Effect of Inhibition of the bc₁ Complex on Gene Expression Profile in Yeast* [S]

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Because the respiratory chain is the major site of oxidation of the reduced equivalents and of energy production in aerobic cells, its inhibition has severe impact on the cells. Communication pathways from the respiratory chain are required to allow the cell to sense the defect and respond to it. In this work, we studied changes in gene expression induced by the treatment of yeast cells with myxothiazol, an inhibitor of the bc₁ complex, an enzyme of the respiratory chain. The pattern and time-course expression of the genes resemble those of the environmental stress response, a common gene expression program induced by sudden changes in the environment. In addition, the changes were, for most of the genes, mediated through the transcription factors Msn2/4, which play a central role in the cellular response to these stresses. By using a mutant with a myxothiazol-resistant bc₁ complex, we showed that the changes of expression of the majority of the genes was caused by the inhibition of the bc₁ complex but that other stresses might be involved. The expression pattern of CTT1, coding for a cytoplasmic catalase, was further studied. The expression of this gene was largely dependent on Msn2/4 and the inhibition of the cytochrome bc₁. Addition of oxidants of NADH was found to decrease the expression of CTT1 induced by myxothiazol treatment, suggesting that the accumulation of NADH caused by the inhibition of the respiratory chain may be involved in the signaling pathway from the mitochondria to the transcription factor.

The mitochondrial respiratory chain is the main site of oxidation of reducing equivalents and of energy production in aerobic cells, both of which are essential for metabolic pathways. Inhibition of the respiratory function is thus expected to have a severe impact on the cell and to cause re-adjustment of cellular metabolism. Herein, we report on the effect of respiratory chain dysfunction on the pattern of gene expression. We chose to study the inhibition of the respiratory chain at the level of the bc₁ complex, because several mutations in this complex have been reported in human diseases and, most interestingly, this enzyme is the target of fungicides or anti-parasite drugs used in agriculture and medicine. Therefore it seemed interesting to understand the cellular events occurring upon treatment.

The mitochondrial bc₁ complex is a membrane-bound enzyme that catalyzes the transfer of electrons from ubiquinol to cytochrome c and couples this electron transfer to the vectorial translocation of protons across the inner mitochondrial membrane. The catalytic mechanism, called the Q-cycle, requires two distinct quinone-binding sites (Q₁, quinol oxidation site, and Q₂, quinone reduction site), which are located on opposite sides of the membrane and linked by a transmembrane electron-transfer pathway. The mitochondrially encoded cytochrome b subunit provides both the Q₁ and Q₂ pockets and the transmembrane electron pathway (via the hemes b). Q₂-specific inhibitors (Q₁) are used as anti-microbial agents in agriculture and medicine. A number of mutations causing or proposed to cause resistance to these inhibitors have been reported in various plant and human pathogens. One target site mutation in cytochrome b in particular, G143A, appears to play a central role in the mechanism of resistance in plant pathogen fungi (1). When introduced in yeast Saccharomyces cerevisiae, this mutation caused a high level of resistance to Q₁ inhibitors such as myxothiazol, axoxystrobin, pyraclostrobin, and atovaquone (2, 3). Several mutations in human cytochrome b have been reported in patients (reviewed in Ref. 4). The mutation G291D, for instance, was proposed to be a primary cause of exercise intolerance (5, 6). The homologous mutation in yeast, G291D, had a clear deleterious effect. It severely impaired respiratory function and altered the assembly of the iron-sulfur protein in the bc₁ complex (7).

In this work, we studied changes in gene expression induced by treatment of yeast cells with myxothiazol and by the disease mutation G291D. Exposure of yeast cells to the Q₁ inhibitor changed the expression of many genes. We show that these changes were in most cases mediated through the transcription factors Msn2/4, which play a central role in the cellular response to various stresses in yeast (8). The transcription factor Yap1, involved in the cellular response to oxidative stress (9), also participated in the changes in gene expression after myxothiazol treatment. We addressed the question of signaling pathway from the inhibited respiratory chain to the transcription factor.

**MATERIALS AND METHODS**

**Yeast Strains and Media**—The S. cerevisiae strains used in this study were: W303-1B/WT (WT), CG291D (G291D), CGW143A (G143A) (2, 7); W303-1B STRE-LacZ, W303-1B STRE-LacZ Δmsn2/4 (ΔMsn2/4), W303-1B Δyap1 (ΔYap1) (kindly provided by P. Piper, University of Sheffield, UK); BY4741, BY4741 Δmsn2/4, BY4741 Δyap1 (from Euroscarf). YPGal (1% yeast extract, 2% peptone, 3% galactose, 0.8 g/liter of a complete supplement mixture (Anachem)) was used as the yeast growth medium.

**RNA Isolation, Target Preparation, and DNA Microarray Analysis**—30 ml of YPGal media was inoculated at an A₆₀₀ of 0.08. After 8 h of growth at 28 °C with shaking (mid-log phase), myxothiazol was added, and the cells were incubated from 2 min to 2 h. Cells were harvested at 1,780 × g at 4 °C for 4 min. The pellet was washed with...
ice-cold water and rapidly frozen in liquid N$_2$ for storage at ~ 80 °C. Total RNA was extracted from frozen cells resuspended in AE buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.3) by a hot phenol method (10), and the absorbance at 260 and 280 nm was used for quantification and purity control. RNA integrity was assessed using the Agilent Technologies 2100 Bioanalyzer. mRNA isolation, cDNA synthesis, in vitro transcription (cRNA synthesis), and cRNA fragmentation were performed using methods described in the Affymetrix Expression Analysis Technical Manual (11). Briefly, after an ethanol precipitation of total RNA, poly(A)$^+$ RNA was enriched in a single round using the Qiagen Oligotex kit, and the double-stranded cDNA was made from RNA using the SuperScript Choice System (Invitrogen) and a modified oligo(dT) primer with a 17 RNA polymerase promoter sequence. After purification and quantification of the double-stranded cDNA with the GeneChip Sample Cleanup Module (Affymetrix), in vitro transcription was performed with T7 RNA polymerase and biotinylated ribonucleotides (BinxArray High Yield RNA Transcription kit, Enzo Diagnostics). Obtained biotinylated cRNA was then purified and subsequently fragmented with the GeneChip Sample Cleanup Module (Affymetrix). All microarray experiments were carried out using a complete Affymetrix GeneChip system. 15 μg of fragmented cRNA was hybridized onto Affymetrix GeneChip Yeast Genome S98 arrays in a rotating hybridization oven at 45 °C for 16 h. This was followed by washing and staining on a GeneChip Fluidics Station 450, and scanning of the GeneChip Scanner 3000 with autoloader. Acquisition and quantification of array images as well as primary data analysis were performed using the GeneChip Operating System software version 1.2 from Affymetrix. Microsoft Excel was used for further statistical analyses. The genomic expression profiles were made using the software Cluster and TreeView (rana.lbl.gov/EisenSoftware.htm).

Triplicates were made for WT cells without treatment (independent cell preparations), and duplicates for WT cells treated for 10 or 30 min with 8 μM myxothiazol (independent cell preparations). For each time point in WT cells, and for the G291D mutant without treatment, the -fold change was relative to the average of the expression level in WT$^1$ cells without treatment. For WT cells after 10 or 30 min of 8 μM myxothiazol inhibition, the -fold change was calculated using the average of the expression level after treatment. For the other cell lines (BY4741, AM2n24, AT2p1, and G143A), the -fold change was relative to the average of the expression level in respective cells without treatment. We assumed that the transcript level was significantly increased or decreased for a specific gene when its Z-score was higher than 1.96 compared with all genes designated as present by the Affymetrix software. The gene classification was made with the Yeast Genome Data ware. The gene classification was made with the Yeast Genome Database (www.yeastgenome.org).

Quantitative Real-time PCR—Total RNA extraction, mRNA purification, and cDNA synthesis were carried out as described previously. Quantitative PCR was performed using CTT1 (AGAAAGAGTTCCGGGAGCCGTG and TCTGGATGAGGGCGCCTAT)- and ACT1 (TTGGAATCCGTTATGAGCGGCGTAT)- specific primers. All real-time PCR reactions were performed in a 25-μl mixture containing 1 μl of cDNA preparation, 1× SYBR green PCR Master Mix (Applied Biosystems), containing SYBR green 1 Dye, AmpliTaq Gold® DNA polymerase, dNTPs with dUTP, passive reference, and optimized buffer components), and 300 nM of each primer. Real-time quantitations were performed using the 7700 Sequence Detector ABI Prism (Applied Biosystems). The fluorescence threshold value was calculated using the Sequence Detector software version 1.7a. ACT1 (encoding actin) reverse transcription-PCR products were used as internal standard. All PCR reactions were in the linear range as attested by comparison to primary calibration experiments. The quantity of mRNA of each gene of interest in each sample, indicated by the threshold cycle (Ct), was therefore normalized by the level of ACT1 RNA: 

$$C_{\text{gene of interest}} - C_{\text{ACT1}} = \Delta C_{\text{r}}.$$ 

Relative quantification of mRNA within the starting material was performed by using the $2^{-\Delta \Delta C_{\text{r}}}$ method ($\Delta C_{\text{sample}} - \Delta C_{\text{non-treated cells}} = \Delta \Delta C_{\text{r}}$; relative quantity = $2^{-\Delta \Delta C_{\text{r}}}$), as suggested by the manufacturer (Applied Biosystems). Each sample was analyzed at least twice.

β-Galactosidase Assay—STRE-LacZ cells were grown in YPGal medium as specified for the microarray analysis. After 8 h of growth, cells were either treated or not with 8 μM myxothiazol and incubated for 10, 60, or 120 min, then cells were harvested at 1,780 $g$ at 4 °C for 4 min. The pellet was washed with ice-cold water, reharvested, and resuspended in ice-cold Z-buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, 50 mM β-mercaptoethanol, pH 7), then the $A_{420}$ was measured. 1 drop of 0.1% SDS and 2 drops of chloroform were added, and the mix was equilibrated at 28 °C for 15 min. o-Nitrophenyl-β-D-galactopyranoside (2.2 μM) was added, and, after incubation at 28 °C for 15 min, the mix was centrifuged at 2,530 $g$ at room temperature for 2 min, and then the $A_{420}$ and $A_{500}$ of the supernatant were measured. β-Galactosidase activity was expressed in the following units: 1000 × ($A_{420} - (1.75 	imes A_{500})$)/ (15 min × culture volume used in the assay in milliliters × $A_{420}$). All experiments were done at least in triplicate.

**RESULTS**

**Myxothiazol Exposure and Genome Expression**—Yeast provides a simple experimental system for testing the effect of dysfunction of the respiratory function because of its capacity for both fermentative and respiratory growth. Galactose (YP-Gal) was supplied to cultures, because this carbon source can be used either for fermentation or for respiration by yeast cells. Contrary to glucose, galactose does not induce the so-called glucose repression of the respiration and mitochondrial biogenesis. To test the effect of the $bc_1$ complex inhibitor on growth, wild type cells (WT) were cultivated in YPGal with increasing concentrations of myxothiazol (Fig. 1). The addition of 0.1 μM myxothiazol had no effect on cell growth. A slight decrease was observed at 0.5 μM inhibitor. At 8 μM, growth was inhibited more than 2-fold. At this concentration the respiratory function was completely inhibited (not shown) but the cells were still able to grow by fermentation. By contrast, the growth of G143A, which harbors a mutation in the cytochrome b $Q_1$ domain causing high resistance to myxothiazol (2), was unaffected by the addition of 8 μM inhibitor.

Changes in gene expression in the WT strain with active respiratory function, suddenly challenged with myxothiazol, were monitored. When WT cells were treated for 2–120 min with 8 μM myxothiazol, the expression of almost 900 genes was altered compared with the non-treated cells. Genes with altered expression were classified in 14 functional categories as shown in Table I (the complete set of data is available in the supplementary material). In each functional category, more overexpressed than repressed genes were generally observed, with the exception of the category "DNA structure, replication, transcription, and translation," where the number of repressed genes was much larger than the number of overexpressed genes. Most of these 238 repressed genes are involved in transcription and translation, and many of them have previously been observed to be repressed in response to different stresses (12). The repressed set includes genes encoding cytoplasmic ribosomal proteins, RNA polymerases I, II, and III, tRNA syn-
HSP82 myxothiazol treatment was monitored. The expression pattern of several genes was analyzed in WT cells treated for 2, 5, 10, 30, 60, or 120 min with 8 μM myxothiazol (Fig. 2A). The maximum overexpression of genes in WT was usually observed after 10- or 30-min treatment with myxothiazol. The analysis of the complete set of data showed that around 25% of the 446 overexpressed genes had their highest expression after 10-min treatment, and around 25% after 30 min. A similar pattern was observed for the repressed genes: 10% of these genes showed their maximum repression at 10-min treatment and 47% at 30-min treatment (data not shown).

A transient change of expression was most clearly seen for CTT1 (Fig. 2D). Within 5 min of exposure to myxothiazol, the expression of CTT1 increased by 10-fold to reach 17-fold after 10 min. After 30 min, the expression decreased to a level closer to pre-stress level. This time-course response is similar to the pattern of expression induced by several environmental changes: immediately after the shift to stressful environment, cells respond with large changes in the expression of genes, and after a while the transcripts return to lower levels (13). Therefore the behavior we observe might represent the first response to sudden inhibitor-induced stress, followed by re-adjustments of the cell metabolism. In the mutant G291D, with the disease mutation causing a permanent respiratory deficiency, the transcript profile was closer to the profile in WT after long treatment with the inhibitor.

**Induction of Gene Expression by Myxothiazol**

We have shown that the addition of myxothiazol induced changes in expression of many genes. We then addressed the question of the specificity of the effect observed, whether the changes were caused by the inhibition of the bc1 complex or by other stresses due to sample handling and/or the addition of the lipophilic compound myxothiazol, independently of, or in addition to, its inhibitory effect on the bc1 complex activity. We monitored the transcription changes after treatment with increasing concentration of myxothiazol and compared the data with the transcript changes observed in the myxothiazol-resistant strain G143A after myxothiazol treatment. The gene expression changes were monitored in WT cells treated for 10 min with 0.1, 0.5, or 8 μM myxothiazol, and in G143A treated for 10 min with 0.5 or 8 μM of myxothiazol. Fig. 2B shows the expression pattern of genes in the three illustrative categories described earlier. In WT cells, the -fold change for the majority of the genes increased with increasing concentration of myxothiazol.

### Inhibition of bc1 Complex and Gene Expression Changes

**Table I**

| Gene number/functional category | Repressed genes after myxothiazol treatment | Overexpressed genes after myxothiazol treatment | Repressed genes in G291D | Overexpressed genes in G291D |
|---------------------------------|---------------------------------------------|-----------------------------------------------|--------------------------|-----------------------------|
| Response to oxidative stress    | 1                                           | 6                                             | 0                        | 1                           |
| Electron transfer and ADP/ATP translocator | 4                                           | 7                                             | 0                        | 2                           |
| Glycolysis, gluconeogenesis, and carbohydrate storage pathway | 11                                           | 74                                            | 5                        | 21                          |
| Peroxisome, fatty acid, and lipid metabolism | 3                                           | 17                                            | 3                        | 5                           |
| Amino acid metabolism, catabolism, and transport | 13                                           | 11                                            | 2                        | 4                           |
| Ion homeostasis                 | 3                                           | 5                                             | 0                        | 2                           |
| Transporter and targeting       | 19                                          | 33                                            | 6                        | 10                          |
| Protein folding and modification (chaperone) | 1                                           | 14                                            | 0                        | 5                           |
| Response to variable stress     | 4                                           | 7                                             | 1                        | 1                           |
| Kinase, phosphorylase, and signal transduction | 4                                           | 31                                            | 0                        | 7                           |
| Cellular organization and biogenesis | 35                                          | 56                                            | 9                        | 14                          |
| DNA structure, replication, transcription, and translation | 238                                         | 32                                            | 3                        | 10                          |
| Other                           | 16                                          | 12                                            | 5                        | 3                           |
| Unknown genes                   | 81                                          | 141                                           | 17                       | 35                          |
| Total                           | 433                                         | 446                                           | 51                       | 120                         |

a The WT cells were treated for 2–120 min with 8 μM myxothiazol. The gene expression of the treated WT cells was compared to the gene expression with untreated WT. The genes repressed or overexpressed after treatment were grouped in functional categories.

b The gene expression in the mutant G291D was compared with the gene expression in the WT cells. The preparation of the samples and data analysis are described under "Materials and Methods."
Inhibition of bc1 Complex and Gene Expression Changes

TABLE II

Overexpressed genes after myxothiazol exposure: activation by bc1 inhibition and by transcription factors Msn2/4 and Yap1

| Activation        | Category                                      | Number of genes |
|-------------------|-----------------------------------------------|-----------------|
| Inhibition of the bc1 complex | Strong ($R_1 \leq 0.25$)               | 24              |
|                    | bc1 inhibition plus other stress (0.25 < $R_1 < 0.75$) | 160             |
|                    | Other stress (0.9 < $R_1$)                    | 3               |
|                    | Unclear (0.75 > $R_1 > 0.9$)                 | 4               |
|                    | Yap1 only (0.25 < $R_2 < 0.75$; 0.9 > $R_3$)  | 3               |
|                    | Msn2/4 only (0.75 < $R_2 < 0.9$; 0.9 > $R_3$) | 115             |
|                    | Yap1 and Msn2/4 (0.75 < $R_2 < 0.9$)         | 49              |
|                    | Other $R_2$ and $R_3 > 0.9$                   | 5               |
|                    | Unclear (0.75 > $R_2$ and $R_3 < 0.9$)        | 19              |

FIG. 2. Changes in gene expression profiles induced by myxothiazol treatment and by the disease-mutation G291D. Changes in gene expression relative to control were monitored in WT, ΔYap1, ΔMsn2/4, and G143A mutants treated with myxothiazol, and in G291D. The expression profiles of genes from three functional categories (response to oxidative stress; electron transfer and ADP/ATP translocator; protein folding and modification (chaperone)) whose transcript levels were significantly increased in at least one treatment or strain are represented. See “Materials and Methods” for further details on data analysis. Each horizontal strip represents a single gene. The fold change is represented by a color (see color bar). A, expression of genes in WT at 2–120 min after addition of 8 μM myxothiazol, compared with gene expression in non-treated G291D; B, induction of genes in WT and G143A at 10 min after addition of increasing concentrations of myxothiazol; C, induction of genes in WT, ΔYap1, and ΔMsn2/4 10 min after addition of 8 μM myxothiazol; D, expression changes in CTTT1.

myxothiazol. In G143A, -fold changes in the presence of 0.5 or 8 μM myxothiazol were very similar, differing by less than 0.5. Therefore, when the expression data were calculated for each gene, assignment of genes into categories utilized the cut-off values for $R_1$, $R_2$, and $R_3$ as indicated in the Table. For most genes, an effect was only observed at concentrations that inhibited growth in YPGal.

In Table II, the 191 genes overexpressed 10 min after addition of the inhibitory dose of myxothiazol (8 μM) were assigned to four categories depending on the stress causing the transcript change. To this end, the ratio -fold change in G143A/-fold change in WT ($R_1$) was calculated for each gene. A $R_1$ value of < 0.25 represents a clear induction of the gene expression by bc1 complex inhibition. 24 genes fell into this category and are listed in Table III. For most of the genes (160 out of 191), the $R_1$ value was between 0.25 and 0.75, indicating that other stresses, in addition to the inhibition of the bc1 complex, could induce the changes in gene expression for these genes, the induction was not due to respiratory inhibition.

Transcription Factors That Regulate the Changes in Gene Expression Caused by Myxothiazol Treatment—To determine the involvement of three major transcription factors, Yap1 and Msn2/4 in the myxothiazol-induced response, we monitored the genomic expression after 10-min treatment with 8 μM myxothiazol in strains with deletion of Yap1 or of Msn2/4. Yap1 is a transcription factor involved in the response to oxidative stress (9), whereas Msn2 and Msn4 are redundant transcription factors involved in the general stress response (8). A double deletion ΔMSN2 ΔMSN4 was used and named ΔMSN2/4. As exemplified in Fig. 2C, for most of the genes the deletion of Msn2/4 decreased the induction by myxothiazol, whereas the changes observed in ΔYap1 were closer to the changes observed in WT. Therefore, Msn2/4, more than Yap1, seem to have a major role in the gene activation caused by myxothiazol. However, there were some exceptions, for example the deletion of YBP1 abolished the activation of the gene CTA1 by myxothiazol. This gene codes for the catalase A involved in the removal of superoxide radicals from the peroxisomal matrix. In Table II, the 191 genes overexpressed 10 min after addition of 8 μM myxothiazol were assigned to five categories depending on the transcription factors regulating the induction. As previously shown, the ratio -fold change in the deletion mutants ΔYap1 and ΔMsn2/4/-fold change in WT ($R_2$ and $R_3$) was calculated for each gene. Most of the genes were found to be activated by Msn2/4 (60%); 26% of the genes were regulated by both Yap1 and Msn2/4; three genes were regulated by Yap1 only; and five genes were not activated by these transcription factors.

These results were confirmed by repeating the experiments with another strain BY4147. The strain W303-1B used in this study harbors mutations in the YBP1 gene. This gene codes for...
a protein Ybp1, which forms a stress-induced complex with Yap1 and might affect its activity (14). The genomic expression analysis was carried out in three strains: BY4147, BY4147Δ yap1, and BY4147Δ msn2/4, under the same conditions described previously. Similar gene expression and regulation were observed, i.e. 44% of genes were activated by Msn2/4, 31% by Yap1 and Msn2/4, 2% by Yap1, and 5% were not activated by these transcription factors (data not shown). So, independently of the strain used, Msn2/4 seems to be involved in the regulation of most of the genes induced by myxothiazol treatment. Yap1 also participates, but to a lower extent, in the activation of the gene expression.

As observed for environmental stresses, the genes showed varying levels of dependence on Msn2/4. The ratio -fold change of the strain used, Msn2/4 seems to be involved in the activation of most of the genes induced by myxothiazol treatment. Yap1 also participates, but to a lower extent, in the activation of the gene expression.

As observed for environmental stresses, the genes showed varying levels of dependence on Msn2/4. The ratio -fold change in ΔMsn2/4/-fold change in WT (Rc in Table II) varied from 0.05 to 0.75 (the cut-off value for Msn2/4 regulation chosen in our study). Some genes are heavily dependent on Msn2/4 such as CTT1 (Table II), others are partially dependent on these factors. Msn2/4 may thus play a different regulatory role for each gene, and other regulators may also be involved.

Translational Changes of an MSN2/4-driven Gene Induced by Myxothiazol Treatment—Msn2/4 bind to the stress response element (STRE) to activate gene transcription (15). The STRE-LacZ reporter gene was used to monitor myxothiazol induction of genes at the protein level. β-Galactosidase activity was measured in untreated and myxothiazol-treated cells as described under “Materials and Methods.” The cells were harvested, and the expression of the reporter gene was followed as indicated under “Materials and Methods.” The controls were as follows: after the 8-h growth at 28 °C, cells were cultivated without myxothiazol for 1 h at 37 °C (C−), or 2 h at 28 °C (C+). The mean of the values obtained for β-galactosidase activity and the (±) S.D. are represented.

Signal between the Myxothiazol Inhibition of the bc1 Complex and the Activation of Msn2/4—Respiratory inhibition by myxothiazol induced the expression of a number of genes through the activity of Msn2/4 and, to a lower extent, Yap1. We next addressed the question of the signal between the inhibition of the bc1 complex and Msn2/4. The sudden inhibition of the respiratory chain by an inhibitor of the bc1 complex might have varying consequences. One immediate effect would be a decrease in the NADH re-oxidation process. The accumulation of NADH might be a signal activating Msn2/4. To test this hypothesis, we monitored the induction of the gene CTT1 by myxothiazol in the presence of oxidants of NADH. CTT1 was
highly induced after myxothiazol treatment; the induction was clearly caused by the inhibition of the bĉ complex and regulated by Msn2/4 (Fig. 2D). Quantitative real-time-PCR experiments (as described under “Materials and Methods”) confirmed the results obtained by microarray analysis for CTT1 gene expression in WT, ΔMsn2/4, and G143A cells 10 min after treatment with 8 μM myxothiazol. As shown in Fig. 4, the relative quantity of CTT1 transcript was low in ΔMsn2/4 and G143A, and much higher in WT.

The electron sinks acetoain or acetaldehyde (16, 17) were then added to WT cells for 10 min prior to 10 min of inhibition with myxothiazol, and the relative quantity of CTT1 transcript was assessed by quantitative real-time-PCR. The addition of these NADH-oxidizing agents decreased by 30% the expression of CTT1 induced by myxothiazol. This suggested that the level of NADH might be involved in the activation of the Msn2/4-regulated gene. The transcript level in the presence of the reductin sink, however, remained higher than the level in the absence of myxothiazol. It can be suggested that the reductive stress due to the accumulation of NADH may be only one factor signalling between myxothiazol inhibition of the respiratory chain and gene activation by Msn2/4.

**DISCUSSION**

**Myxothiazol Treatment and Stress Response**—In this work, we have studied, in yeast, changes in gene expression induced by myxothiazol, a cytochrome bĉ-specific inhibitor. After 2- to 120-min treatment with an inhibitory dose of myxothiazol, the expression of nearly 900 genes changed. Some 200 genes overexpressed in our conditions were also overexpressed under other stresses (13, 18–20), and these may belong to a set of genes involved in the common response to environmental changes, for instance, genes from the category “protein folding and modification (chaperone) such as HSP26, HSP42, and HSP78; genes from the category “glycolysis, gluconeogenesis and carbohydrate storage pathway,” such as GPH1 coding for a glycerogen phosphorylase, FBP2 coding for a fructose-2,6-bisphosphatase, and TPS2 coding for a trehalose-6-phosphate phosphatase; and genes from the category “electron transfer and ADP/ATP translocator” for example CYC7, coding for iso-2-cytochrome c, and COX5B coding for cytochrome c oxidase chain Vb. Some 100 genes repressed in our conditions were also down-regulated by other stresses, and most of them belong to the category “DNA structure, replication, transcription, and translation.” We also observed that the pattern of expression of many genes induced by myxothiazol treatment resembled the time-course response of genes induced by sudden environmental changes. In addition, we have shown that Msn2/4 regulated the expression of most of the genes altered in response to myxothiazol treatment and that the so-called STRE was activated by Ms2/4 in the presence of the inhibitor. Msn2 and -4 are known to be key regulators of the general response to stress (12). They activated a large number of genes after sudden environmental changes, such as glucose starvation, pH change, temperature upshift, etc. Msn2 and -4 are CysHxHis zinc finger proteins normally resident in the cytoplasm, but, in stress conditions, they are located in the nucleus where they bind specifically to STRE (8, 21). The cis-acting STREs are found in the promoters of a large set of genes induced by a variety of stress signals. It is thus most likely then that the sudden inhibition of the respiratory function initiates a similar stress response.

**Signaling Pathways from the Mitochondria**—By comparing the changes in gene expression induced by myxothiazol treatment in WT and in G143A, harboring a mutation at the inhibitor target that hinders its binding, we showed that for several genes, such as CTT1, the expression change was clearly initiated by the inhibition of the bĉ complex. We then addressed the question of the signal from the inhibited respiratory chain to Msn2/4 and the consequent induction of the stress-induced genes. How could the sudden inhibition of the electron flux in the respiratory chain activate Msn2/4?

Mitochondria play an important role in many cellular processes. Pathways of communication from mitochondria to the nucleus are needed in response for instance to mitochondrial dysfunctions, because cellular metabolism needs to be readjusted to compensate for respiratory defects. A pathway well studied is the so-called retrograde response (for review see Ref. 22). In respiratory-deficient cells, the tricarboxylic acid cycle fails to operate as a full cycle. This limits the production of oxaloacetate, and in turn α-ketoglutarate, the direct precursor to glutamate. To compensate, respiratory-deficient cells induce the expression of many genes whose products function in pathways that would re-supply mitochondria with oxaloacetate and acetyl-CoA. Thus, for example, there is an increase of the peroxisomal fatty-acid oxidation that results in an increase in acetyl-CoA production. There is an increase in the expression of genes of the glyoxylate cycle, such as CIT2 coding for the citrate synthase, and in genes involved in transport. In addition, the genes required for the three first steps of the tricarboxylic acid cycle undergo an alteration in their regulation system and come under the control of retrograde pathway regulatory factors. In our study, myxothiazol treatment induced the overexpression of 17 genes belonging to the retrograde response. For example, CRC1 and CAT2, coding for a carnitine transporter and a carnitine acetyl-CoA transferase, respectively, and CIT2 were induced after 2 h of treatment. However, this response involving CIT2 seems to occur, in our conditions, after a long treatment with myxothiazol, whereas most of the genes were induced more rapidly. In addition it has been shown that in W303-1A, a strain very similar to the strain used in this study, CIT2 expression was unresponsive or only mildly responsive to mitochondrial dysfunction (23).

Another signaling pathway needs thus to be invoked to explain our results. We have shown that the myxothiazol-induced
stress functioned mainly through the Msn2/4 regulation. We hypothesized that NADH, which could no longer be reoxidized because of the sudden inhibition of the electron flux through the respiratory chain, could induce a stress that might be the signal activating the transcription factors. To test this hypothesis, we have used two NADH-oxidizing agents, acetoin and acetaldehyde. These compounds are known to relieve the growth inhibition that is caused by the intracellular accumulation of NADH, under anoxic conditions in a yeast mutant with deletion of GPD1 and GPD2 (16, 17). These genes encode the isoenzymes of NAD-dependent glycerol-3-phosphate dehydrogenase involved in glycerol production. We have shown here that the addition of these redox sinks decreased CTT1 overexpression caused by bc1 complex inhibition. Thus, NADH may be involved in the response to sudden respiratory inhibition. One of the questions to be addressed now is how an increase in NADH could activate Msn2/4. NADH may not become restricted by lack of NAD+. In yeast, NADH is re-oxidized by the external and internal NADH dehydrogenases, the entry point of the respiratory chain. When the respiratory chain is inhibited, glycerol production and alcoholic fermentation serve as redox valves to dispose of excess reducing power (for review see Ref. 24). In addition to the cytochrome-containing respiratory pathway, higher plants, some protists, and many fungi possess an alternative cyanide-resistance respiratory pathway. This pathway is mediated by an alternative oxidase, which bypasses cytochromes bc1 and oxidase by accepting electrons from the ubiquinone pool and reducing oxygen to water. The alternative oxidase would thus be a way to re-oxidize NADH and provide the NAD+ needed for metabolic processes when the cytochrome pathway does not function. Increases in alternative oxidase activity have been observed in plant cells after exposure to a number of stress conditions, including low temperature, wounding, and inhibition of the cytochrome pathway of electron transport (for review see Ref. 25). In fungi also, the alternative oxidase is overexpressed when pathogens are treated with fungicide Qo inhibitors such as stroblurins and provide a stroblurin-insensitive pathway for electron flux (for review see Ref. 26). The induction of alternative oxidase expression by respiratory inhibitors is thought to be mediated by reactive oxygen species arising from the inhibition of the cytochrome pathway (27, 28).

We showed here that, in yeast, the sudden inhibition of the respiratory chain activated the Msn2/4 and Yap1 pathways. The activation of the stress response may be mediated, at least partly, by the NADH level. More work will now be needed to determine the precise mechanism of Msn2/4 and Yap1 activation initiated by respiratory chain inhibition. That will help elucidate the molecular basis of the pathways of communication between mitochondria and nucleus.

REFERENCES

1. Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. (2002) Pest Manag. Sci. 58, 859–867
2. Fisher, N., Brown, A. C., Sexton, G., Cook, A., Windass, J., and Meunier, B. (2004) Eur. J. Biochem. 271, 2264–2271
3. Fisher, N., and Meunier, B. (2005) Pest Manag. Sci., in press
4. Fisher, N., and Meunier, B. (2001) Eur. J. Biochem. 268, 1155–1162
5. Bouzidi, M. F., Carrier, H., and Godinot, C. (1996) Biochim. Biophys. Acta 1317, 199–209
6. Dumoulin, B., Sagnot, I., Ferlin, T., Bezon, D., Stepiani, G., and Mousson, B. (1996) Mol. Cell. Probes 10, 389–391
7. Fisher, N., Castleden, C. K., Bourges, I., Brasseur, G., Dujardin, G., and Meunier, B. (2004) J. Biol. Chem. 279, 12951–12958
8. Görner, W., Durschlag, E., Martinez-Pastor, M. T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H., and Schuller, C. (1997) Genes Dev. 11, 586–597
9. Kuge, S., Jones, N., and Nomoto, A. (1997) EMBO J. 16, 1710–1720
10. Schmitt, M. E., Brown, T. A., and Trumpower, B. (1990) Nucleic Acids Res. 18, 3093–3099
11. Ter Linde, J. J., and Steensma, H. Y. (2002) Yeast 19, 825–840
12. Causton, H. C., Ren, B., Koh, S. S., Harbison, C. T., Kanin, E., Jennings, E. G., Lee, T. I., True, H. L., Lander, E. S., and Young, R. A. (2001) Mol. Biol. Cell 12, 3233–3337
13. Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000) Mol. Biol. Cell 11, 4241–4257
14. Veal, E. A., Ross, S. J., Malakasi, P., Peacock, E., and Morgan, B. A. (2003) J. Biol. Chem. 278, 30896–30904
15. Estruch, F., and Carlson, M. (1993) Mol. Cell. Biol. 13, 3872–3881
16. Valadi, A., Granath, K., Gustafsson, L., and Adler, L. (2004) J. Biol. Chem. 279, 39677–39685
17. Ansell, R., Granath, K., Hohmann, S., Thevelein, J. M., and Adler, L. (1997) EMBO J. 16, 2179–2187
18. DeRisi, J. L., Luhr, V. R., and Brown, P. O. (1997) Science 278, 680–686
19. Palkova, Z., Devaux, F., Ianovska, M., Minarikova, L., Le Crom, S., and Jacq, C. (2002) Mol. Biol. Cell 13, 3901–3914
20. Tai, S. L., Boer, V. M., Daran-Lapujade, P., Walsh, M. C., De Winde, J. H., Daran, J. M., and Pronk, J. T. (2004) J. Biol. Chem. 280, 437–447
21. Martinez-Pastor, M. T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H., and Estruch, F. (1996) EMBO J. 15, 2227–2335
22. Butow, R. A., and Avadhani, N. G. (2004) Mol. Cell. 14, 1–15
23. Kirchman, P. A., Kim, S., Lai, C. Y., and Jazwinski, S. M. (1999) Genetics 152, 179–190
24. Bakker, B. M., Overkamp, K. M., van Maris, A., Kotter, P., Luttik, M. A. H., van Dijken, J. P., and Pronk, J. T. (2001) FEBS Microbiol. Lett. 25, 15–17
25. Day, D. A., Dry, I. B., Soole, K. L., Wiskich, J. T., and Moore, A. L. (1991) Plant Physiol. 95, 945–953
26. Wood, P. M., and Hollox, D. W. (2003) Pest Manag. Sci. 59, 499–511
27. Minagawa, N., Kaga, S., Nakane, M., Sakajo, S., and Yoshimoto, A. (1992) FEBS Lett. 320, 217–219
28. Wagner, A. M. (1995) FEBS Lett. 368, 339–342