translation initiation / elongation) MEF1 MTO1 TUF1
	(others) MSK1 GRS1
Amino acid metabolism (3 genes)	(amino acid biosynthesis) BAT1 ILV3 LEU4
TCA cycle (1 gene)	LSC1
Lipid / fatty acid metabolism (1 gene)	ERG13
Unknown (1 gene)	SWS1

GO terms were refered to for a description of the function of proteins localized in mitochondria. Among 36 heat-repressed genes, 2(*) are categolized in multiple functional groups. [#], arbitrarily set functional categories.

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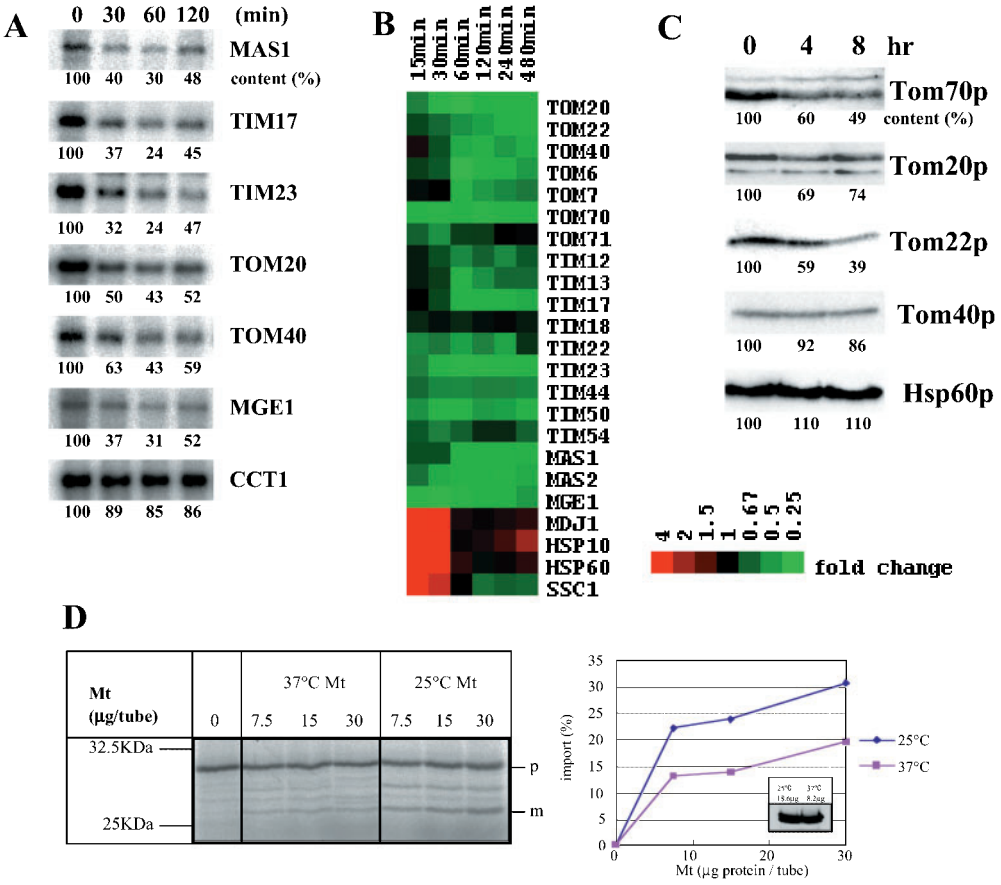

Fig. 3. **Response of genes involved in mitochondrial biogenesis after mild heat stress.** (A) Northern blot analysis of several genes involved in mitochondrial preprotein import. The conditions were the same as described in the legends to Fig. 2. (B) Response of genes involved in mitochondrial preprotein import as revealed by the "Horizontal Strip". (C) Repression of the mitochondrial import receptors as revealed by immunoblotting. Total proteins extracted from 0.1 OD_{600} cells were analyzed by SDS-PAGE and subsequent immuno-blotting with the indicated antibodies. Band intensities were quantified and shown as percentages, with the intensity value at time 0 set to 100%. (D) Comparison of preSU9-DHFR import activity between mitochondria isolated from cells grown in 25°C and 37°C. The import activity as a function of total mitochondrial protein is shown in the right panel.

II, III, IV, and V were significantly down-regulated. It should be noted in this regard, that the NDH2 gene driven by the STRE-upstream promoter was up-regulated (Fig. 2A).Considering that Δ*ndh1* yeast cells exhibit the same phenotype as Δ*ndh1* × *ndh2* cells (*29*), however, the down-regulation of NDH1 and NDI1 seem to eventually compromise the function of Complex I. Thus, these results suggest that overall mitochondrial respiratory function is down-regulated by mild heat stress.

To confirm this possibility, the growth rate of yeast cells at 25°C or 37°C under fermentable or non-fermentable conditions was examined (Fig. 2C). In a non-fermentable carbon source (YPEG), growth at 37°C began to decline after 4 h. In a fermentable carbon source (YPD), the growth rate at 37°C was not affected. Similar results were obtained with other yeast strains, SEY6210 and FY1679 (data not shown). Taken together, these results indicate that heat-treatment compromises mitochondrial respiratory function.

Repression of Genes Involved in Mitochondrial Biogenesis—Seven genes associated with mitochondrial biosynthesis were significantly repressed after heat treatment (sub-category "mitochondrial translocation" in Table 5). TOM6, TOM20, and TOM70, and TIM17 and TIM23 encode components of the preprotein translocase of the outer membrane (TOM complex) and inner membrane (TIM complex) (*30–32*). Mas1p is a subunit of the mitochondrial processing peptidase, which removes the presequences of mitochondrial preproteins in the matrix space (*33*). MGE1 is a nucleotide exchange factor for mHsp70 family proteins and cooperates with them to drive the translocation of preproteins into the matrix space (*32*, *34*).

Northern blot analysis also confirmed the repression of several of the above genes and TOM40 (Fig. 3A and data not shown). In addition, the gene response as revealed by the "Horizontal Strip" presentation indicated that genes encoding the other components of the TOM and TIM complexes were also repressed, *i.e.*, TOM22, TOM7, TIM12, TIM13, TIM44, TIM50, and MAS2 (Fig. 3B).

To examine whether these results were reflected at the cellular protein level, we performed immuno-blot analysis for several components of the TOM complex in 0.1 OD600 cells. The levels of Tom70p, Tom22p, and Tom20p were significantly reduced after the heat stress, whereas Hsp60 was unaffected (Fig. 3C). Unexpectedly, the Tom40p protein level was only slightly affected, although the TOM40 transcript was reduced under heat stress (compare Fig. 3, A and C). Tom20 is the central preprotein import receptor, and Tom22 functions as both an the import receptor and channel regulator (*35*). Tom70p functions as a transport receptor for a subset of preproteins, including an inner membrane ADP/ATP carrier and Tim54p, a component of the Tim23 translocase system (*36*). Furthermore, the deletion of both TOM70 and TOM20 induces synthetic lethality (*37*). Therefore, these results suggest that mild heat treatment compromises mitochondrial preprotein import capacity. We examined this point using an *in vitro* preprotein import system. Mitochondria isolated from cells grown at 37°C in YPEG

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imported preSU9-DHFR at lower efficiency than those from cells grown at 25°C (Fig. 3D). Western blot analysis of the isolated mitochondria revealed that the specific content of Tom40 in mitochondria from 37°C-grown cells was approximately 2.3-fold of that in mitochondria from 25°C-grown cells, suggesting that an the import step at a level other than Tom40 level is limiting.

Gene Response to Mild Heat Stress for Metabolic Remodeling—A notable feature of mild heat-stress was the up-regulation of 12 genes for carbohydrate metabolism (categorized as "carbohydrate metabolism" in Table 1). This category contains ENO1 and ENO2, encoding enolase isoforms 1 and 2, respectively, GPM1, encoding phosphoglycerate mutase, and PFK26, encoding 6-phosphofructose-2 kinase. 6-phosphofructose-2 kinase catalyzes the synthesis of fructose 2, 6-bisphosphate, which is an allosteric activator of one of the rate-limiting enzymes in the glycolytic pathway, Pfk1/2p (*38*). TKL2 encodes transketolase, which is a component of the pentose phosphate cycle that indirectly activates glycolysis (*39*). Although not strictly included in the same category, most of the genes related to the glycolytic pathway, including TDH1 (glyceraldehyde 3-phosphate dehydrogenase), PGK1 (phosphoglycerate kinase), and CDC19 (pyruvate kinase) were up-regulated to a significant extent within 30 min after the onset of heat stress (Fig. 4, A and C). These results indicate that glycolysis is up-regulated by heat treatment. In view of the fact that oxidative phosphorylation-related genes are de-repressed when cells undergo transition from glucose fermentative to oxidative metabolism ["diauxic shift" (*7*)], the glycolytic genes seemed to be induced in response to the decreased ATP levels after the repression of respiratory genes. In fact, the data of Epstein *et al*. demonstrated that a set of glycolysis-related genes is induced by treating yeast cells with the respiratory inhibitors antimycin A, oligomycin, or carbonyl cyanide m-chlorophenylhydrazone (409). Microarray analysis revealed that HXK1, TDH1, PGK1, and ENO1 and 2 were upregulated more than 2-fold in the presence of the respiratory inhibitor antimycin (Fig. 4A, see "AA-120min"). Taken together, these results suggest that defects in the mitochondrial respiratory system lead to the upregulation of the glycolytic pathways.

Glutamate is a precursor of nucleotides and other amino acids, and is synthesized directly from the TCA cycle intermediate α-ketoglutaric acid, whose level is regulated by the supply of OAA and its condensing partner, acetyl-CoA. As described above, because genes encoding the succinate dehydrogenase complex (Complex II) and a chaperone protein, Tcm62p, involved in its assembly were down-regulated ("carbohydrate metabolism" in Table 2, and Fig. 2A), OAA regeneration in the TCA cycle should not proceed. Furthermore, the LSC1 gene encoding succinyl-CoA ligase, which catalyzes succinate synthesis, was also repressed ("TCA cycle" in Table 5 and Fig. 4D). Therefore, OAA and acetyl-CoA must be supplied to mitochondria by another route to maintain glutamate synthesis for biosynthetic reactions. Epstein *et al*. (*40*) demonstrated that the production of OAA and acetyl-CoA in β-oxidation and carbohydrate metabolism are up-regulated in rho0 petite mutants. In our microarray data, a set of genes associated with β-oxidation in peroxisomes were likely to be up-regulated (Fig. 4, B and D): POX1/FOX1 (actl-CoA oxidase, the first and rate-limiting enzyme of β-oxidation), POT1/FOX3 (3-ketoacyl-CoA thiolase), and FAA1 (long-chain fatty acid-acetyl CoA lyase). In addition to β-oxidation, the acetyl-CoA biosynthetic pathway from ethanol fermentation [ADH5 (alcohol dehydrogenase) and ACS1 (acetyl-CoA synthase)] was also upregulated (Fig. 4, B and D). Although the behaviors of ALD2 and 3 are not known because of their absence from our arrays, they are reported to be up-regulated 10-fold through the action of the Msn2/4p activator after mild heat stress (*41*). Furthermore, fumarate, a precursor of OAA in the TCA cycle, can also be supplied from the urea cycle as a by-product of arginine biosynthesis. According to our microarray data, ARG1 was upregulated ("amino acid metabolism" in Table 1, and Fig. 4D). All other enzymes in this cycle (ARG3 and ARG4) tended to be up-regulated on the "Horizontal Strip" display (Fig. 4, B and D). Taken together, these data suggest that the metabolic flux into glutamate is maintained during heat-induced mitochondrial dysfunction.

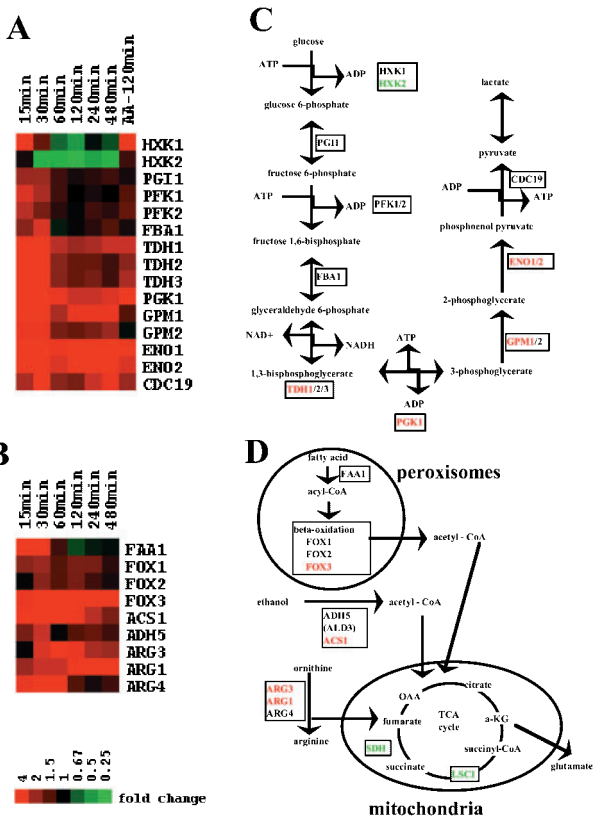

Fig. 4. **Heat induction of genes involved in glycolysis and acetyl-CoA production.** Expression profiles of glycolysis-related genes (A) and genes involved in acetyl-CoA production pathways (B). (C) Schematic representation of the glycolysis pathway. (D) The genes involved in acetyl-CoA production in peroxisomes and cytosol, and those involved in the transport of acetyl-CoA into mitochondria. The genes denoted in red and green letters are those induced and repressed, respectively.

Damage to mitochondrial electron transport and the subsequent glutamate insufficiency leads to activation of the RTG response through the activation of responsible transcription factors, Rtg1/3p (*42*). In our array data, the typical RTG-response genes, CIT2 and DLD3, were up-

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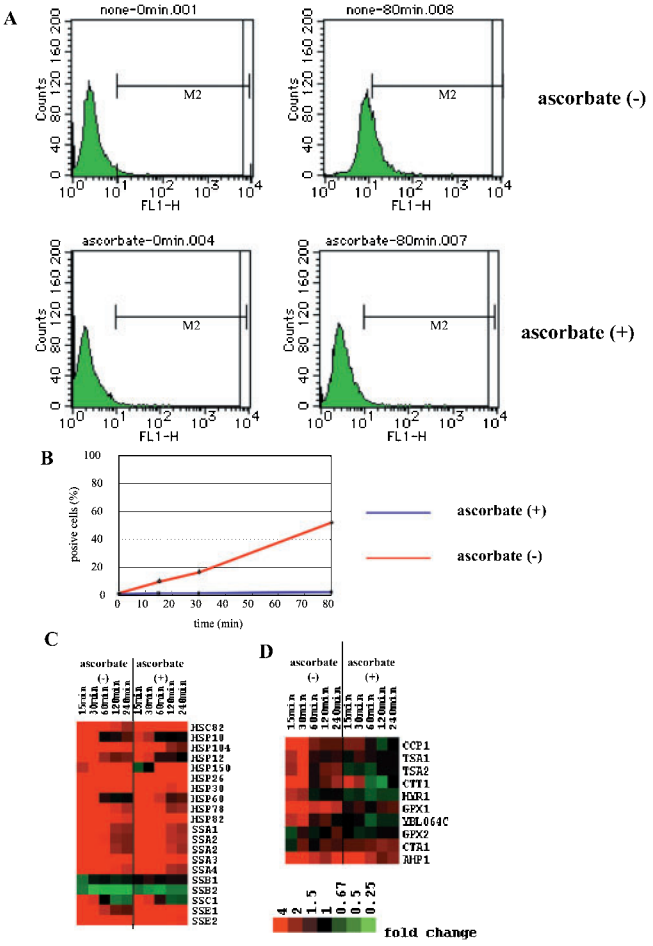

Fig. 5. **Effect of the ROS-scavenger ascorbate on the mild heat-induced gene expression profiles.** (A) Yeast cells grown at 25°C to 1.0 OD_{600} were shifted to 37°C and cultured in the presence (+) or absence (–) of 10 mM ascorbate. Before the temperature shift, the cells were incubated in 40 μg/ml H2Rhodamine 123 for 30 min. The cells were harvested at time 0 or 80 min and the fluorescence of H2Rhodamine123 was detected by FACScan with a fluoroisothiocyanate filter. (B) Time-course of ROS production in yeast cells in the presence or absence of ascorbate. Percentage of positive cells within the M2-range at each time point is shown. (C) and (D) Expression profiles of HSP genes and peroxidase-family genes in mild heat stress in the presence (+) or absence (–) of 10 mM ascorbate, respectively. Cells were cultured as in (A). Other conditions were as described in the legend to Fig. 1 and in "MATERIALS AND METHODS."

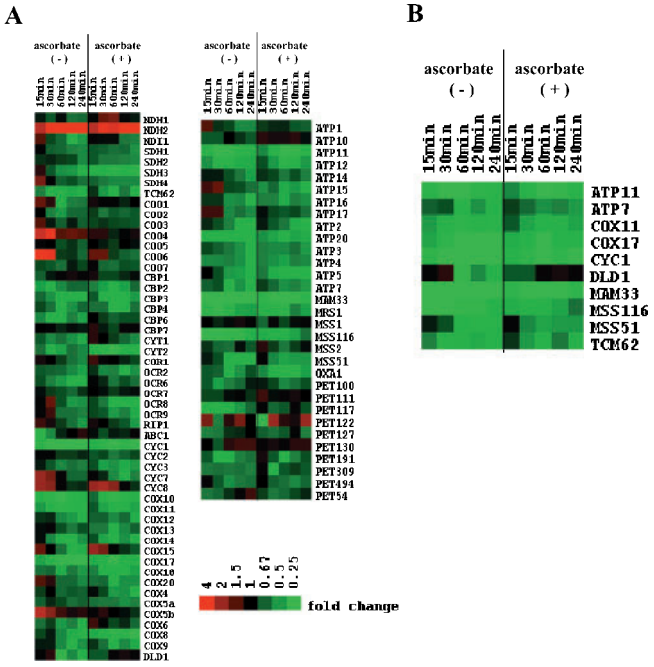

Fig. 6. **Effect of ascorbate on the heat-induced repression of mitochondrial respiratory chain-related genes.** (A) Expression profile of mitochondrial respiratory chain-related genes in the presence or absence of 10 mM ascorbate. (B) Expression profile of the heat-repressible genes extracted from (A) ("Energy generation" genes in Table 5) in the presence or absence of ascorbate. Preparation of cells and gene expression analyses were as described in the legend to Fig. 5 and in "MATERIALS AND METHODS."

regulated, suggesting that the RTG-signaling system was activated, probably *via* mitochondrial dysfunction under the present conditions (data not shown).

Heat Stress-Associated ROS Generation Is Involved in Heat-Repression of a Limited Number of Mitochondrial Biogenesis–Related Genes—Mitochondria-driven ROS generation is mainly responsible for heat-induced oxidative stress, and affects the heat-sensitivity of cells (*4*, *5*). We therefore examined whether ROS are involved in the observed gene repression. ROS generation was detected by a peroxidase-dependent fluorescent reagent, dihydrorhodamine123 (*43*), using a cell sorter (Fig. 5, A and B). Incubation of yeast cells at 37°C induced a time-dependent accumulation of ROS, and 10 mM ascorbate strongly inhibited this production (Fig. 5B). We then examined the gene expression profile under mild heat treatment in the presence of 10 mM ascorbate. The expression profile of HSP genes was not significantly affected by ascorbate (Fig. 5C), probably because the heat shock transcription factor (Hsf1p) is directly activated by heat (*44*). In contrast, heat-induction of most peroxidase and catalase genes, which are responsible for ROS-detoxification, was attenuated in the presence of ascorbate (Fig. 5D). These results indicate that cells respond to heat stress via multiple upstream signals. Although heat-driven ROS production was strongly decreased in the presence of ascorbate, heat-repression of most genes involved in the mitochondrial respiratory chain was affected only weakly or not at all (Fig. 6A). In fact, none of the heat-repressible mitochondrial respiratory genes listed in Table 5, except DLD1, were affected (Fig. 6B). These results suggest that most of the genes involved in mitochondrial respiration are not under the regulation of heat-inducible oxidative stress.

Similarly, ascorbate affected the heat-repression of only a limited number of genes involved in mitochondrial protein translocation (Fig. 7A). The heat-repression of MAS1, MGE1, and TOM70 was relieved significantly by ascorbate (Fig. 7B, upper panel), whereas that of TOM20, TOM6, TIM17, and TIM23 was not (Fig. 7B, lower panel). The other genes involved in mitochondrial biogenesis were affected only slightly or not at all (Fig. 7A), suggesting that heat-driven oxidative stress is partially responsible for the heat-repression of mitochondrial biogenesis genes.

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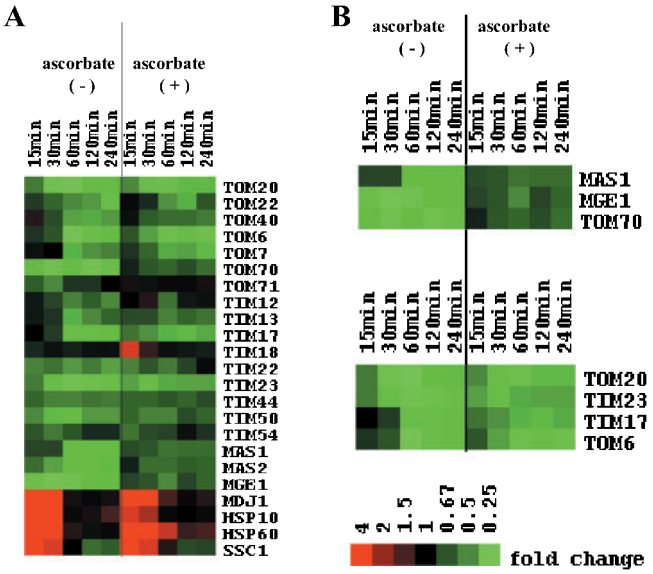

Fig. 7. **Effect of ascorbate on the heat repression of mitochondria biogenesis-related genes.** (A) Expression profile of mitochondria biogenesis-related genes in the presence or absence of ascorbate. (B) Expression profile of the heat-repressible genes extracted from (A) ("Protein translocation" genes in Table 5) in the presence or absence of ascorbate. Other conditions were as described in the legend to Fig. 6.

DISCUSSION

The global gene expression pattern in yeast cells under mild heat stress conditions was analyzed. Most notably, these experiments reveal that the genes associated with mitochondrial respiration as well as those associated with mitochondrial biogenesis are down-regulated. This repression was confirmed by Northern blot and Western blot analyses for several of the genes. Consistent with these observations, yeast cell growth was repressed under heat stress in a non-fermentable carbon source, but not in a fermentable carbon source. Furthermore, mitochondria isolated from cells treated at 37°C exhibited compromised preprotein-import activity compared with those from cells treated at 25°C. We, therefore, speculate that heat stress represses mitochondrial function by repressing mitochondrial oxidative phosphorylation and mitochondrial biogenesis. In contrast, in addition to most stress-responsible genes, the expression of a set of genes involved in glycolytic pathways and the pathway that supplies both OAA and its condensing partner acetyl-CoA in the TCA cycle were up-regulated; the latter genes might thus maintain the metabolic flux into glutamate, the precursor for nucleotides and amino acids.

Taken together, we speculate that the following events occur in cells exposed to non-lethal mild heat stress. Heat stress directly activates HSE- and STRE-regulated cell stress genes through transcription activators Hsf1p and Msn2/4p, respectively (*44*). Most molecular chaperone genes are activated by these transcription factors and the gene products prevent denatured proteins from aggregating, or promote their refolding. Heat stress induces ROS from the compromised mitochondrial electron transport system, provoking oxidative stress in the cells (*5*). This upregulates genes encoding many peroxidases, SODs, and enzymes with anti- oxidant functions. Most of the cytoplasmic ribosomal proteins are repressed by heat through the repressor activity of Rap1p. This seems to be a response to maintain the fidelity of protein translation and folding at the expense of the rate of protein synthesis. On the other hand, mitochondrial biosynthesis is down-regulated, which might contribute to minimizing ROS production. The repression of mitochondrial function also leads to the up-regulation of genes involved in glycolysis, acetyl-CoA, and OAA biosynthesis to compensate for mitochondrial dysfunction in ATP synthesis and glutamate synthesis: the metabolic remodeling response. In this way, yeast cells alleviate heat-damage via ROS and adapt to the stress at the expense of cell growth. Comparison of our data with those of Epstein *et al.* (*40*) in response to mitochondrial dysfunction reveals that 54 out of 145 genes are included among the oligomycin-repressed genes. These results suggest that the heat-repression of these genes may occur, in part, via mitochondrial dysfunction.

The upstream signal for the repression of mitochondrial function remains to be determined. Because heat-damaged mitochondria produce ROS, which are mainly responsible for the cellular oxidative stress, the cells probably respond to repress mitochondrial function to minimize cellular damage due to ROS production. ROS production was induced within 20 min after the temperature-shift in the present study, and the genes for peroxidases that catalyze the reduction of hydrogen peroxide were transiently induced within 15 min (Fig. 5B and data not shown). The genes for enzymes involved in the mitochondrial respiratory chain and mitochondrial protein import responded rather quickly, within 30 to 60 min, after the onset of heat stress (see Figs. 2 and 3). We, therefore, examined the possibility that the repression of genes involved in the mitochondrial respiratory chain and preprotein translocation is caused primarily by the mitochondrial-driven ROS. The heat-induced repression of the respiratory genes, however, was not affected by ascorbate (Fig. 6). Similarly, heat-repression of most mitochondrial biogenesis-related genes, except for MAS1, MGE1, and TOM70, was not affected by ascorbate (Fig. 7). These results suggest that ROS are not directly involved in the repression of most of these genes. The upstream regulator(s) of the above-mentioned genes under this mild heat-induced stress remains to be analyzed.

A search of the upstream promoter regions of the heat-repressible, mitochondrial biogenesis-related genes for consensus sequences revealed a conserved 12-bp sequence in eight mitochondrial biogenesis-related genes (Sakaki *et al.*, unpublished results). Identification and characterization of such responsible *cis*-elements and transcription factors will answer the current question of how mitochondrial function is modified for adaptation under heat-stress conditions.

We thank T. Endo and G. Schatz for providing us with antibodies against Tom20P, Tom22p, and Tom40p, and antibodies against Tom70p, respectively. We also thank A. Enomoto and K. Hayashi for instruction in DNA microarray experiments, and S. Rospert, N. Ishihara, and M. Sakaguchi for valuable

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discussions. This work was supported by grants from the Ministry of Education, Science, and Culture of Japan, Takeda Science Foundation, and Core Research from Evolutional Science and Technology to K.M.

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