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# Yeast Adapt to Near-Freezing Temperatures by STRE/Msn2,4-Dependent Induction of Trehalose Synthesis and Certain Molecular Chaperones

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## Summary

**Virtually nothing is known about the biochemical adaptations in eukaryotic cells that may enhance survival at low temperatures or upon freezing. Here we demonstrate an adaptive response in yeast that is activated below 10°C and increases tolerance to low temperatures and freezing. This response involves a dramatic accumulation of the chemical chaperone trehalose and induction of trehalose-synthesizing enzymes (Tps1, Tps2) and certain heat shock proteins (Hsp104, Hsp42, Hsp12, Ssa4). mRNAs for these proteins increase dramatically below 10°C and even at 0°C. Their expression requires Msn2,4 transcription factors but also involves marked mRNA stabilization. Upon return to 30°C, *TPS1, TPS2,* and *HSP104* mRNAs, trehalose levels and tolerance to freezing fall dramatically within minutes. Mutants lacking trehalose or Msn2,4 die more rapidly at 0°C and upon freezing. Thus, below 10°C, yeast show an adaptive response that sustains viability at low or freezing temperatures, which are commonly encountered in natural environments and laboratory refrigerators.**

## Introduction

Although transcriptional and translational adaptations to increases in ambient temperature have been extensively studied (i.e., the heat shock response), the biochemical responses of cells to low temperatures and mechanisms that may protect against freezing have received very little attention. Yet most organisms are often exposed to near-freezing or freezing temperatures in their natural environments. In fact, microorganisms probably encounter rapid temperature downshifts much more often than they encounter heat shock both in the laboratory settings (e.g., upon being shifted from growth conditions to the refrigerator or freezer) and also in natural habitats (e.g., day-night and seasonal temperature changes). Changes in gene expression in response to a drop from the optimal growth temperatures to 10°C–18°C were first described in *E. coli* and termed the "cold shock response" (Jones and Inouye, 1994). After such a downshift, cells undergo a temporary growth arrest and a transient induction of a specific set of "cold shock proteins," many of which participate in transcription or translation and are important for cell growth at 10°C–16°C (Kandror and Goldberg, 1997; Thieringer et al., 1998; Phadtare et al., 1999). In yeast, several cold shock proteins (also called LOT, *LO*w *T*emperature Response proteins) are also induced upon shift to 10°C–18°C, but their functions and mode of regulation are largely unknown (Kondo and Inouye, 1991; Kondo et al., 1992; Zhang et al., 2001). However, no prior studies have addressed the cellular responses to near-freezing temperatures (below 10°C) that might protect against loss of viability at 0°C–4°C and upon freezing.

During studies of the adaptation of *E. coli* to the cold (10°C–16°C), we found that these cells produce large amounts of the disaccharide trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) through the induction of biosynthetic enzymes, OtsA and OtsB (Kandror et al., 2002). This sugar was previously shown to accumulate dramatically in stationary phase (Lillie and Pringle, 1980) and upon heat shock (Hottiger et al., 1987), osmotic shock (Hounsa et al., 1998), or oxidative stress (Wonisch et al., 1997). More importantly, unlike the production of major cold shock proteins, production of this sugar is actually critical for the survival of *E. coli* upon a further temperature downshift to 0°C–4°C (Kandror et al., 2002).

The present study was initially undertaken to test whether the production of trehalose may play a similar protective role in eukaryotes, specifically in *Saccharomyces cerevisiae*. In contrast to *E. coli*, we observed no trehalose induction in yeast during shift to temperatures above 10°C (i.e., during the cold shock response). However, below 10°C and surprisingly even at 0°C, this molecule was found to accumulate dramatically due to increased transcription and translation of trehalose biosynthetic enzymes. Moreover, this accumulation of trehalose markedly enhanced the cells' viability at 0°C and upon freezing.

The observation that trehalose-synthetic enzymes are induced even at 0°C was quite unexpected and led us to investigate whether other genes are coordinately induced at these very low temperatures. These studies led to another surprising discovery—that a number of "heat shock proteins," which function as molecular chaperones, are also markedly induced at 0°C–4°C. The present study thus describes a transcriptional response in yeast to very low temperatures, which we term the "near-freezing response," and which is clearly distinct from the previously described cold shock response.

In yeast, there are three major transcriptional control elements that are activated under different stressful conditions: heat shock elements (HSE) which bind the heat shock transcription factor (HSF); stress response elements (STREs) which are controlled by Msn2,4 transcription factors; and AP-1-responsive elements (APEs) which bind Gcn4 and Yap transcription factors (Ruis and Schuller, 1995). The present studies have investigated whether any of these transcription factors trigger induction of this stress response at 0°C, whether mRNA stabilization may also be important in adaptation to 0°C, and how important are the induction of trehalose and these

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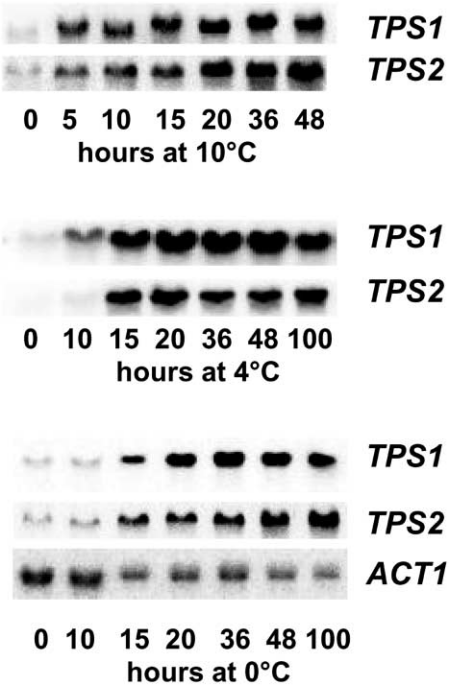


Figure 1. Increase in *TPS1* and *TPS2* mRNA Content after Temperature Shift from 30°C to 10°C, 4°C, and 0°C

Cultures were grown to mid-log phase and shifted to the indicated temperatures. Before and at different times after the shift, samples were taken, and total RNA was isolated and analyzed by Northern blot.

Hsps for survival at near-freezing temperatures or upon freezing.

## Results

### *TPS1* and *TPS2* Transcription Is Induced below 10°C and Even at 0°C

Trehalose synthesis in yeast is catalyzed by two enzymes, trehalose-6-phosphate-synthase (Tps1) and trehalose-6-phosphate phosphatase (Tps2), which together with Tsl1 or Tps3 proteins form the trehalose synthase complex (Bell et al., 1998). To test whether, as in *E. coli*, transcription of genes for trehalose synthesis in eukaryotes is upregulated in response to a fall in temperatures, yeast, growing exponentially at 30°C, were shifted to 16°C or 10°C, and at various times, total RNA was isolated and analyzed by Northern blot. As found in *E. coli*, exponentially growing cells showed very low levels of *TPS1* and *TPS2* mRNAs, but in contrast to our observations in *E. coli*, the content of *TPS1* and *TPS2* mRNA was barely detectable after the shift to 16°C. At 10°C, however, their levels rose markedly by 5 hr of incubation, and by 20 hr had increased about 20-fold (Figure 1). This marked increase was missed in early studies of the cold shock response, which focused on changes during the first several hours at 10°C (Kondo and Inouye 1991; Kondo et al., 1992; Zhang et al., 2001), but recent analysis of gene expression after shift to 10°C also noted induction of these genes by 8 hr (Sahara et al., 2002).

We then tested whether transcription of trehalose-synthetic enzymes could also increase at temperatures below 10°C, where growth ceases and loss of viability can occur. Surprisingly, by 15–20 hr at 4°C and even at 0°C, cellular content of *TPS1* and *TPS2* mRNAs also increased more than 20-fold, and these levels were maintained for up to 85 hr at these temperatures (Figure 1). These large increases in the relative amounts of *TPS1* and *TPS2* mRNAs reflect absolute increases in their cellular content since loading on the Northern blots was normalized to total RNA content which did not change during incubation at 30°C, 10°C, or 4°C. Only after 50 hr at 0°C we observed up to a 30% decrease in total RNA, but by that time *TPS1* and *TPS2* transcripts were already induced at least 20-fold (Figure 1). As an internal control, we also measured the levels of actin 1 (*ACT1*) mRNA in the same blot. In contrast to *TPS1* and *TPS2*, the levels of *ACT1* mRNA decreased with time at 0°C (Figure 1).

### Synthesis and Cellular Content of Tps1 and Tps2 Proteins Increase at 0°C

Our subsequent studies focused on the processes that occur at 0°C, since dramatic changes in gene expression and cell composition at near-freezing temperatures were totally unexpected. Overall protein synthesis drops dramatically upon shifting the yeast to 0°C, and growth ceases. To test whether Tps1 and Tps2 may nevertheless be preferentially synthesized at this temperature, cells at mid-logarithmic phase were shifted to 0°C for 24 hr (when levels of *TPS1*and *TPS2* mRNAs are near maximal) and then incubated with $^{35}$S-methionine. In 6 hr at 0°C, total $^{35}$S incorporation was about five times lower than that in 15 min at 30°C. While overall synthesis thus appears to decrease about 100-fold, several proteins were clearly synthesized at higher relative rates at 0°C than at 30°C (Figure 2A). Two of the highly induced proteins were identified as Tps1 and Tps2 by Western blot with an anti-Tps polyclonal rabbit antibody (kindly provided by Dr. J. Londesborough [VTT, Espoo, Finland]) (Figure 2A). Thus, even though overall protein synthesis is strongly reduced at 0°C, the relative rates of expression of trehalose-synthesizing enzymes is enhanced. The total levels of Tps1 and Tps2 proteins in the cell also increased 2-fold by 48 hr at 0°C (Figure 2B). Since this increase in the protein levels is much less dramatic than the 20-fold increase in mRNAs levels (Figure 1), translation appears to be rate limiting at near-freezing temperatures, and induction of trehalose-synthesizing enzymes is mainly determined by the levels of mRNAs.

### Trehalose Accumulates in the Cells below 10°C

An important question is how the expression of Tps1 and Tps2 might influence production and accumulation of trehalose at these very low temperatures. As shown in Figure 3, trehalose content was barely detectable in logarithmically growing cells at 30°C and rose only when the culture reached saturation. However, when logarithmically growing cells were shifted to 10°C, growth rate significantly decreased, and after 12 hr trehalose content started to increase dramatically, and by 30 hr showed a 40-fold increase over the 30°C levels. Surprisingly, temperature shift to 4°C, or even 0°C (where

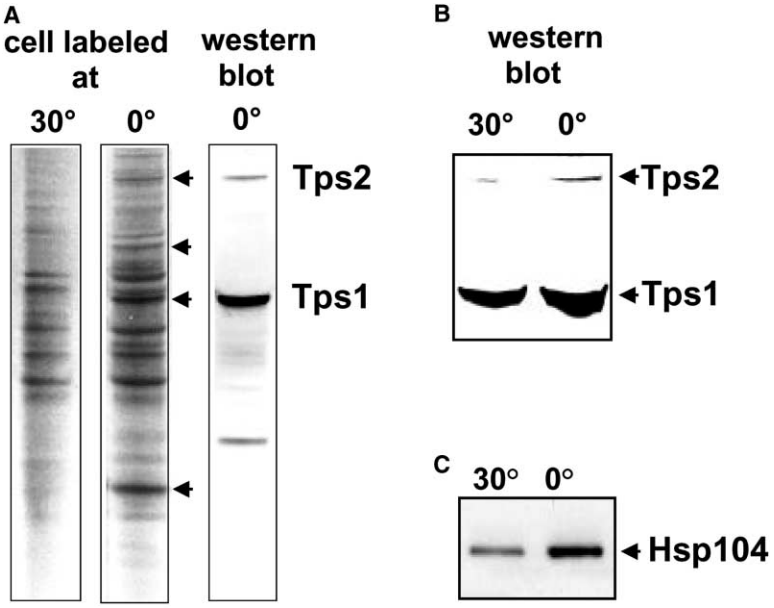


Figure 2. Synthesis and Cellular Content of Tps1 and Tps2 Proteins Increase at 0°C

(A) Wild-type cells were grown at 30°C in minimal media lacking methionine until mid-log phase, when half of the culture was transferred to 0°C. Thirty milliliter aliquots were labeled for 15 min at 30°C or for 6 hr after 24 hr incubation at 0°C. Thirty microliters (0.3 mCi) of methionine (Amersham) was used in each labeling experiment. Cells were harvested by centrifugation, washed with 50 ml ice-cold water, and stored at −80°C. Equal amounts of cell extracts (by radioactivity) were loaded on the gel and analyzed by PAGE, followed by Western blot with anti-Tps antiserum and autoradiography.

(B and C) Cells were grown until mid-log phase at 30°C and shifted to 0°C. Cell extracts were prepared before the shift and after 48 hr at 0°C. Equal amounts of the extracts (by protein) were loaded onto PAGE and analyzed by Western blot with anti-Tps or anti-Hsp104 antiserum.

growth stopped completely, as estimated both by $OD_{600}$ and colony-forming ability), also resulted in very large increases in trehalose content. Between 25 and 50 hr at 0°C or 4°C, trehalose levels increased up to 25- to 50-fold and were then maintained at these levels for at least another 50 hr (as long as we made observations) (Figure 3). Thus, trehalose accumulates in yeast cells at temperatures below 10°C, and even at 0°C, when growth stops. Those observations are different from our results obtained in *E. coli,* where trehalose accumulation did not occur below 10°C (Kandror et al., 2002).

### The Pattern of Gene Expression at 0°C

As shown in Figure 2A, the relative rates of synthesis of several proteins were higher at 0°C than at 30°C. To obtain a more comprehensive view of these changes in gene expression upon the shift to 0°C, we performed a transcriptional microarray analysis. This approach has been applied to yeast under a number of environmental stresses, including heat shock (Gasch et al., 2000), osmotic stress (Gasch et al., 2000), and cold shock (shift to 18°C [Gasch et al., 2000; Lashkari et al., 1997] or to 10°C [Sahara et al., 2002] where cell growth continues), but not at near-freezing temperatures where growth stops.

mRNA samples from yeast growing at 30°C and from cells incubated at 0°C for 24 hr were therefore analyzed by whole-genome microarray hybridization. Each microarray contained about 6000 yeast genes. About 600 mRNAs were either increased or decreased by more than 3-fold at 0°C. In accord with the Northern blot analysis (Figure 1), the microarray data showed that multiple genes for trehalose synthesis (not only *TPS1* and *TPS2*, but also *TPS3*, and to a lesser extent *TSL1*) were induced up to 7-fold after 24 hr at 0°C (Table 1). It is noteworthy that the increases in *TPS1* and *TPS2* mRNAs were higher than those of the two major "cold shock genes," *TIP1* and *TIR1*, whose levels increased about 2- to 4-fold at 0°C (Table 1). Aside from *TIP1* and *TIR1*, the patterns of genes induced at 0°C differed dramatically from the pattern of genes previously reported to be induced at 18°C (Lashkari et al., 1997) or 10°C (Sahara et al., 2002). In particular, most of the major heat shock genes markedly upregulated at 0°C are strongly suppressed both at 18°C and 10°C.

The mRNAs for *HSP42*, *HSP104*, and *SSA4* (a member of the Hsp70 family) were increased 5- to 8-fold at 0°C (Table 1). Thus, the expression of trehalose-synthesizing enzymes and certain molecular chaperones are coordinately induced during the near-freezing response. Accordingly, the levels of *HSP104* mRNA increased at 0°C, 4°C, and 10°C, with a time course that closely followed those of *TPS1* and *TPS2* (data not shown). By 48 hr at 0°C, cellular content of Hsp104 (as shown by Western blot analysis) also increased 3-fold in a similar manner to Tps1 and Tps2 (Figure 2).

The overall changes in gene expression during the near-freezing response clearly resemble in many ways those characteristic of the heat shock response (Lashkari et al., 1997; Gasch et al., 2000), although the absolute changes in these mRNAs in the nongrowing cells at 0°C were less marked and slower in onset than those in growing yeast shifted to 37°C (Table 1). The induction of Hsp104 at 0°C is especially surprising since it plays a key role in cell survival at high temperatures (Sanchez and Lindquist, 1990; Lindquist and Kim, 1996). However, it is also noteworthy that many of the genes that are part of the heat shock response are not induced at near-freezing temperatures (e.g., the levels of *HSP82*, *HSP78*, *HSP26*, *HSP30*, *SSA2*, the *SSA3* mRNAs either did not change or increased less than 2-fold at 0°C). Thus, near-freezing response is a distinct transcriptional response in yeast.

### The Near-Freezing Response Is Controlled by Msn2,4 Transcription Factors

To test whether any of the known stress-activated transcription factors are involved in the induction of the

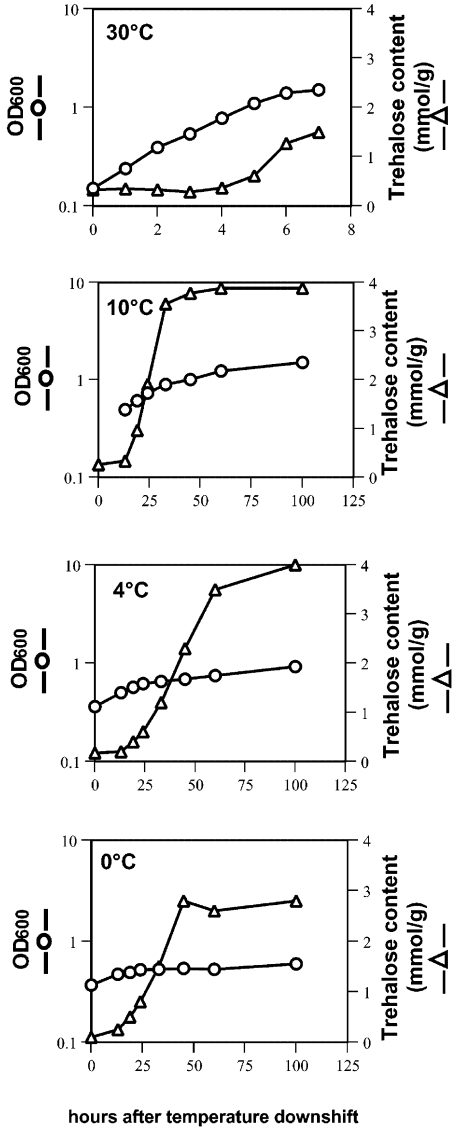


Figure 3. Yeast Growth and Trehalose Accumulation at Different Temperatures

Wild-type cells were grown at 30°C to mid-log phase and shifted to designated temperatures, and growth rate (circles) and trehalose content (triangles) were followed for up to 100 hr after the shift (0′ time).

near-freezing response, we used mutants lacking one or more of these factors (kindly provided by Kevin Struhl, Harvard Medical School). Wild-type and mutant cells lacking Gcn4, Yap1, Yap1,2,5, and Msn2,4 were grown at 30°C until mid-log phase, and then shifted for 24 hr to 0°C, and the levels of *TPS1*, *TPS2*, and *HSP104* mRNAs were determined by Northern blot analysis before and after temperature shift. The strains carrying deletions in *Gcn4* or the *Yap* family members (*YAP1*, *YAP2*, *YAP1,2,5*) induced these genes at 0°C in a fashion similar to the wild-type cells (Figure 4A). By contrast, the Δ*MSN2,4* double mutant showed very little induction (Figure 4A). These results strongly suggest that induction of the near-freezing response is mediated by STRE elements via the Msn2,4 pathway. STRE elements are

Table 1. Ratio of Gene Expression

| Genes | Near-Freezing[a] 0°C/30°C | Heat Shock[b] 37°C/30°C | Cold Shock[b] 18°C/37°C |
|---|---|---|---|
| *TPS1* | 5.6 | 11 | 1.1 |
| *TPS2* | 5.9 | 10.6 | 1.1 |
| *TPS3* | 6.8 | 1.1 | −1.4 |
| *TSL1* | 1.6 | 29 | 0.4 |
| *HSP104* | 7.2 | 28 | −1.7 |
| *HSP42* | 8.1 | 71 | 1.4 |
| *HSP12* | 3.6 | 58 | 3.9 |
| *HSP78* | 1.6 | 15.8 | −3.3 |
| *HSP26* | 1.2 | 10.8 | −1.2 |
| *HSP30* | 1.0 | 11.7 | −1.2 |
| *SSA1* | 2.6 | 10.1 | 1.2 |
| *SSA2* | 1.6 | 2.8 | 1.1 |
| *SSA3* | 1.7 | 7.6 | 0.7 |
| *SSA4* | 6.4 | 58 | 0.8 |
| *TIP1* | 4.2 | 2.1 | 1.5 |
| *TIR1* | 1.8 | 1.4 | 1.3 |

Comparison of expression rates of genes for trehalose synthesis, heat shock proteins, and cold shock proteins during near-freezing response, heat shock (Gasch et al., 2000), and cold shock (Gasch et al., 2000). mRNA samples from yeast growing at 30°C or incubated at 0°C for 24 hr were analyzed by whole-genome microarray hybridization. Genes whose expression changed most dramatically during near-freezing response are presented in this table, and their levels of expression compared to those during heat (shift from 30°C to 37°C) and cold shock (shift from 37°C to 18°C).

[a]This study.

[b]Data from Gasch et al. (2000).

indeed present in the promoter areas of several genes induced at 0°C (e.g., *TPS1*, *TPS2*, *HSP104*, *HSP12*, *HSP42*, *SSA4*; data not shown).

### Dramatic Stabilization of *TPS1*, *TPS2*, and *HSP104* mRNAs at 0°C

The involvement of Msn2,4 in the induction of these genes at 0°C indicates that their transcription is activated (Figure 4A). However, these results do not rule out the possibility that stabilization of specific mRNAs in the cold may also contribute to their accumulation at near-freezing temperatures. In fact, in *E. coli*, the induction of cold shock genes involves mRNA stabilization (Fang et al., 1997; Mitta et al., 1997). To test whether a similar mechanism functions in yeast, wild-type cells were grown at 30°C to mid-log phase and shifted to 0°C for 2 days to increase the cellular content of mRNAs for near-freezing genes. Samples were then taken at 0°C and at different times after the transfer back to 30°C, and the content of *TPS1*, *TPS2*, and *HSP104* mRNAs was analyzed by Northern blot. Although extremely stable at 0°C (data not shown), after the shift back to 30°C the levels of these mRNAs decreased dramatically. *TPS1* and *TPS2* mRNAs fell to basal levels within 5 min at 30°C, while *HSP104* mRNA disappeared by 15 min. In contrast, the content of *ACT1* mRNA did not decrease at all in 1 hr at 30°C (Figure 4B). Thus, some (and perhaps all) of the RNAs induced at near-freezing temperatures are extremely unstable at 30°C ($t_{1/2} < 5$ min), but appear completely stable at 0°C. Thus, as was found in *E. coli* (Fang et al., 1997; Mitta et al., 1997), this dramatic stabilization must contribute to the increase in their levels at very low temperatures.

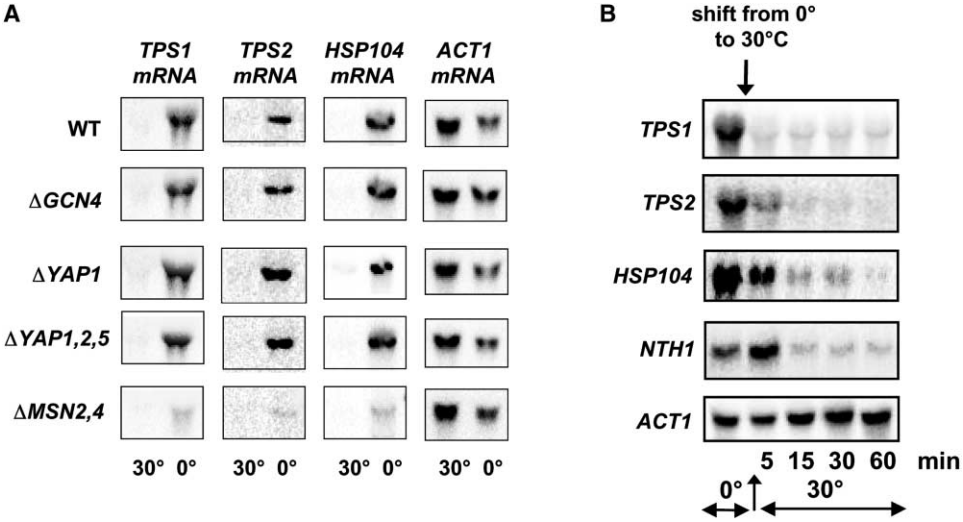


Figure 4. A Number of Near-Freezing mRNAs Are Induced at 0°C and Disappear within Minutes upon Return to 30°C

(A) Increase in *TPS1*, *TPS2*, and *HSP104* mRNA levels at 0°C is Msn2,4 dependent. Wild-type and Δ*GCN*, Δ*YAP1*, Δ*YAP1,2,5*, and Δ*MSN2,4* deletion strains were grown until mid-log phase and shifted to 0°C. Before and 24 hr after the shift, samples were taken, and total RNA was isolated and analyzed by Northern blot with *TPS1*, *TPS2*, and *HSP104* probes. *ACT1* was used as a control.

(B) A number of near-freezing mRNAs are rapidly degraded upon return to 30°C. Wild-type cells were grown until mid-log phase at 30°C, shifted for 2 days to 0°C, and then returned to 30°C. Samples were taken after 2 days at 0°C, and at different times after the shift to 30°C, total RNA was isolated and analyzed by Northern blot with *TPS1*, *TPS2*, *HSP104*, *NTH1*, and *ACT1* (control) probes. Similar results were obtained when 1,10-phenanthroline (100 μg/ml) was added before the shift to 30°C to block mRNA synthesis.

It is noteworthy that the rates of disappearance of these mRNAs at 30°C were the same in the presence of 1,10-phenanthroline, which blocks transcription in yeast by more than 90% (Parker et al., 1991). Therefore, the rapid degradation of these mRNAs accounts for their disappearance, and their synthesis must cease almost immediately after shift from 0°C to 30°C.

### The Msn2,4-Dependent Activation of Transcription Is Important for Viability at 0°C and upon Freezing

To learn whether the induction of the near-freezing response is important in enhancing survival at very low temperatures, the viability of the wild-type and mutants lacking different stress-activated transcription factors was studied at 0°C. As shown in Figure 5, the wild-type cells were quite viable at 0°C, and less than 10% died in 3 weeks. There was no difference in the viability at 0°C of the wild-type and the mutants carrying deletions in *GCN4* or *YAP* genes. By contrast, only 10% of the Δ*MSN2,4* mutant survived this exposure to 0°C (Figure 5). Thus, Msn2,4 function is critical for survival at near-freezing temperatures.

To determine which Msn2,4-dependent gene product(s) is especially important for survival at this temperature, mutants carrying deletions in either of the most strongly induced chaperone genes, *HSP104* and *SSA4*, or genes for trehalose synthesis, *TPS1* and *TPS2*, were tested for viability at 0°C. The cells lacking *HSP104* or *SSA4* were as viable at 0°C as the wild-type; however, the viability of the trehalose-deficient mutant, Δ*TPS1,2* (a generous gift from J. Thevelein and S. Hohmann, Katholieke Universiteit, Leuven, Belgium), in which no trehalose accumulation could be measured, was even lower than that of Δ*MSN2,4* mutant (Figure 5). Thus, the capacity to produce trehalose is critical for cell viability at 0°C. The *TPS1,2*-deficient strain died more rapidly at 0°C than did the cells lacking the whole Msn2,4 response, presumably because the latter cells retained some ability to produce trehalose (Figure 5).

In natural environments, temperatures often fall to near-freezing and eventually go below zero. In addition to enhancing viability at 0°C, the induction of these genes at 0°C–10°C may have evolved to protect cells against freezing. To test this possibility, wild-type cells and the mutant strains (Δ*TPS1,2*, Δ*MSN2,4*, Δ*HSP104*, Δ*SSA4*, Δ*GCN*, Δ*YAP1,2,5*) were transferred to −20°C, and at different times, samples were thawed and their colony-forming ability was assayed. The first sample was taken 60 min after the shift to −20°C to determine the effects of simple freezing-thawing on the cells, and then after 1 and 3 days at −20°C to examine the effects of these mutations on cell survival during prolonged periods in the frozen state. Although all yeast strains showed some loss of viability upon freeze-thaw, this loss was significantly larger for Δ*MSN2,4* and Δ*TPS1,2* mutants. In a typical experiment only 50% of Δ*MSN2,4* and 30% of Δ*TPS1,2* survived 1 hr at −20°C, in comparison to about 80% of the wild-type cells and other mutants (Δ*HSP104*, Δ*SSA4*, Δ*GCN*, Δ*YAP1,2,5*) (Figure 5). Also, these strains survived at −20°C better than Δ*MSN2,4* and Δ*TPS1,2* cells, all of which had died by 3 days at −20°C, while at least 25% of all other strains survived longer storage (Figure 5). In other words, in addition to rapid loss of viability upon freezing, there was an exponential loss of viability at −20°C that was faster in Δ*TPS1,2* mutant than in Δ*MSN2,4* and wild-type. These findings confirm the prior observation that Δ*TPS1* strain is more sensitive to freezing and freeze-drying than the wild-type (Diniz-Mendes et al., 1999).

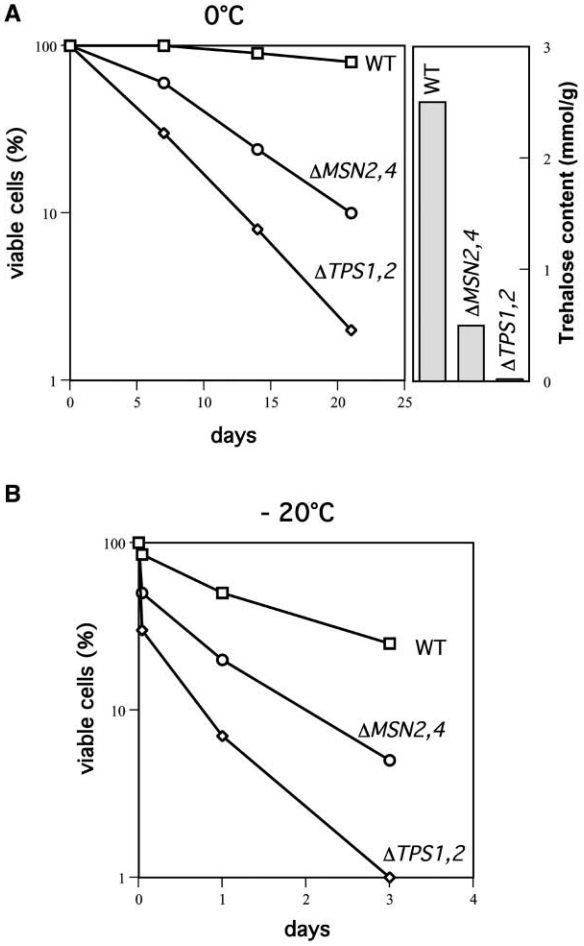


Figure 5. Mutant Strains Carrying Deletions in *TPS1,2* or *MSN2,4* Genes Are Less Viable at 0°C (A) and −20°C (B)

(A) Exponentially growing wild-type and mutant cultures (Δ*TPS1,2*, Δ*HSP104*, Δ*SSA4*, Δ*MSN2,4*, Δ*YAP1,2,5*, Δ*GCN4*) were shifted to 0°C. Trehalose levels were determined after 3 days at 0°C. In Δ*HSP104*, Δ*SSA4*, Δ*YAP1,2,5*, and Δ*GCN4,* they were similar to those of the wild-type. Before and every 7 days after the shift, aliquots of suitable dilutions were plated onto YPGal agar plates. After incubation at 30°C for 3 days for a wild-type and 5 days for mutant, colonies were counted, and the percent of viable cells was measured.

(B) Wild-type and mutant strains were grown at 30°C until mid-log phase. Equal amounts of cells ($OD_{600}$) were spotted onto sterile filter paper (1 $cm^2$) and shifted to −20°C. Before freezing, and then 60 min, 1 day, and 3 days after freezing, filters were incubated for 15 min in 2 ml YPGal media and plated on YPGal plates, and the percent of viable cells was determined. Viability of Δ*HSP104*, Δ*SSA4*, Δ*YAP1,2,5*, and Δ*GCN4* mutants at 0°C and −20°C was similar to that of the wild-type.

Since other component of the near-freezing response were induced in the Δ*TPS1,2* mutant, these components alone (e.g., molecular chaperones) cannot provide protection at near-freezing temperatures and during freezing.

### Trehalose Accumulation during Cell Adaptation at 0°C–4°C Increases Freeze Tolerance

These data suggest that in natural environments, as the temperature falls below 10°C, trehalose production rises and protects cells upon further temperature drop below 0°C. To test this idea, wild-type cells and mutants unable to make trehalose were grown to the mid-log phase at 30°C and shifted for 48 hr to 0°C–4°C to induce near-freezing response. Then the cells were frozen at −20°C, and their ability to survive freezing was compared with that of cells shifted directly from 30°C to −20°C.

After 5 days at −20°C, about 70% of the cells pre-adapted at 4°C were still alive, while only 25% of the control cells survived this incubation (Figure 6A). Thus, adaptation to near-freezing temperatures (i.e., induction of near-freezing response) enhances the viability of the cells undergoing freezing, and preadaptation at 4°C consistently showed a better protective effect than preadaptation at 0°C (data not shown). When similar experiments were done with the Δ*TPS1,2* mutant, surprisingly, incubation at 0°C–4°C resulted in even greater killing upon freezing. All of the trehalose-deficient cells which had been incubated at 4°C were dead after 5 days at −20°C, while 10% of the control cells shifted directly from 30° to −20°C were still alive (Figure 6A). Thus, trehalose accumulation is the critical component of the near-freezing response, without which cells do not acquire freeze tolerance.

### Trehalose Content and Freeze Tolerance Decrease Quickly upon Return to 30°C

Although trehalose is clearly important for the cells to survive freezing, it remains uncertain whether this disaccharide provides protection during the actual freezing or during readaptation of the cells to 30°C (i.e., when the cells are removed from the freezer and incubated at 30°C to determine viability). To distinguish between these two possibilities, we followed trehalose content in cells shifted back to 30°C after incubation for 2 days at 0°C. As shown in Figure 6B, high levels of trehalose accumulated at 0°C decreased dramatically within 5 min after the shift back to 30°C, and by 1 hr, trehalose content reached the low level characteristic of the cells growing at 30°C. Thus, the high levels of trehalose found at near-freezing temperatures are no longer present in the cell by the time growth is reinitiated (after 1 hr at 30°C). These findings suggest that it is important for the cell to destroy trehalose rapidly when the cell returns to temperatures that allow growth. (Similar rapid elimination of trehalose has been observed when heat shocked yeast are returned to 30°C [Singer and Lindquist 1998a, 1998b].) Interestingly, the gene for neutral trehalase *NTH1* (enzyme responsible for trehalose degradation) is also slightly induced in the cold and achieves maximal induction immediately after the shift back to 30°C (Figure 4B), apparently to allow rapid degradation of trehalose. These results indicate that trehalose accumulation protects cells against near-freezing and freezing temperatures and is not required for readaptation to 30°C.

The degree of freeze tolerance acquired at 4°C (Figure 6) closely correlates with the trehalose content. After 2 days at 4°C, when the maximal levels of trehalose were generated, cells acquired the highest resistance to freezing (Figure 6B). Following the shift back to 30°C, trehalose content fell dramatically and so did acquired freeze tolerance (Figure 6B). These results, together with those presented in Figure 6A, demonstrate a direct

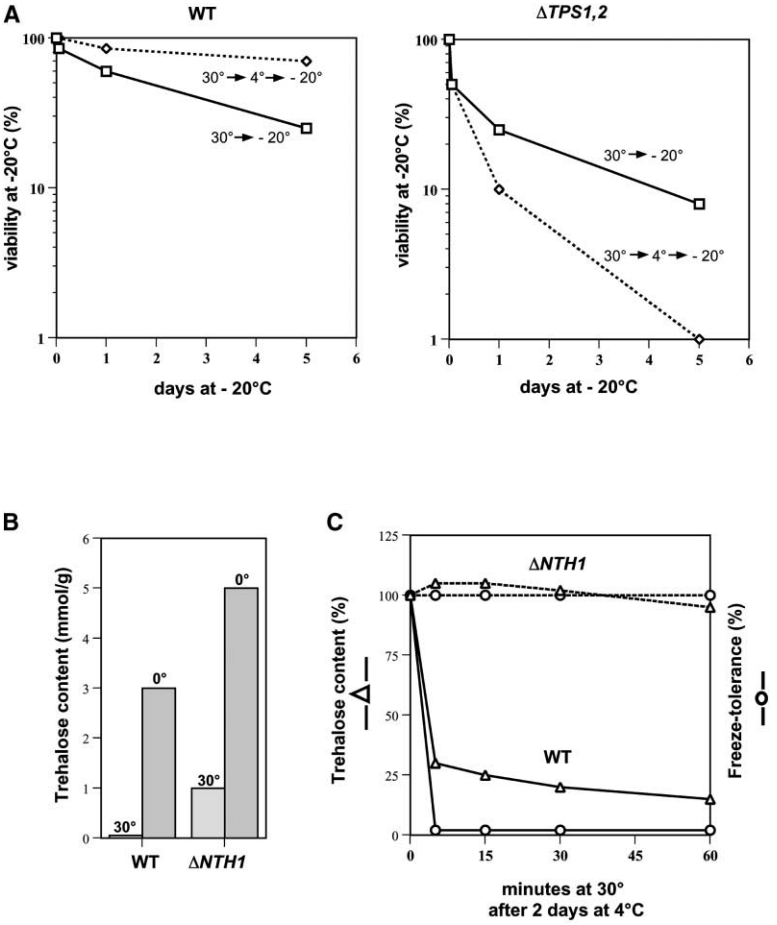


Figure 6. Freeze Tolerance Closely Correlates with Cellular Trehalose Content

(A) Adaptation of wild-type (but not Δ*TPS1,2*) cells to 4°C increases their resistance to freezing. Wild-type and Δ*TPS1,2* cells were grown until mid-log phase at 30°C, and each culture divided in two. One portion was shifted to 4°C, and after 2 days, cells were frozen at −20°C, and the percent of viable cells was determined after different times at −20°C (as in Figure 5). The other portion was transferred from 30°C directly to −20°C, and the percent of viable cells was determined.

(B) Trehalose content in the wild-type and Δ*NTH1* mutant at 30°C and after 2 days at 0°C.

(C) Trehalose content and acquired freeze tolerance decrease rapidly in the wild-type (but not in Δ*NTH1* mutant) upon return to 30°C. The wild-type and Δ*NTH1* mutant were grown until mid-log phase at 30°C, shifted to 4°C for 2 days, and then returned to 30°C. Samples were taken for assay of trehalose content and sensitivity to freezing (as in Figure 5) before the shift to 4°C, after 2 days at 4°C, and at various times after the shift to 30°C. The increase in the amount of cells resistant to freezing due to adaptation at 4°C (termed "acquired freeze tolerance") was taken as 100%.

involvement of trehalose in the acquisition of freeze tolerance.

### Trehalose Content Determines Tolerance to Freezing

To test more directly whether trehalose content determines freeze tolerance, we used a mutant unable to degrade trehalose because it lacks neutral trehalase Nth1 (kindly provided by Susan Lindquist, MIT). At 30°C, trehalose content of Δ*NTH1* cells was much higher than in the wild-type (Figure 6B). Nevertheless, after shift to 0°C, trehalose content of the mutant increased markedly, and by 2 days was 60% higher than in the wild-type (Figure 6B). The sensitivity to freezing of the Δ*NTH1* was similar to wild-type at 30°C (25% of the wild-type and 30% of Δ*NTH1* cells were still alive after 5 days at −20°C). Upon incubation at 4°C for 48 hr, resistance of the Δ*NTH1* to freezing increased to an even greater extent than in the wild-type (90% of Δ*NTH1* cells and 70% of wild-type survived 5 days of freezing) presumably because of its higher content of trehalose. These findings confirm the prior observation in yeast *Schizosaccharomyces pombe* that accumulation of trehalose by overexpression of *TPS1* (especially in the strain lacking neutral trehalase) increased its resistance to freezing (Soto et al., 1999).

As expected, trehalose levels in the Δ*NTH1* mutant, unlike those in the wild-type, did not decrease rapidly upon return from 0°C to 30°C and remained constant for at least an hour (Figure 6C). Moreover, its tolerance to freezing remained high throughout this period, while in the wild-type, it dramatically decreased within minutes (Figure 6C). Thus, the cell's trehalose content directly determines its resistance to freezing.

Use of the Δ*NTH1* mutant also allowed us to clarify the importance of trehalose synthesis and hydrolysis in the regulation of trehalose content during the near-freezing response. Because trehalose is quite stable in Δ*NTH1* mutant at 30°C as well as at 0°C, its marked accumulation at 0°C must occur primarily through accelerated synthesis. By contrast, rapid elimination of trehalose in the wild-type upon return to 30°C is due to hydrolysis by neutral trehalase (Figure 6C). Thus cellular trehalose content is determined by the balance between its synthetic and degradative rates, both of which are precisely regulated.

## Discussion

### Trehalose and Tolerance to Near-Freezing and Freezing Conditions

The present studies have uncovered an adaptive response in yeast, which may also function in many other eukaryotic organisms at very low temperatures. It is very likely that this response is important for viability of yeast in most parts of the globe where near-freezing or freez-

ing conditions are common, at least during winter. Moreover, in the laboratory, storage of yeast and other microorganisms in refrigerators and freezers is a standard practice and is generally assumed to preserve organisms in their prior state—not to induce specific adaptive changes. This response may appear relatively slow in onset as compared to the rapid responses that yeast show upon heat or osmotic shock (e.g., more than 20 hr was required for maximal accumulation of trehalose). However, immediate temperature decreases of 30°C, as used here, are not encountered in natural environments, where such large decreases in temperature typically occur over many days or with seasonal changes. Thus, adaptations requiring 1–3 days can have major protective consequences.

Although a number of coordinate changes in gene expression were found to occur at 0°C–4°C, including the production of many chaperones, the most important component in enhancing viability at 0°C or upon freezing is clearly the production of high levels of trehalose. The inability to synthesize this sugar resulted in a rapid loss of viability at 0°C, while trehalose accumulation ensured long-term survival at this temperature and also during freezing. Also, we have shown that preadaptation at 4°C enhances resistance to freezing, and that a key process leading to freeze tolerance is accumulation of trehalose (Figure 6). In contrast, the rapid loss of this adaptation upon shift back to 30°C correlates with and is a consequence of the rapid digestion of trehalose by neutral trehalase (Nth1) (Figure 6). The importance of trehalose accumulation in enhancing tolerance to freezing can account for the earlier observation that exposure to other stressful conditions (heat and oxidative shocks) that cause trehalose accumulation helps to protect yeast against freezing (Lewis et al., 1995).

In addition to microorganisms and prokaryotes, trehalose production occurs in insects and plants, but not mammals or birds. One possible reason why mammals may have lost the ability to synthesize trehalose is that they are warm-blooded, and their intracellular temperatures are maintained far above freezing, even in very cold environments. In contrast, "cold-blooded" multicellular organisms may have to rely on trehalose production to withstand very low (and high) ambient temperatures. In fact, our recent observations suggest that higher eukaryotes (e.g., *Drosophila*) may also induce trehalose synthesis in response to near-freezing temperatures (unpublished data).

### The Induction of Stress Proteins at Near-Freezing Temperatures

Quite remarkable is the finding that yeast can undergo gene transcription, RNA processing, and translation of trehalose-synthesizing enzymes at 4°C and even 0°C, even though overall protein synthesis at such temperatures is greatly reduced. It is also surprising that at near-freezing temperatures, where most enzymatic activities are barely detectable, trehalose-synthetic enzymes can with time generate very high levels of trehalose. This dramatic accumulation of trehalose at 0°C is due primarily to its accelerated synthesis since it also occurred in the Δ*NTH1* mutant, in which trehalose was very stable at both 0°C and 30°C. It is interesting that the absolute levels of Tps1 and Tps2 proteins increased only 2-fold by 2 days at 0°C, while trehalose content increased at least 20-fold. Presumably, the marked stabilization of trehalose (in the wild-type) at 0°C helps account for its dramatic accumulation. However at 0°C, other components of the trehalose-synthase complex, Tps3 and Tsl1 (Bell et al., 1998), are also induced (Table 1) and may enhance production of the disaccharide.

The coordinate induction of trehalose-synthesizing enzymes and certain heat shock proteins at 0°C–10°C differs dramatically from the changes in gene expression seen upon downshift to 10°C–18°C, which had been termed the cold shock response. Yeast grow well at 18°C and even at 10°C, which therefore should not be viewed as highly stressful conditions. Accordingly, genes for trehalose synthesis and *HSP104* are not induced by such a temperature drop; on the contrary, downshift from 30°C to 18°C for 1 hr resulted in about 3-fold repression of their transcription (Lashkari et al., 1997). At 10°C, the maximal induction of *TPS1*, *TPS2*, and *HSP104* occurs only by 10–15 hr after the shift, in contrast to cold shock genes (e.g., *TIP1*, *TIR1*, *NSR1*), which are induced during the first 2–6 hr. Moreover, the levels of *HSP104*, *TPS1*, and *TPS2* mRNAs and trehalose, once elevated, were maintained at these levels for up to 50–80 hr, while the induction of cold shock proteins is a transient response (Kondo and Inouye, 1991; Kondo et al., 1992; Zhang et al., 2001). Thus, the adaptation to near-freezing temperatures is a distinct adaptive response.

Quite unexpected was the finding that a number of heat shock genes are highly induced at near-freezing temperatures, especially since temperature downshift to 10°C–18°C resulted in their specific repression both in *E. coli* and yeast (Jones and Inouye, 1994; Lashkari et al., 1997). The induction of *HSP104* at 0°C is particularly surprising, since this chaperone is the most important heat shock protein providing thermotolerance in yeast (Sanchez and Lindquist, 1990; Lindquist and Kim, 1996). The induction of *SSA4* at 0°C is also striking since it had been argued that the Hsp70 family of chaperones is unlikely to function at near-freezing temperatures (Romisch and Matheson, 2003), and a different family member (Ssb1) had been associated with mild cold shock response (Maneu et al., 2000). It remains unclear how chaperones, like Hsp104 and Ssa4, which function through ATP hydrolysis at high temperature, can do so at 0°C or 4°C.

### The Transcriptional Regulation of Near-Freezing Response

The similarities in the expression patterns of genes for trehalose synthesis and *HSP104* in response to different temperature downshift conditions (e.g., their repression at 18°C or 16°C and coordinate induction at 10°C, 4°C, and 0°C) strongly suggest that a single mechanism activates their expression in response to near-freezing temperatures. This conclusion was clearly supported by the finding that the stress-activated transcription factors, Msn2 and Msn4, are essential in this response. It is noteworthy that more than 80% of the genes reported to be induced in an Msn2,4-dependent manner in response to heat, $H_2O_2$ (Gasch et al., 2000), or acid (Caus-

ton et al., 2001) are also upregulated during the near-freezing response. It is unclear why some of these Msn2,4-dependent genes are not induced at 0°C. Some Msn2,4-activated genes lack a classical STRE element (Causton et al., 2001) which may be important for induction at low temperatures. In addition, Msn2,4 may bind to other upstream consensus sequences or act in concert with additional factors that are stress specific.

The major heat shock proteins contain several different positive stress response elements in their promoter regions that ensure proper expression under extreme conditions. For example, increased transcription of *HSP104* during heat shock is usually obtained through cooperation of Msn2,4/STRE and HSF/HSE systems, although each factor can alone activate the *HSP104* promoter (Grably et al., 2002). Induction of *HSP104*, *TPS1*, and *TPS2* at 0°C is strictly dependent on Msn2,4/STRE, since this response is greatly reduced in the Δ*MSN2,4* mutant and is not affected in strains lacking other transcriptional activators (e.g., Gcn4 or Yap family members; the involvement of HSF could not be tested directly since this factor is essential even under non-stressful conditions). Similarly, while activation of *TPS2* transcription during heat shock or exposure to cycloheximide was shown to require Yap1 (Gounalaki and Thireos, 1994), its activation at 0°C is clearly dependent on Msn2,4 and is not affected in a triple *YAP1,2,5* deletion mutant. It is also noteworthy that although the *YAP1* deletion results in a cold-sensitive phenotype (i.e., reduced growth at 16°C) (Fernandes and Rodrigues-Pousada et al., 1997), *YAP* mutants showed no decrease in viability at 0°C or upon freezing. These observations are further evidence that adaptations to 16°C (cold shock) and to near-freezing temperatures are distinct responses involving different transcriptional activators and having distinct physiological consequences.

The changes in the mRNA content seen below 10°C, however, are not caused by the transcriptional adaptations alone. Dramatic stabilization of *TPS1*, *TPS2*, and *HSP104* mRNAs is clearly occurring at near-freezing temperatures since these mRNAs were degraded within minutes upon return from 0° to 30°C (Figure 4B). The mechanism for rapid destruction of these mRNAs at 30°C, but not *ACT1* mRNA, is obviously a fundamental problem for future investigation. In any case, stabilization of these, and presumably other near-freezing, mRNAs clearly complements the changes in Msn2,4-dependent transcription and contributes to their large induction at low temperatures. It is noteworthy that the induction of cold shock genes in *E. coli* is also associated with differential stabilization of mRNAs (Fang et al., 1997; Mitta et al., 1997).

## Possible Mechanisms of Cell Protection by Trehalose

Since the accumulation of trehalose at near-freezing temperatures is very important for viability both at 0°C and upon freezing, an important goal for future work will be to understand the reasons for cell death under such conditions and the possible mechanisms of trehalose protective action. Early work on trehalose had assumed that this sugar protected cell membranes during heat shock or freezing (Hochachka and Somero, 2002). However, the involvement of the major molecular chaperones in adaptations to near-freezing temperatures makes it likely that at these temperatures there is damage to certain cell proteins and suggests possible mechanisms for the protective action of trehalose. Hsp104 and trehalose have synergistic effects in protecting proteins against heat denaturation (Singer and Lindquist, 1998a, 1998b). Upon heat shock, trehalose rises rapidly and appears to reduce protein aggregation until the microaggregates can be disassembled, and these proteins can be refolded by Hsp104 (Singer and Lindquist, 1998a, 1998b). Since Hsp104 and trehalose are induced coordinately at 0°C, they may also function together at near-freezing temperatures, where certain cell proteins or membranes may be damaged (Privalov, 1990; Kaul et al., 1992) Trehalose was also shown in vitro to act synergistically with the small heat shock protein, p26, in preventing heat denaturation and aggregation of a model protein (Viner and Clegg, 2001). This observation seems especially interesting since the p26 homolog, Hsp42, and another small heat shock protein, Hsp12, were markedly induced at 0°C. It is also possible that trehalose stabilized proteins in a fashion similar to another protective osmolyte, trimethylamine N-oxide (TMAO), which accumulates in the cells of elasmobranches and raises the free energy of the denatured state of proteins, shifting the equilibrium in favor of the native state (Bolen and Baskakov, 2001; Baskakov and Bolen, 1998). In fact, in related experiments (unpublished data), we have found that trehalose can prevent cold-induced denaturation and aggregation of certain pure proteins, but the generality of these effects and their relationship to cold and freeze tolerance remain to be established.

These findings and previous results thus indicate a more general role for heat shock proteins and suggest that trehalose and several molecular chaperones act synergistically during adaptation to near-freezing temperatures. The molecular mechanisms by which trehalose and heat shock proteins can protect cell proteins at 0°C–4°C and the identity of the cellular constituents that are susceptible to such damage are important questions for future research that may have practical applications in enhancing resistance of organisms to low temperatures and freezing.

## Experimental Procedures

All strains were grown in yeast extract peptone-galactose medium (YPGal) except for labeling experiments where minimal media lacking methionine was used.

Trehalose was assayed as described previously (Kienle et al., 1993; Lee and Goldberg, 1998). Samples were normalized by the amount of protein in the extract.

### Preparation of Cell Extract

Cells were collected, resuspended in 0.5 ml prewarmed (60°C) complete cracking buffer (8 M Urea, 5% SDS, 40 mM Tris-HCl [pH 6.8], 0.1 mM EDTA, 0.4 mg/ml bromephenol blue, 1% β-mercaptoethanol, 5 mM PMSF, protease inhibitors cocktail), and broken with glass beads.

### Northern Blot Analysis

RNA electrophoresis was performed according to Oligotex. RNA was extracted by acid phenol method. Loading was normalized by the amount of total RNA. Samples were run in 0.8% formaldehyde

agarose gel, blotted onto Zeta-Probe Blotting membrane (BIO-RAD), and crosslinked with UV light.

Probes for Northern blot were prepared by PCR using yeast DNA as a template. Primers were designed to amplify 400 nucleotide fragments of *TPS1* (5′-GGCCGTTATTCCATTACCATCCTG-3′ and 5′-GGCCCCTACTTAACGAATCTGC-3′), *TPS2* (5′-AGGCAAAGACC GATACTACTCA and 5′-GGCCCCAACAAGACCAT), *ACT1* (5′-GCC GGTTTTGCCGGTGAC-3′ and 5′-GGAAGATGGAGCCAAAGC-3′), *HSP104* (5′-AGGACGACGCTGCTAACATCTTG and 5′-GGTGGAGT CGGCATCTTCATCT).

Probes were labeled by using random primers Stratagene Prime-it II kit. Hybridization was carried out as previously described in (Church and Gilbert, 1984). Northern blots were visualized by Molecular Imager FX.

### Transcriptional Microarray Analysis

mRNA samples from yeast growing at 30°C or incubated at 0°C for 24 hr were analyzed by whole-genome microarray hybridization. Each microarray contained PCR products of 6218 yeast genes. The results of two independent biological replicates were filtered. The signals whose absolute values in both channels in at least one hybridization were less than two standard deviations from the background were discarded. Hybridizations were of very high quality since 6120 of the 6218 genes passed the threshold. According to the Rosetta resolver error model (Hughes et al., 2000) used here, the 4257 genes with a $p < 0.05$ can be considered highly significant. About 670 of these were either up- or downregulated more than 3-fold at 0°C.

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