Rox3 and Rts1 Function in the Global Stress Response Pathway in Baker's Yeast

Carlos C. Evangelista, Jr.,[1] Ana M. Rodriguez Torres,[2] M. Paullin Limbach and Richard S. Zitomer

Department of Biological Sciences, State University at of New York, Albany, New York 12222

Manuscript received August 21, 1995
Accepted for publication December 23, 1995

ABSTRACT

Yeast respond to a variety of stresses through a global stress response that is mediated by a number of signal transduction pathways and the *cis*-acting STRE DNA sequence. The *CYC7* gene, encoding iso-2-cytochrome c, has been demonstrated to respond to heat shock, glucose starvation, approach-to-stationary phase, and, as we demonstrate here, to osmotic stress. This response was delayed in a the *hog1-Δ1* strain implicating the Hog1 mitogen-activated protein kinase cascade, a known component of the global stress response. Deletion analysis of the *CYC7* regulatory region suggested that three STRE elements were each capable of inducing the stress response. Mutations in the *ROX3* gene prevented *CYC7* RNA accumulation during heat shock and osmotic stress. *ROX3* RNA levels were shown to be induced by stress through a novel regulatory element. A selection for high-copy suppressors of a *ROX3* temperature-sensitive allele resulted in the isolation of *RTS1*, encoding a protein with homology to the B′ regulatory subunit of protein phosphatase $2A_0$. Deletion of *RTS1* caused temperature and osmotic sensitivity and increased accumulation of *CYC7* RNA under all conditions. Over-expression of this gene caused increased *CYC7* RNA accumulation in *rox3* mutants but not in wild-type cells.

ALMOST all cells have the ability to respond to environmental stresses such as sudden elevated temperatures, changes in osmotic pressure, starvation conditions, and more. One set of responses takes the form of changes in the transcriptional program to synthesize proteins that protect the cell against damage. The yeast *Saccharomyces cerevisiae* regulates these responses in a variety of ways including the near ubiquitous specific heat shock response (CRAIG *et al.* 1993), specific responses to starvation for glucose through catabolite repression (RONNE 1995), nitrogen through nitrogen repression (MAGASANIK 1992), and amino acids through the general control pathway (HIINEBUSCH 1990). In addition, there is a more global response to a wide variety of stresses. This latter system has been documented for the induction of the *CTT1* gene, encoding catalase, in response to nitrogen starvation, osmotic stress, heat shock, and oxidative stress (MARCHLER *et al.* 1993); the *HSP104* gene, the *GAC1* gene, a encoding a putative phosphatase type 1 regulatory subunit, and the *UBI4* gene, encoding polyubiquitin, by osmotic stress (SCHULLER *et al.* 1994); the *DDR2* gene in response to heat shock, DNA damage (KBOAYASHI and MCENTEE 1993), and osmotic stress (SCHULLER *et al.* 1994); and the *TPS2* gene, encoding trehalose phosphate phosphatase, in response to a variety of metabolic inhibitors, heat shock, and osmotic stress (GOUNALAKI and THIREOS 1994).

Corresponding author: Richard S. Zitomer, Department of Biological Sciences, SUNY, 1400 Washington Ave., Albany, NY 12222.
E-mail: rz144@cnsvax.albany.edu

[1] *Present address:* Department of Genetics, University of Washington, Seattle, WA 98195.

[2] *Permanent address:* Departamento de Biologia Celular y Molecular, Universidad de La Coruna, Campus de La Zapateira sln. 15071-La Coruna, Spain.

This global response depends upon the cell's ability to sense and signal different stress conditions. For example osmotic stress is signalled through a mitogen-activated protein (MAP) kinase cascade (BREWSTER *et al.* 1993; MAEDA *et al.* 1994), and the starvation conditions of stationary phase is signalled through the cAMP-RAS-protein kinase A pathway (WERNER-WASHBURNE *et al.* 1993). These different pathways apparently send the signal to a 140-kDa putative transcriptional activator protein that binds to the *cis*-acting STRE element, CCCCT, found in the upstream region of genes that are induced by this global stress response (KBOAYASHI and MCENTEE 1993; MARCHLER *et al.* 1993; SCHULLER *et al.* 1994; GOUNALAKI and THIREOS 1994). In addition, a second transcriptional activator, Yap1p, the yeast homologue of mammalian Ap1, has been implicated in this response (GOUNALAKI and THIREOS 1994).

Studies on the regulation of the *CYC7* gene have indicated that it is induced by some of these stresses. Yeast contains two genes encoding the cytochrome c protein; *CYC1* encodes iso-1-cytochrome c protein, comprising 95% of the cytochrome c in respiring cells, and *CYC7* encodes iso-2-cytochrome c protein (SHERMAN and STEWART 1971). The *CYC7* expression is subject to glucose regulation and to a weak oxygen induction (ZITOMER *et al.* 1987). In addition, *CYC7* expression is induced by heat shock and low cAMP levels (PILLAR and BRADSHAW 1991) and in the approach-to-stationary phase (LAZ *et al.* 1984) suggestive of the global stress response. In this study, we demonstrated that *CYC7* also responds to osmotic stress, and this response as well as the heat shock response is probably mediated through three CCCCT elements dispersed throughout the *CYC7* regulatory region.

In cells carrying a *CYC1* deletion, the low level of *CYC7* expression is insufficient to support cell growth on the nonfermentable energy source lactate, and this growth defect has been used extensively in selections for mutations that cause *CYC7* over-expression (CLAVILIER *et al.* 1969; ROTHSTEIN and SHERMAN 1980). A variety of regulatory factors have been identified in this way, including Rox3 (ROSENBLUM-VOS *et al.* 1991). Rox3 is an essential nuclear protein whose function is unknown. In our attempts to elucidate its function, we have implicated Rox3 in the stress response and isolated the *RTS1* gene as a high-copy suppressor of a *ROX3* temperature-sensitive allele. The *RTS1* gene product shows a high degree of homology to the rabbit skeletal B′ subunit of phosphatase $2A_0$ (ZOLNIERWICZ *et al.* 1994; CSORTOS *et al.* 1995), suggesting a role for this phosphatase in the stress response.

MATERIALS AND METHODS

Strains, media, cell growth, and transformations: The following *S. cerevisiae* strains were used in this study. RZ53-6 is *MATα, trp1-289, leu2-3, -112, ura3-52, ade1-100* (BALASUBRAMANIAN *et al.* 1993), and RZ53-6Δ*rts1* was constructed by replacement of the wild-type *RTS1* allele with the *rts1::URA3* disruption. Both RZ71-7 (*MATα, trp1, leu2-3, -112, ura3-52, ade1-100, lys2*, gal$^-$, *rox3::LEU2*) and RZ68-2 (*MATa, trp1, leu2-3, -112, ura3-52, ade1-100, rox3::LEU2*) were described previously; they contain a deletion of *ROX3* that is lethal, and consequently, these strains must carry a *ROX3* allele on a plasmid (ROSENBLUM-VOS *et al.* 1991). MZ18-19C (*MATα, trp1, leu2-3, -112, ura3-52, ade1-100, rox3::LEU2, rts1::URA3* plus YEp(112)*rox3-182*) was derived from a mating between RZ53-6Δ*rts1* and RZ68-2 transformed with YEp(112)*rox3-182*. AH12-7 is *MATα, trp1-1, leu2-3, -112, his4-589, cyc1-1*, gal$^-$, and AH12 is a derivative of AH12-7 with a *LEU2* replacement of the *CYC7* chromosomal allele (HEALY *et al.* 1987). The *hog1-Δ1* mutant JBY13 (*MATa, ura3-52, lys2-801, ade2-101, leu2-Δ1, his3-Δ200, hog1-Δ1::TRP1*) and the corresponding wild type, YPH102, have been described (SCHULLER *et al.* 1994).

Yeast cells were grown in either rich YPD media or for selective growth in synthetic media lacking the appropriate nutrient (ROSE *et al.* 1990). Growth in liquid was carried out at 30° with vigorous shaking unless otherwise specified. Transformations were carried out as described (KLEBE *et al.* 1983; CHEN *et al.* 1992).

The *Escherichia coli* strain HB101 was used for all plasmid constructions; cells were grown and transformed as described (AUSUBEL *et al.* 1994).

Plasmids: The plasmid constructions described below were carried out using general procedures as described (AUSUBEL *et al.* 1994). Enzyme reactions were carried out as recommended by the manufacturer (New England Biolabs). DNA fragments were purified from agarose gels using the GeneClean kit (Bio101).

CYC7 derivatives: The *TRP1-CEN3* plasmid containing the wild-type *CYC7* gene, YCp*CYC7*(2), and its derivatives, B297, B192, X194, and Δ41, carry deletions in the *CYC7* regulatory region as indicated in Figure 3 (WRIGHT and ZITOMER 1984). YCp*CYC1/7* was constructed by insertion of the 573-bp *Bam*HI-*Xho*I fragment from YCp*CYC7*(2) into the *Bam*HI, *Xho*I-digested YCp*CYC1*(2.4) (LOWRY *et al.* 1983). This resulted in a *TRP1-CEN3* plasmid containing the upstream region of *CYC7* (−142 to −715) fused to the *CYC1* coding region plus TATA elements.

ROX3 derivatives: YCp(22)*ROX3*H was constructed by insertion of the 2.7-kb *Hin*dIII fragment containing the *ROX3* gene (ROSENBLUM-VOS *et al.* 1991) into *Hin*dIII-digested YCplac22 (GIETZ and SUGINO 1988). This plasmid contained the entire *ROX3* coding sequence plus 1.4 kb of 5′ and 0.6 kb of 3′ flanking sequences. YCp(22)*rox3-Pv* was constructed by insertion of a 786-bp *Pvu*II fragment from YCp(22)*ROX3*H into the *Sma*I site of YCplac22. This resulted in a plasmid that contained codons 1–118 of the *ROX3* coding sequence and 432 bp of 5′ flanking sequences. YEp*rox3-182* was described previously (ROSENBLUM-VOS *et al.* 1991). It contains the 2.7-kb *ROX3* fragment carrying the mutant *rox3-182* allele in the *Hin*dIII site of YEplac112 (GIETZ and SUGINO 1988). This mutant allele contains a nonsense triplet at codon 129.

CYC1/ROX3 fusions: The plasmid YCp*CYC1*ΔSX was constructed by digestion of YCp*CYC1*(2.4) with *Sma*I and *Xho*I, followed by a fill-in reaction with Klenow fragment of DNA polymerase I and ligation. This regenerated *Xho*I site and created a plasmid lacking all *CYC1* UAS elements. The synthetic DNA fragments containing the sequences from the *ROX3* regulatory region −169 to −155, 5′TCGACGA_{10}GGAACG and 5′TCGACGTTCCT_{10}CG were inserted into this *Xho*I site. This generated several plasmids used in this study: YCp*CYC1/R3-1X* contained one copy of the sequence with the A_{10} sequence in the coding strand; YCp*CYC1/R3-2X* contained two copies tandemly repeated with the A_{10} sequence in the coding strand; and YCp*CYC1/R3-A* was identical to YCp*CYC1/R3-1X* except that it contained one less A residue in the run of 10 A's that apparently arose either as a mistake during DNA synthesis or as a mutation in transformed cells.

RTS1 derivatives: Restriction maps of the plasmids described in this section are presented in Figure 9. YEp*RTS1* was obtained from the YEp24 genomic library (constructed by M. ROSE and D. BOTSTEIN). YEp(195)*RTS1*XB was constructed by the insertion of the 3.4-kb *Bam*HI-*Xho*I fragment from YEp*RTS1*XB into the *Bam*HI-*Sal*I sites of YEplac195 (GIETZ and SUGINO 1988). YEp(195)*RTS1*BX was constructed by the insertion of the 2.2-kb *Bam*HI-*Xho*I fragment from YEp*RTS1* into the *Bam*HI-*Sal*I sites of YEplac195. YEp(195)*RTS1*XBΔE and YEp(195)*RTS1*XBΔH were constructed by digestion of YEp(195)*RTS1*XB with either *Eco*RI or *Hin*dIII, respectively, and religation. p*rts1::URA3* was constructed by digestion of YEp(195)*RTS1*XB with *Hpa*I-*Cla*I and insertion of the 1.1-kb *Sma*I-*Cla*I *URA3* fragment from YEp24 (BOTSTEIN *et al.* 1979). This generated a partial deletion allele of *RTS1* in which codons from 375 through the end of the coding sequence plus 242 bp of 3′ flanking sequences were deleted. For replacement of the *RTS1* chromosomal allele with this mutant allele, the plasmid was digested with *Bam*HI and *Xba*I.

RNA blots: RNA was prepared, and the blots were carried out as described (ZITOMER *et al.* 1987). The DNA probes were prepared from the plasmids pBS*ACT1*, pYe*CYC1*(2.4), pYe*CYC7*(2), and pBS*ROX3*H as described (ZITOMER *et al.* 1987; ROSENBLUM-VOS *et al.* 1991). The *RTS1* probe was prepared as a *Bam*HI-*Xho*I fragment from YEp(195)*RTS1*XB. The radioactivity in the hybridization bands was quantitated using a Betascan. The numbers presented in the text represent a normalization to actin mRNA that was used as a control in every blot. All RNA blots were repeated at least once.

DNA sequence analysis: DNA sequence analysis was carried out by the method of SANGER *et al.* (1977) using Sequenase (U.S. Biochemicals). Oligonucleotide primers were synthesized using an Applied Biosystems DNA Synthesizer (Perkin Elmer).

RESULTS

***CYC7* expression is stress induced:** The expression of the *CYC7* gene has been shown to be induced by heat shock and during the approach to stationary phase. In

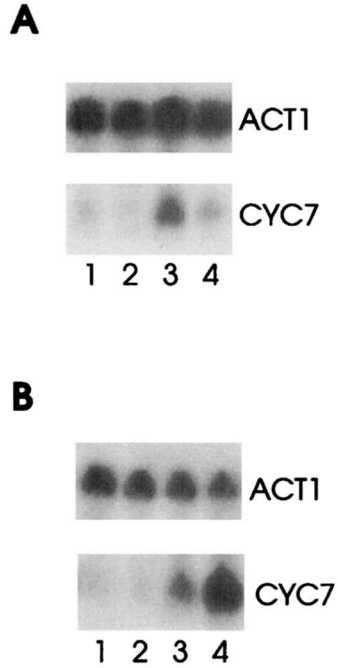

FIGURE 1.—Accumulation of *CYC7* RNA under stress. RNA was prepared from the yeast strain RZ53-6 and subjected to an RNA blot. The RNA was hybridized with radioactively labeled *CYC7* and *ACT1* DNA. (A) Cells were grown to midexponential phase at 30° under normal (vigorous aeration) conditions (lane 1), then subject to one of the following stress conditions: hypoxia induced by bubbling nitrogen through the culture (lane 2), heat shock by shifting the incubation temperature to 37° for 60 min (lane 3), osmotic stress for 60 min induced by the addition of NaCl to a final concentration of 1 M (lane 4). (B) Cells were grown to midexponential phase at 30° under normal conditions (lane 1), then NaCl was added to a final concentration of 1 M. Incubation was continued for 30 min (lane 2), 60 min (lane 3), and 120 min (lane 4).

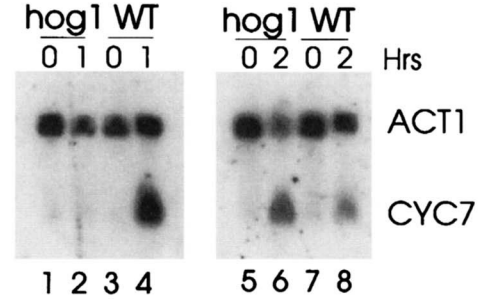

FIGURE 2.—The effect of the *hog1-Δ1* mutation on *CYC7* RNA accumulation under osmotic stress. RNA was prepared from midexponential phase YPH102, *HOG1* wild-type cells (lanes 3, 4, 7, and 8) and JBY13 *hog1-Δ1* cells (lanes 1, 2, 5, and 6) and subjected to an RNA blot. Cells were harvested before (lanes 1, 3, 5, and 7) or 1 (lanes 2 and 4) or 2 hr (lanes 6 and 8) after the addition of NaCl to a final concentration of 1 M. The RNA was hybridized with radioactively labeled *CYC7* and *ACT1* DNA.

addition, expression is induced upon a shift from glucose to a poorer energy source, and while glucose repression is a characteristic of the expression of many genes encoding respiratory functions, *CYC7* appears to respond through a different pathway, independent of the Hap2/3/4 transcriptional activators (PREZANT *et al.* 1987). Also, while wild-type *CYC7* expression is not induced by the stress of hypoxia, in derivatives carrying mutations in the Hap1 binding site, an hypoxic induction is evident (CERDAN and ZITOMER 1988). This pattern of stress induction is suggestive that *CYC7* is part of the global stress response. To further explore this possibility, an RNA blot was performed on cells subjected to heat shock and osmotic stress. A *cyc1* deletion strain was used to avoid cross-hybridization of the *CYC7* probe with the more highly expressed homologue. The results are shown in Figure 1 and clearly indicate that *CYC7* RNA levels were increased in response to these two stresses. While *CYC7* RNA was barely detectable under normal aerobic and anaerobic growth conditions (A, lanes 1 and 2, respectively), after 1 hr at 37° or in 1 M NaCl, RNA levels increased five- and twofold, respectively. A time course of the osmotic stress induction indicated that *CYC7* RNA levels increased steadily to a fivefold increase over 2 hr (B).

The osmotic stress response for many yeast genes is mediated through the *HOG1*-dependent pathway. To determine if this were also the case for *CYC7*, a *hog1* deletion mutant and its congenic parent were subjected to osmotic stress, and *CYC7* RNA levels were measured (Figure 2). The increase in *CYC7* RNA levels seen in wild-type cells grown in 1 M NaCl for 1 hr was absent in the *hog1-Δ1* mutant. Interestingly, the kinetics of the response was different in this strain background; the response in wild-type cells peaked at 1 hr, while the *hog1* mutant did show some induction after 2 hr. SCHULLER *et al.* (1994) reported that while the osmotic stress induction of *CTT1* and *DDR2* was completely lost in a *hog1* deletion strain, that of *GAC1* and *UBI4* was only delayed. Thus, the Hog1-dependence of the osmotic stress response appears to vary, perhaps dependent upon the other transcriptional signals that control the expression of a given gene. Our results indicate that the osmotic stress response of *CYC7* falls into this latter class. Nonetheless, from these results, it is clear that *CYC7* is a stress-induced gene, and that the osmotic stress is mediated, at least in part, through the *HOG1* MAP kinase pathway.

The stress-response of *CYC7* is mediated through multiple STRE elements: We had previously carried out a deletion analysis of the *CYC7* upstream region that identified the Hap1 binding site (UAS_{Hap1}), a repression region (URS_{1-3}), and a Rox1 operator as indicated in Figure 3 (WRIGHT and ZITOMER 1984). The analysis was carried out under glucose repressed and derepressed conditions, but other stress conditions were not assayed. Given subsequent findings, we decided to reassess the effect of some of the deletions on the *CYC7* stress response.

An inspection of the *CYC7* upstream region indicated the presence of three copies of STRE, 5′-CCCCT (Figure 3). The effect of deleting one or more of these

```
-420
   CCGTCATCGTTGATATTATT ATTATCATTCCGCCTGAAGA AAAAGAAACGAAAAAAGAAA

-360
   TGGATCAGCAGCCGGGTTAT AGCGCCCCTTATTGTATTAT TTTCCTTCGTGCCTTCTCTG
                                OpROX1                  OpA
                                                        |-------------
-300
   AGAAGGGTCTGCAGTCCCCC GCCGAGGGGTCTTTTCCCAC CTTCTCAAAGCTAATAGCGA
B297--| OpB                      OpC
   ---------Δ41---------------|

-240
   TAATAGCGAGGGCATTTATT CAAGTTCCAACTACTATAAG TGGCCGCAAGGGGCAAAGAC
     UAS_HAP1                                  B192---|
                                                      |----------------
-180              TATA Box
   AAAGGCACACAACATATATA TATATCGTGTTGTGAAGCTC GAGAAGATTAGATCGGAATA

   -------X194--------------------------|

-120
   GTTCTCTTTTTGTTGAGGTT GAAACAAAATCAAAGACTTA TACAAGAAGATCACATACAA

 -60
   GCATTTATTCACATTACTTT AAGTAAACTTCAGTAAACTA CATTACATCATAAACAAAAC


   ATG GCT AAA GAA AGT ACG GGA TTC
   Met Ala Lys Glu Ser Thr Gly Phe
```

FIGURE 3.—Sequence of the *CYC7* regulatory region. The sequence of the region 5′ to the *CYC7* coding sequence plus the first eight codons is presented. Bases are numbered in positive integers from the A in the translational initiation codon 3′-ward, and in negative integers 5′-ward. The regulatory elements are presented in shaded boxes for the Rox1 binding site (OpROX1), the negative elements (OpA, B, and C) and the Hap1 binding site (UAS_{HAP1}). The TATA box in underlined. The three proposed stress response elements are presented in larger letters. The deletions used in this study are indicated by dashed lines underneath the sequences deleted for Δ41 and X194 or by just the 3′ endpoint for B297 and B192.

elements on *CYC7* RNA accumulation during osmotic stress was determined and the results are presented in Figure 4A. The deletions indicated in Figure 3 were carried on an centromeric plasmid that was transformed into a strain carrying deletions of the genomic *CYC1* and *CYC7* genes. Deletion B297 lacked the 5′ STRE plus the Rox1 operator and part of the repression elements. Consequently, the *CYC7* RNA level was increased over that in wild type during normal growth (basal level) but was induced 4.5-fold further by osmotic stress. The deletion Δ41 lacked the middle STRE as well as the URS elements. Like B297 this deletion gave an increased basal level of RNA that was further induced fourfold upon osmotic stress. The deletion X194 lacked the 3′ most STRE plus the TATA box. An alternate, cryptic TATA box gave a wild-type-like basal RNA level (HEALY *et al.* 1987), and this level was further induced threefold by osmotic stress. The deletion B192 lacked all the *CYC7* regulatory elements except the 3′-most STRE; four of the five basepairs of this element were retained. This deletion still showed the a weak osmotic induction of *CYC7* RNA. The four deletions combined removed all the *CYC7* regulatory sequences through the TATA box; the only common element that remained in each was the STRE, and in each case the osmotic stress response was observed. The same results were obtained for glucose repression (WRIGHT and ZITOMER 1984) and heat shock (data not shown).

The results of this deletion analysis suggested that the STRE elements were responsible for the stress response of the *CYC7* gene but did not rule out the possibility that the response was due to a region 3′ to −142 that remained intact in all the deletions. To rule out this possibility, the *CYC7* upstream region from −142 through −716 was fused to a *CYC1* derivative containing only the coding region and TATA box and lacking the UAS region. A UAS-less *CYC1* did not respond to stress

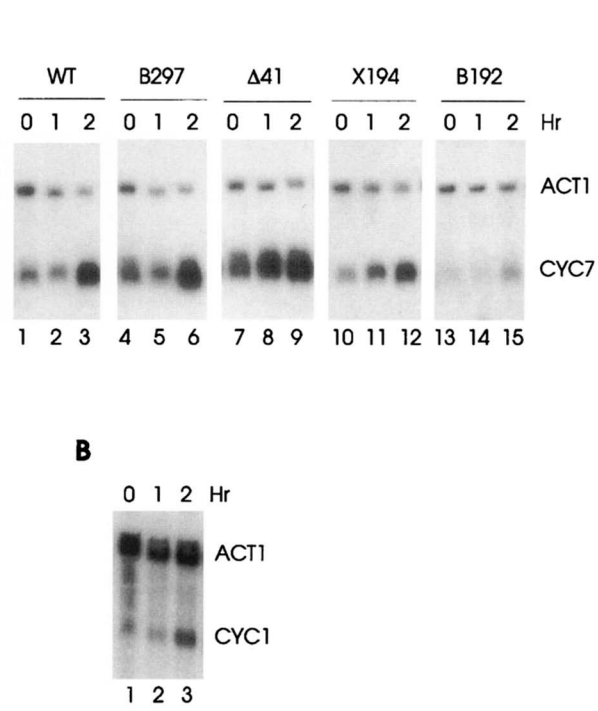

FIGURE 4.—Effect of deletions in the regulatory region on *CYC7* RNA accumulation under osmotic stress. (A) RNA was prepared from AH12 cells transformed with YCp*CYC7*(2) (WT, lanes 1–3), or the deletion derivatives B297 (lanes 4–6), Δ41 (lanes 7–9), X194 (lanes 10–12), or B192 (lanes 13–15) and subjected to an RNA blot. The cells were grown to midexponential phase then harvested before (lanes 1, 4, 7, 10, and 13) or 60 (lanes 2, 5, 8, 11, and 14) or 120 min (lanes 3, 6, 9, 12, and 15) after the addition of NaCl to a final concentration of 1 M. The RNA was hybridized to radioactively labeled *CYC7* and *ACT1* DNA. (B) RNA was prepared from AH12 cells transformed with YCp*CYC1/7* and subjected to an RNA blot. The cells were grown to midexponential phase then harvested before (lanes 1) or 60 (lanes 2) or 120 min (lanes 3) after the addition of NaCl to a final concentration of 1 M. The RNA was hybridized to radioactively labeled *CYC1* and *ACT1* DNA.

(see Figure 7, lanes 14–16). The fusion gene was transformed into the *cyc1, cyc7* deletion strain. Transformants carrying this plasmid were subjected to osmotic stress, and RNA accumulation was determined. The results presented in Figure 4B show that the ability to respond to this stress was carried within the upstream region of *CYC7,* and combined with the results of the deletion analysis, implicate the STREs of *CYC7* in the response.

The *rox3-Pv* mutation affects the stress response of CYC7: The *ROX3* gene was initially identified through mutations that caused increased aerobic *CYC7* expression. The Rox3 function is essential to the cell, and, interestingly, all the mutant alleles sequenced encoded truncated versions of the protein (ROSENBLUM-VOS *et al.* 1991). We have subsequently found that the increased *CYC7* expression in these mutants is highly strain dependent, and that no affect or even a slight decrease in *CYC7* expression has been observed when these alleles were moved into different backgrounds. We have no explanation at present for these affects. We also observed that in most strain backgrounds these mutants were temperature sensitive for growth, suggesting that they might play a role in the cell's heat shock response. To investigate this possibility, we assayed *CYC7* RNA levels in samples prepared from a *ROX3* deletion strain carrying either a wild-type *ROX3* plasmid or a plasmid carrying the truncated allele *rox3-Pv* grown under stress conditions. This mutation gave no apparent increase in *CYC7* RNA levels under normal nonstress growth conditions in this strain background but dramatically diminished both the osmotic stress (Figure 5A) and the heat shock (Figure 5B) responses. Thus it appears that Rox3 may be involved in part of the stress response of the *CYC7* gene.

We also tested the ability of wild-type and the *rox3-Pv* cells to form colonies on plates containing rich medium, rich medium plus 1 M NaCl, or rich medium lacking glucose and containing 2% glycerol. The mutants were unable to grow under either glucose starvation or osmotic stress conditions. Since these phenotypes, as well as the temperature sensitivity of *rox3-Pv* mutants, are not characteristic of an inability of cells to express the *CYC7* gene, a *cyc7* deletion mutant has no phenotype, they strongly suggest that Rox3 is an essential component of the global stress response pathway, and the inability of cells to fully induce other essential stress functions is responsible for the lack of growth.

A novel stress induction element regulates *ROX3* transcription: We previously reported that the expression of *ROX3* is increased during the stress of anaerobiosis (ROSENBLUM-VOS *et al.* 1991). Since the *rox3-Pv* mutation affected the *CYC7* response to osmotic stress and heat shock, we decided to investigate further *ROX3* expression under various stress conditions. As can be seen in the RNA blot presented in Figure 5, *ROX3* RNA levels in wild-type cells were increased 2.5-fold above the high basal level in response to oxygen starvation (90 min growth under nitrogen) and twofold in response to heat shock and osmotic stress. No RNA from the *rox3-Pv* cells was detectable with the *ROX3* probe that is probably a result of the lack of transcriptional termination signals 3′ to the coding sequence.

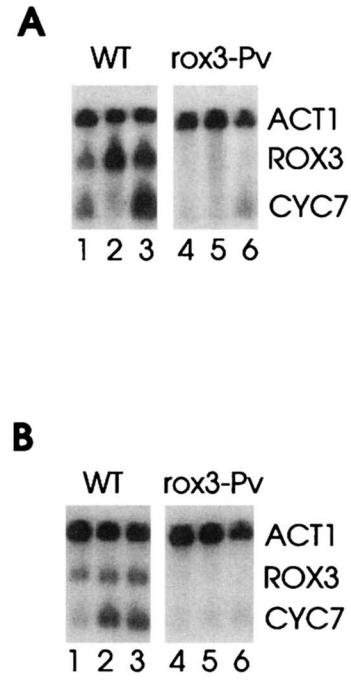

FIGURE 5.—Effect of the *rox3-Pv* allele on the stress-induced accumulation of *CYC7* RNA. RNA was prepared from RZ71-7 cells transformed with either YCp(22)*ROX3*H (WT, lanes 1–3) or YCp(22)*rox3-Pv* (rox3-Pv, lanes 4–6) and subjected to an RNA blot. The RNA was hybridized to radioactively labeled *CYC7, ROX3,* and *ACT1* DNA. (A) Cells were grown to midexponential phase and harvested before (lanes 1 and 4) or after 90 min during which nitrogen was bubbled through the culture to induce hypoxia (lanes 2 and 5) or 60 min after shifting the culture temperature to 37° (lanes 3 and 6). (B) Cells were grown to midexponential phase and harvested before (lanes 1 and 4) or 60 (lanes 2 and 5) or 120 min (lanes 3 and 6) after the addition of NaCl to a final concentration of 1 M.

An inspection of the upstream region of *ROX3* revealed no sequences obviously similar to the heat shock element or STRE, so we carried out a deletion analysis of this region. The pattern of *ROX3* expression resulting from a variety of deletions was complex (data not shown), but the data suggested that a repeated element, $5'GA_{10}GGAA$, appeared to be responsible for the anaerobic induction. This element is present three times in the *ROX3* upstream region (Figure 6) that was in part responsible for the complexity of the deletion analysis. Therefore, to analyze the transcriptional activation and regulatory activity of this sequence, we constructed a number of plasmids containing upstream regions of *ROX3* inserted upstream of the *CYC1* gene that lacked its two UAS's. A strain carrying *cyc1* and *cyc7* deletions was transformed with these constructs, and the transformants were grown to mid-exponential phase aerobically at 30° then either shifted to 37° for 1 or 2 hr to

```
                                -511
                                 AAGCCCACTCT CCAGACGGAAACCATACAAT
-480
 GCCTCCGCTGGCTGAATTGT CGCGCCCCGCCCAACGACGG TTTACCGACAGCTGCTAGCT
-420
 GGGCTCAACAGGTGGTTAGC CCACCAATTCCCCTGTCGCT CTTCGCTCTGAATGTGACGG
-360
 CAAATTTCGACCCGTTGTTC CTGTTCCTTTTTTTTTCAA TTGGACTGAAAAAAAAAAAG
-300
 AACCGAATCTGGAAAGATAC ACCCAAACATACATAGAATG TACGGATGCATGATTGTCTC
-240
 AGCCTCGTTTGGCTCATCGT TCTTCATTTCTTTTTCCTAA TTTTGATAGAGACAATAGAT
-180
 AGACGTGGAAGGAAAAAAAA AAGGAAAGCCCAACAATATT GAGAAACGAAGAGGTGTATT
-120
 TGGTTTAAATAGAGCCTCTT CATTCCTTTCCTGATCTGAC AACAGGGTGGAACATAAAAT
-60
 ATAGATCTGTAGTGAGTGCG AATAGCAATAGTAAGTGAAC GAAAAAGGAATACGATAATA
1                                                              48
ATG GCT TCT AGA GTG GAC GAA ACT ACA GTC CCC TCA TAC TAC TAT TAC
Met Ala Ser Arg Val Asp Glu Thr Thr Val Pro Ser Tyr Tyr Tyr Tyr
```

FIGURE 6.—Sequence of the *ROX3* regulatory region. The sequence of the *ROX3* locus from 511 bp 5′ of the translational initiation codon through codon 48 is presented. The three putative stress-induced transcriptional activation sequences are indicated in the shaded boxes.

induce heat shock or grown under nitrogen for 90 min to induce hypoxia. RNA was prepared from these transformants, and the results of an RNA blot probed with the *CYC1, ROX3,* and *ACT1* coding sequences is presented in Figure 7. The control lanes (13–16) contained RNA from cells transformed with a plasmid lacking any insert and showed no transcriptional activation under any conditions. Lanes 1X and 2X contained RNA from cells transformed with plasmids containing one or two copies, respectively, of a synthetic DNA containing the 5′GA_{10}GGAA sequence from −169/−155 of *ROX3.* A single copy of this sequence activated transcription under normal growth conditions (30°, aerobic), and two copies increased RNA levels twofold further. Under hypoxic and heat shock conditions, one copy of the sequence caused a twofold increase in *CYC1* RNA levels, while two copies caused a 3.5- and twofold increase, respectively. On the other hand, RNA prepared from cells carrying a construct with a single copy of this sequence containing a deletion of a single A residue (5′GA_9GGAA) accumulated twofold less *CYC1* RNA under normal conditions, and no stress induction was observed. These results clearly demonstrated that the sequence 5′GA_{10}GGAA can support basal levels of transcription that are induced upon both hypoxia and heat shock. In each case where one or two copies of this sequence was present, the *CYC1* RNA levels mimicked those of *ROX3.*

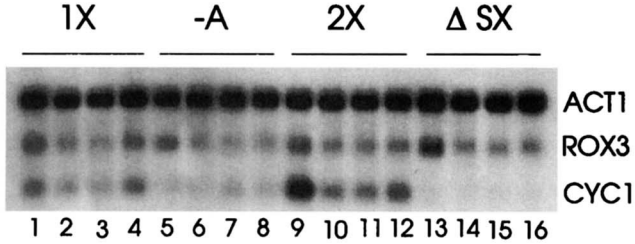

FIGURE 7.—Transcriptional activation activity of the *ROX3* stress element. RNA was prepared from AH12 cells transformed with one of the following plasmids: YCp*CYC1*/R3-1X (1X, lanes 1–4); YCp*CYC1*/R3-A (-A, lanes 5–8); YCp*CYC1*/R3–2X (2X, lanes 9–12); and YCp*CYC1*ΔSX (ΔSX, lanes 13–16). Cells were grown to midexponential phase and harvested before (lanes 2, 6, 10, and 14) or after 90 min of hypoxia (lanes 1, 5, 9, and 13), or 60 (lanes 3, 7, 11, and 15) or 120 min (lanes 4, 8, 12, and 16) after shifting the temperature to 37°. The RNA was hybridized to radioactively labeled *CYC1, ROX3,* and *ACT1* DNA.

High-copy suppression of the *rox3-Pv* allele: The effect of *ROX3* mutations on the stress response of *CYC7* as well as the temperature-sensitive, osmotic-sensitive, and glycerol-minus phenotype caused by these mutations suggested that Rox3 plays a role in the global stress response. Unfortunately, the sequence of the Rox3 protein provided no clues as to its function, and DNA binding studies using the *CYC7* upstream region and recombinant Rox3 protein were negative. In an attempt to obtain some indication of Rox3 function, we carried out suppression analyses to identify genes that either interact with Rox3 or bypass its function. We were unable to isolate any suppressors of the lethality of the *rox3* null allele either by mutagenesis or by high-copy suppression. However, we isolated a number of high copy suppressors of the *rox3-Pv* temperature-sensitive allele. A transformant carrying this allele on a centromeric plasmid and a chromosomal deletion of the *ROX3* locus was transformed with a yeast genomic library constructed in the *URA3* episomal plasmid YEp24, and the transformants were plated at 37°. All rapidly growing transformants contained the wild-type *ROX3* gene, but a number of novel genes were isolated from slower growing colonies. One contained a gene now designated *RTS1* (*ROX Three Suppressor*). This plasmid could not complement a *rox3* deletion; all attempts to isolate a ura^+, trp^- segregant that would carry the *URA3-RTS1* but not the *rox3-Pv-TRP1* plasmid failed.

A deletion analysis of the YEp*RTS1* plasmid localized the suppressing region to a 3.3-kb fragment (Figure 8). The sequence of this fragment was determined, and a large open reading frame encoding a protein of 758 amino acids was found. (The DNA sequence is listed in the various data bases under Accession No. U06630). This putative protein is 50% identical to the several copies of the rabbit skeletal phosphatase $2A_0$ regulatory subunit B′ (Database Accession Numbers: U37769, U37770, U38190-4) (ZOLNIEROWICZ *et al.* 1994;

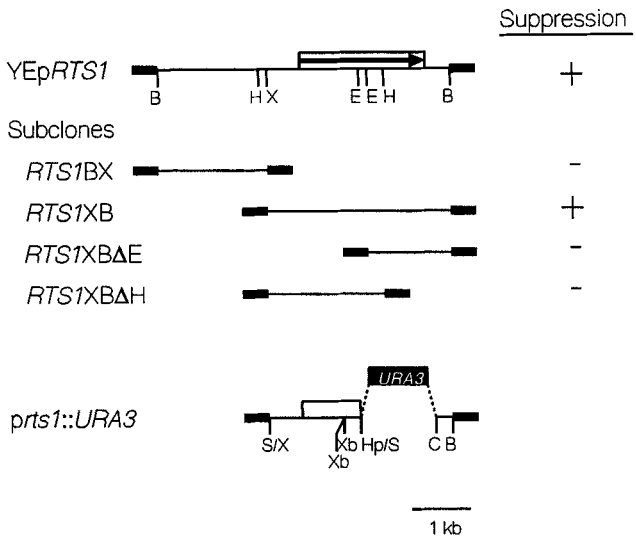

FIGURE 8.—Localization of the *RTS1* gene. The plasmid YEp*RTS1* is diagrammed at the top. The thin line represents sequences from the *RTS1* locus; the box with the arrow represents the *RTS1* coding sequence and the direction of transcription; the thick line represents vector sequences. The subclones represented below are described in MATERIALS AND METHODS. The plasmid used to disrupt and partially delete the *RTS1* chromosomal gene is presented at the bottom. The filled box represents the *URA3* locus. The restriction sites are as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; S, *Sal*I; Sm, *Sma*I; X, *Xho*I; Xb, *Xba*I. The column on the right represents the ability of the various subclones to suppress the temperature-sensitive growth phenotype (+) when transformed into the strain RZ71-7 carrying the YCp(22)*rox3-Pv* plasmid.

CSORTOS *et al.* 1996) strongly suggesting that it plays a similar role in yeast cells.

An *RTS1* deletion allele affects the stress response: A deletion allele of *RTS1* was constructed by replacing the sequences from 1123 to 2512 with the *URA3* gene (see Figure 8), then displacing the wild-type chromosomal allele with this construct in the strain RZ53-6 to generate RZ53-6Δ*rts1*. The correct integration event was confirmed by Southern analysis (data not shown). This strain was temperature sensitive (unable to grow at 37°), sensitive to osmotic pressure (grew poorly in media containing 1 M NaCl), and glycerol$^-$ (unable to grow with glycerol as an energy source). These phenotypes were characteristic of the *rox3-Pv* allele as described above. To ascertain that these phenotypes resulted from the *rts1::URA3* allele, the displacement of the wild-type allele was repeated, this time in a homozygous recessive *ura3* diploid strain to generate a *RTS1/rts1::URA3* heterozygote (confirmed by Southern analysis). The diploid was sporulated and subjected to tetrad analysis. In the five full tetrads obtained, all showed two ura$^+$, temperature sensitive to two ura$^-$, wild-type growth phenotypes demonstrating linkage between the *URA3* allele and temperature sensitivity and indicating that the temperature-sensitive phenotype resulted from the *rts1* deletion allele.

To determine whether the combination of the temperature-sensitive *rox3-182* mutant allele (containing a nonsense mutation at codon 129) and the *rts1::URA3* deletion allele showed synthetic lethality, RZ53-6Δ*rts1* was mated with RZ71-1 (YEp(112)*rox3-182*). This latter strain carried a *rox3::LEU2* deletion allele in the chromosome and the *rox3-182* allele on a *TRP1*-containing plasmid. The various alleles could be followed during tetrad analysis by nutritional markers: *rts1::URA3/RTS1* by ura$^\pm$, *ROX3/rox3::LEU2* by leu$^\pm$, and *rox3-182*/no plasmid by trp$^\pm$. After sporulation and dissection, ura+ (*rts1::URA3*)-, leu+ (*rox3::LEU2*)-, trp+ (YEp(112)-*rox3-182*)-viable haploid segregants were found; these were all temperature sensitive as expected. Thus, the *rox3-182* and *rts1* mutations did not show synthetic lethality. In addition, the combination of the *rox3-182* and *rts1* mutations did not suppress the temperature-sensitive phenotypes of the individual mutations.

An RNA blot was carried out to determine the effect of the *rts1* deletion allele on the accumulation of *CYC7* RNA under stress conditions (Figure 9, A and B). The results indicated that the mutation caused a 1.5-fold increase in expression of *CYC7* under normal growth conditions (compare Figure 9, A and B, lanes 1 and 4 in each). Also, the mutation had the opposite effect of that of *rox3* mutations on *CYC7* RNA accumulation during heat shock (compare Figure 9A, lanes 3, 6, and 9) and osmotic stress (compare Figure 9B, lanes 1–4, 5–8, and 9–12); *CYC7* RNA levels were elevated twofold in the mutant relative to the wild-type cells under both stress conditions.

RTS1 was cloned based upon the ability of high copy number to suppress the temperature sensitivity of the *rox3-Pv* mutant, so it was of interest to determine the effect of high copy on *CYC7* expression. *ROX3* wild-type cells carrying multiple copies of *RTS1* and over-expressing the *RTS1* RNA accumulated similar levels of *CYC7* RNA under normal and osmotic stress conditions as did cells carrying a single copy of the gene (compare Figure 9C, lanes 1–3 and 7–9). Similar results were observed for the induction of *CYC7* under heat shock (data not shown). However, in a *rox3-Pv* strain, high-copy expression of *RTS1* resulted in greatly elevated *CYC7* RNA levels under stress conditions compared to the same cells with a single copy of *RTS1* (compare Figure 9C, lanes 4–6 with 10–12). This restoration of the *CYC7* stress response in *rox3* mutant cells by *RTS1* over-expression can explain the suppression of the temperature sensitivity of the *rox3-Pv* strain.

The blot in Figure 9, A and B was also probed for *ROX3* RNA to determine whether the effect of the *rts1* mutation or *RTS1* over-expression was through changes in *ROX3* RNA levels. The results indicated that *ROX3* RNA levels were not significantly altered in the mutant.

Although the *RTS1* gene was cloned based upon high copy suppression of a *rox3* temperature-sensitive mutation, it was not a bypass mutation; the high copy number of the *RTS1* gene could not suppress a *rox3* null allele. Therefore, no conclusions could be drawn from

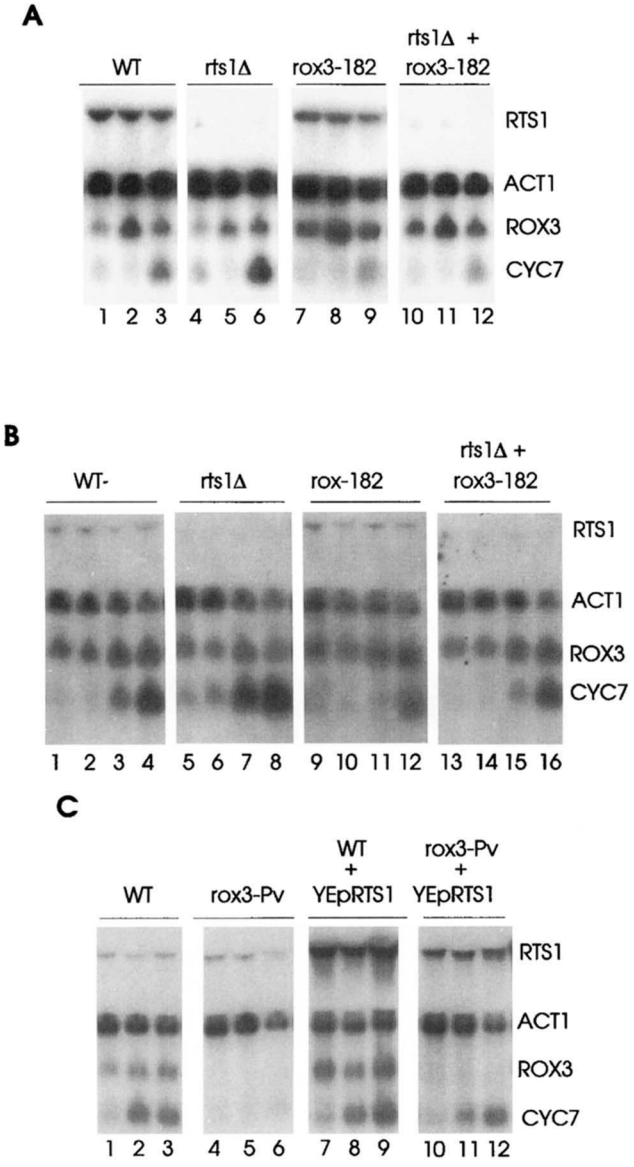

FIGURE 9.—Effect of *RTS1* deletion and overexpression on the accumulation of *CYC7* and *ROX3* RNA. For all three panels, the RNA blot was hybridized with radioactively labeled *CYC7, ROX3, ACT1,* and *RTS1* DNA. (A) RNA was prepared from RZ53-6 (WT, lanes 1–3), RZ53-6Δ*rts1* (rts1Δ, lanes 4–6), RZ68-2 transformed with YEp(112)*rox3-182* (rox3-182, lanes 7–9), and MZ19-18C transformed with YEp(112)*rox3-182* (rts1Δ + rox3-182, lanes 10–12). Cells were grown to midexponential phase and harvested before (lanes 1, 4, 7, and 10) or after 90 min of hypoxia (lanes 2, 5, 8, and 11), or 60 min (lanes 3, 6, 9, and 12) after shifting the temperature to 37°. (B) RNA was prepared from the same strains as indicated in A. Cells were grown to midexponential phase and harvested before (lanes 1, 5, 9, and 13) or 30 (lanes 2, 6, 10, and 14), 60 (lanes 3, 7, 11, and 15), or 120 min (lanes 4, 8, 12, and 16) after the addition of NaCl to a final concentration of 1 M. (C) RNA was prepared from RZ71-7 cells transformed with YCp(22)*ROX3*H (WT, lanes 1–3), YCp(22)*rox3-Pv* (rox3-Pv, lanes 4–6), YCp(22)*ROX3*H plus YEp*RTS1* (WT + YEpRTS1, lanes 7–9), or YCp(22)*rox3-Pv* plus YEp*RTS1* (rox3-Pv + YEpRTS1, lanes 10–12). Cells were grown to midexponential phase and harvested before (lanes 1, 4, 7, and 10) or 60 min after shifting the temperature to 37° (lanes 2, 5, 8, and 11) or 120 min after the addition of NaCl to a final concentration of 1 M (lanes 3, 6, 9, and 12).

the suppression data alone as to whether the gene products functioned in the same pathway, and if so, in what order they functioned. To answer this question, the epistatic relationship between the opposite acting *rox3* and *rts1* mutations was determined. RNA was prepared from a double mutant before and after stress induction and subjected to an RNA blot. The results are presented in Figure 9, A and B. For both heat shock and osmotic stress, the double mutant accumulated less *CYC7* RNA than did the wild-type or *rts1* mutant. These results suggest that *rox3* is epistatic to *rts1* and, therefore, Rox3 may act downstream of Rts1 in the same pathway. This conclusion, however, must be tempered by the knowledge that the *rox3-182* mutation does contain partial function; a null allele is lethal.

***RTS1* RNA levels are not regulated by stress:** To determine whether *RTS1* RNA levels were regulated in response to stress, the RNA blot presented in Figure 9 was also probed with the *RTS1* coding sequence. *RTS1* RNA levels were unchanged by heat shock, hypoxia, or osmotic stress. In addition, RNA levels were unaffected by the *rox3-182* mutation.

DISCUSSION

It is clear that *CYC7* is a stress response gene. The induction of expression by glucose depletion, heat shock, and approach-to-stationary phase have been previously documented (LAZ *et al.* 1984; WRIGHT and ZITOMER 1984; PILLAR and BRADSHAW 1991), and we have demonstrated here that expression is induced by osmotic stress through the *HOG1* kinase pathway. Our findings strongly implicate the previously defined STRE, found in three copies in the *CYC7* upstream regulatory region, in this response; constructs containing any one of these elements can mediate the stress response. These results add to the already complicated combination of regulatory elements that govern *CYC7* expression (WRIGHT and ZITOMER 1984; ZITOMER *et al.* 1987; LOWRY and ZITOMER 1988). There is a poor UAS for heme-dependent Hap1 activation and an antagonistic operator for heme-dependent repression by Rox1. There is a set of general repression elements, the function of which is not known. Adding the STRE to this mix suggests a rather sophisticated level of control over the levels of iso-2-cytochrome c.

The reason for this extensive overall control and the stress response in particular of *CYC7* is not entirely apparent. The dramatic burst of *CYC7* expression as cells approach stationary phase might be explained in terms of a requirement for a higher respiratory rate and a need to increase cytochrome components as oxygen and a fermentable energy source become limiting. There is an hypoxic form of subunit V of cytochrome oxidase, encoded by the *COX5b* gene (HODGE *et al.* 1989), and it has been reported that the iso-2-cytochrome c-Cox5b-containing cytochrome oxidase complex has a higher turnover number than the aerobic

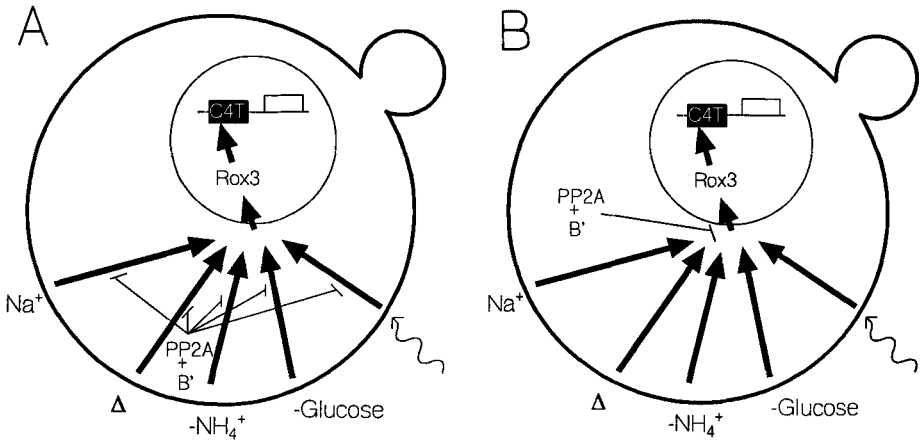

FIGURE 10.—The roles of Rox3 and Rtsl in the stress response. The thicker circular line represents the cell wall, the thinner circle represents the nucleus. The thick arrows pointing from the cell wall to the nucleus represents kinase cascades. Arrows heads represent stimulation, lines with bars at the ends represent inhibition. C4T represents the STRE element. The external signals that initiate the stress response are represented as follows: Na^+, osmotic stress; Δ, heat shock; $-NH_4$, nitrogen starvation; −glucose, glucose starvation; wavy arrow, DNA damage.

complex (WATERLAND *et al.* 1991). However, it is less obvious why other stress conditions, such as heat and osmotic stress, should induce *CYC7* expression. Experiments with *cyc7* deletion strains indicated no decreased survival rates at elevated temperatures, under osmotic stress, glucose limitation, or the transition into or from anaerobiosis under laboratory growth conditions (M. P. LIMBACH and R. S. ZITOMER, unpublished results). Perhaps competitive growth studies or studies on the properties of the cytochrome chain with iso-1 *vs.* iso-2-cytochromes c under these conditions might shed some light on this question.

The role of Rox3 and Rtsl in the stress response: While the stress-mediated transcriptional activation may occur through a common element, the signal transduction pathways for different stresses are clearly different. The osmotic stress response is mediated through a MAP kinase pathway of which the *HOG1* gene product is a member (BREWESTER *et al.* 1993; MAEDA *et al.* 1994; SCHULLER *et al.* 1994). Here we showed that in a *hog1* mutant *CYC7* gave a more limited response to osmotic stress. The approach-to-stationary-phase response is mediated through the cAMP-ras pathway and the attendant protein kinases (BISSINGER *et al.* 1989; PILLAR and BRADSHAW 1991; MARCHLER *et al.* 1993). The pathway for the heat shock response of *CYC7* is not known. Presumably these different stress pathways ultimately feed into the same signal at the level of transcriptional activation through the 5′CCCCT sequence. As indicated here, mutations in both *ROX3* and *RTS1* affect the heat shock, osmotic stress, and glucose starvation response of *CYC7*, suggesting that their gene products are involved in mediating multiple signals.

Two lines of evidence suggest that Rox3 and Rtsl do not directly interact in carrying out their respective functions. First, attempts to measure an interaction in the dihybrid system failed. Second, Rox3 is localized in the nucleus while Rtsl is in the cytoplasm (SHU and HALLBERG 1995). This latter finding supports the epistatic studies that suggest that Rox3 acts downstream from Rtsl. As the environmental signals work their way from the cell surface to the nucleus, it is likely that cytoplasmic Rtsl functions before the nuclear Rox3. The function of Rox3 remains a mystery.

RTS1 has homology to the recently cloned rabbit skeletal B′ subunit of protein phosphatase $2A_0$, $PP2A_0$ (CSORTOS *et al.* 1996), and expression of a rabbit cDNA clone in the *rts1* deletion strain described here complemented the temperature-sensitive phenotype (Y. ZHAO, G. BOGUSLAWSKI, C. C. EVANGELISTA, JR., R. S. ZITOMER and A. A. DEPAOLI-ROACH, unpublished results) providing very strong evidence that Rtsl functions as a regulatory subunit of the yeast $PP2A_0$. PP2A is one member of the family of serine/threonine phosphatases (COHEN 1989). It is comprised of a catalytic subunit and two regulatory subunits designated A and B. Four genes encoding alternate forms of the catalytic subunit, *PPH3, 21,* and *22,* and *SIT4,* have been identified in *S. cerevisiae* (ARNDT *et al.* 1989; SNEDDEN *et al.* 1990; RONNE *et al.* 1991; MAEDA *et al.* 1993), as well as genes encoding the A (*TPD3*) (VAN ZYL *et al.* 1992) and B regulatory subunits (*CDC55*) (HEALY *et al.* 1991). Cells carrying deletions of any one of the *PPH* genes have no reported phenotype, but mutants with deletions of both *PPH21* and *22* grow poorly and deletion of all three *PPH* genes is lethal (SNEDDEN *et al.* 1990). Mutations in *SIT4* were originally isolated as suppressors of a UAS-less *HIS4* transcriptional defect (ARNDT *et al.* 1989). These mutants were temperature sensitive and were subsequently found to arrest cells late in G1 at nonpermissive temperatures (SUTTON *et al.* 1991). A deletion mutant of *TDP3* is both temperature sensitive and cold sensitive, deficient in growth on nonfermentable energy sources, and has defects in transcription by RNA polymerase III (VAN ZYL *et al.* 1992). A *cdc55* deletion mutant is cold sensitive (HEALY *et al.* 1991). The latter two deletion mutants accumulate multinucleated multibudded cells at low temperatures, a similar appearance to cells over-expressing some of the catalytic subunits. If Rtsl functions as an alternate B subunit to Cdc55, it would explain why deletion of the A subunit gene results in both temperature- and cold- sensitive phenotypes, while deletion of the B subunit gene results in only the latter phenotype. The protection against heat shock (and other

global stresses) is the function of the Rts1-containing $PP2A_0$, while low temperature require the Cdc55-containing PP2A form.

We believe that Rts1 functions with PP2A to help control the stress response, and we envision two possible models by which it may do so (Figure 10). In these models we assume that the phosphatase affects all stress responses; the *rts1* deletion affected the three we tested here. Also, we assume that it is the protein kinases that actually sense and modify their activities in response to the signals (as in the case of the Hog1 and ras-cAMP pathways), and the phosphatases function constitutively. Finally, we assume that the different signals all merge on a single transcriptional activator, since a unique-sized protein binds to STRE in yeast extracts. However, until the gene for this activator is cloned, it is still formally possible that there are a family of proteins. For the first model, the phosphatase antagonizes each of the kinase signals for the different stresses, while for the second, the phosphatase acts after the signals have merged. Obviously a combination of the two is possible. We have placed Rox3 at a point after the merging of the signals because it is a nuclear protein, appears to act after the phosphatase, and affects all the stress responses we tested.

While our studies neared completion, we learned that SHU and HALLBERG (1995) also cloned the *RTS1* gene as a high-copy suppressor of a temperature-sensitive allele of *HSP60,* encoding a mitochondrial chaperonin. (They designated the gene *SCS1,* Suppressor of Chaperonin Sixty *1*; we have retained the *RTS1* designation because it is listed in the data base under that name, and there are already two other genes designated *SCS1.*) They found, in agreement with our results, that a deletion of *RTS1* was temperature sensitive. However, the deletion allele caused a decrease in the heat shock response of *HSP60* and two other mitochondrial chaperonins, *MGE1* and *CPN10,* an opposite effect to what we observed for *CYC7.* Also, while over-expression of *RTS1* caused increased expression of *MGE1* and *CPN10* in a strain carrying the *hsp60* temperature-sensitive allele, it had little affect on gene expression in wild-type cells. These differences suggest that these genes are not regulated through the global stress response pathway, and, in agreement with this conclusion, there is no perfect matches to STRE in the published upstream regions for these three heat shock inducible chaperonin genes (READING *et al.* 1989; ROSPERT *et al.* 1993; LALORAYA *et al.* 1994). Thus, as might be expected, $PP2A_0$ probably functions in more than one pathway in the cell.

We are still uncertain as to what causes the lethal phenotype of the *rox3* deletion. Perhaps some of the stress response genes are required during normal growth, and Rox3 is required for their basal level transcription, but we have no evidence for this hypothesis yet. The temperature sensitivity of the *rts1* deletion may not be due to the increased expression of the STRE responsive genes but rather its role in mediating the expression of the heat shock-inducible mitochondrial chaperonins described above. Nonetheless, the osmotic-sensitive phenotype of the *rts1* deletion does suggest that an increased response to stress is detrimental. Since the *rox3* mutations caused a loss of the stress response and the *rts1* deletion caused an increase, it is tempting to speculate that, as with people, a lack of response to stressful situations is detrimental, but an over-response is not good either.

The regulation of *ROX3* expression: *ROX3* RNA levels are increased by anaerobiosis, heat shock, and osmotic stress. This response is not a result of an STRE, but rather a novel sequence $GA_{10}GGAA$. A deletion of a single AT base pair resulted in a loss of transcriptional activation. The function of this sequence differs from that of STRE in that it showed a high level of basal expression and was not responsive to osmotic stress. It is not yet known whether a single transcriptional activator is responsible for both basal level transcription and stress activation or whether there are multiple factors involved. Runs of dA:dT can act as constitutive activation sequences in yeast, but such sequences are generally longer (RUSSELL *et al.* 1983; STRUHL 1985; ROTENBERG and WOOLFORD 1986; LUE *et al.* 1989).

We thank MARJORIE A. ZITOMER for her technical assistance and RICHARD HALLBERG and ANA DEPAOLI-ROACH for sharing their unpublished results with us. This work was supported by an U.S. Public Health Service Grant GM-26061.

LITERATURE CITED

ARNDT, K. T., C. A. STYLES and G. R. FINK, 1989 A suppressor of *HIS4* transcriptional defects encodes a protein with homology to the catalytic subunit of protein phosphatases. Cell **56:** 527–537.

AUSUBEL, F. M., R. BRENT, R. E. KINGSTON, D. M. MOORE, J. G. SEIDMAN *et al.*, 1994 *Current Protocols in Molecular Biology,* John Wiley and Sons, Inc., New York.

BALASUBRAMANIAN, B., C. V. LOWRY and R. S. ZITOMER, 1993 The Rox1 repressor of the *Saccharomyces cerevisiae* hypoxic genes is a specific DNA-binding protein with a high-mobility-group motif. Mol. Cell. Biol. **13:** 6071–6078.

BISSINGER, P. H., R. WIESER, B. HAMILTON and H. RUIS, 1989 Control of *Saccharomyces cerevisiae* catalase T gene (*CTT1*) expression by nutrient supply via *RAS*-cyclic AMP pathway. Mol. Cell. Biol. **9:** 1308–1315.

BOTSTEIN, D., S. C. FALCO, S. STEWART, M. BRENNAN, S. SCHERER *et al.*, 1979 Sterile hosts yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene **8:** 17–24.

BREWSTER, J. L., T. DE VALOIR, N. C. DWYER, E. WINTER and M. C. GUSTIN, 1993 An osmosensing signal transduction pathway in yeast. Science **259:** 1760–1763.

CERDAN, M. E., and R. S. ZITOMER, 1988 Oxygen-dependent upstream activation sites of *Saccharomyces cerevisiae* cytochrome c genes are related forms of the same sequence. Mol. Cell. Biol. **8:** 2275–2279.

CHEN, D.-C., B.-C. YANG and T.-T. KUO, 1992 One step transformation of yeast in stationary phase. Curr. Genet. **21:** 83–84.

CLAVILIER, L., G. PERE-AUBERT and P. P. SLONIMSKI, 1969 Mise en evidence de plusieurs loci independants impliques dans la synthese de l'iso-2-cytochrome c chez la levure. Mol. Gen. Genet. **104:** 195–218.

COHEN, P, 1989 The structure and regulation of protein phosphatases. Annu. Rev. Biochem. **58:** 453–508.

CSORTOS, C., S. ZOLNIEROWICZ, E. BAKO, S. D. DURBIN and A. A. DEPAOLI-ROACH, 1996 J. Biol. Chem. **271:** 2578–2588.

CRAIG, E. A., B. D. GAMBILL and R. J. NELSON, 1993 Heat shock proteins: molecular chaperones of protein biogenesis. Microbiol. Rev. **57:** 402–412.

GIETZ, R. D., and A. SUGINO, 1988 New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:** 527–534.

GOUNALAKI, N., and G. THIREOS, 1994 Yap1p, a yeast transcriptional activator that mediates multidrug resistance, regulates the metabolic stress response. EMBO J. **13:** 4036–4041.

HEALY, A. M., T. HELSER and R. S. ZITOMER, 1987 Sequences required for transcriptional initiation of the *Saccharomyces cerevisiae CYC* gene. Mol. Cell. Biol. **7:** 3785–3791.

HEALY, A. M., S. ZOLNIEROWICZ, A. E. STAPLETON, M. GOEBL, A. A. DEPAOLI-ROACH *et al.,* 1991 *CDC55,* a *Saccharomyces cerevisiae* gene involved in cellular morphogenesis: identification, characterization, and homology to the B subunit of mammalian type 2A protein phosphatase. Mol. Cell. Biol. **11:** 5767–5780.

HINNEBUSCH, A. G., 1990 Transcriptional and translational regulation of gene expression in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae.* Prog. Nucleic Acid Res. Mol. Biol. **38:** 195–240.

HODGE, M. R., G. KIM, K. SINGH and M. G. CUMSKY, 1989 Inverse regulation of the yeast *COX5* genes by oxygen and heme. Mol. Cell. Biol. **9:** 1958–1964.

KBOAYASHI, N., and K. MCENTEE, 1993 Identification of cis and *trans* components of a novel heat shock stress regulatory pathway in *Saccharomyces cerevisiae.* Mol. Cell. Biol. **13:** 248–256.

KLEBE, R. J., J. V. HARRISS, Z. D. SHARP and M. G. DOUGLAS, 1983 A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. Gene **25:** 333–341.

LALORAYA, S., D. B. GAMBILL and E. A. CRAIG. 1994 A role for a eukaryotic GrpE-related protein, Mge1p, in protein translocation. Proc. Natl. Acad. Sci. USA **91:** 6481–6485.

LAZ, T. M., D. F. PIETRAS and F. SHERMAN, 1984 Differential regulation of the duplicated iso-cytochrome c genes in yeast. Proc. Natl. Acad. Sci. USA **81:** 4475–4479.

LOWRY, C. V., and R. S. ZITOMER, 1990 *ROX1* encodes a heme-induced repression factor regulating *ANB1* and *CYC7* of *Saccharomyces cerevisiae.* Mol. Cell. Biol. **8:** 4651–4658.

LOWRY, C. V., J. L. WEISS, D. A. WALTHALL and R. S. ZITOMER, 1983 Modulator sequences mediate the oxygen regulation of *CYC1* and a neighboring gene in yeast. Proc. Natl. Acad. Sci. USA **80:** 151–155.

LUE, N. F., A. R. BUCHMAN and R. D. KORNBERG, 1989 Activation of RNA polymerase II transcription by a thymidine-rich upstream element *in vitro.* Proc. Natl. Acad. Sci. USA **86:** 486–490.

MAEDA, T., A. Y. TSAI and H. SAITO, 1993 Mutations in a protein tyrosine phosphatase gene (*PTP2*) and a protein serine/threonine phosphatase gene (*PTC1*) cause a synthetic growth defect in *Saccharomyces cerevisiae.* Mol. Cell. Biol. **13:** 5408–5417.

MAEDA, T., S. M. WURGLER-MURPHY and H. SAITO, 1994 A two-component system than regulates an osmosensing MAP kinase cascade in yeast. Nature **369:** 242–245.

MAGASANIK, B., 1992 Regulation of nitrogen utilization, pp. 283–317, in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression,* edited by E. W. JONES, J. R. PRINGLE and J. R. BROACH. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

MARCHLER, G., C. SCHULLER, G. ADAMS and H. RUIS, 1993 A *Saccharomyces cerevisiae* element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J. **12:** 1997–2003.

PILLAR, T. M., and R. E. BRADSHAW, 1991 Heat shock and stationary phase induce transcription of the *Saccharomyces cerevisiae* iso-2-cytochrome c gene. Curr. Genet. **20:** 185–188.

PREZANT, T., K. PFEIFER and L. GUARENTE, 1987 Organization of the regulatory region of the yeast *CYC7* gene: multiple factors are involved in regulation. Mol. Cell. Biol. **7:** 3252–3259.

READING, D. S., R. L. HALLBERG A. M. MYERS, 1989 Characterization of the yeast *HSP60* gene coding for a mitochondrial assembly factor. Nature **337:** 655–659.

RONNE, H, 1995 Glucose repression in fungi. Trends Genet. **11:** 12–17.

RONNE, H., M. CARLBERG, G. Z. HU and J. O. NEHLIN, 1991 Protein phosphatase 2A in *Saccharomyces cerevisiae:* effects on cell growth and bud morphogenesis. Mol. Cell. Biol. **11:** 4876–4884.

ROSE, M. D., F. WINSTON and P. HIETER, 1990 *Methods in Yeast Genetics, A Laboratory Course Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

ROSENBLUM-VOS, L. S., L. RHODES, C. C. EVANGELISTA, K. A. BOAYKE, P. WICK *et al.,* 1991 The *ROX3* gene encodes an essential, nuclear protein involved in *CYC7* gene expression in *Saccharomyces cerevisiae.* Mol. Cell. Biol. **11:** 5639–5647.

ROSPERT, S., T. JUNNE, B. S. GLICK and G. SCHATZ, 1993 Cloning and disruption of the gene encoding yeast mitochondrial chaperonin 10, the homologue of *E. coli* groES. FEBS Lett. **335:** 358–360.

ROTENBERG, M. O., and J. L. WOOLFORD, 1986 Tripartite upstream promoter element essential for the expression of *Saccharomyces cerevisiae* ribosomal protein genes. Mol. Cell. Biol. **6:** 674–687.

ROTHSTEIN, R. J., and F. SHERMAN, 1980 Genes affecting the expression of cytochrome c in yeast: genetic mapping and genetic interactions. Genetics **94:** 871–889.

RUSSELL, D. W., M. SMITH, D. COX, V. M. WILLIAMSON and E. T. YOUNG, 1983 DNA sequences of two yeast promoter-up mutants. Nature **304:** 652–654.

SANGER, F., S. NICKLEN and A. R. COULSON, 1977 DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:** 5463–5467.

SCHULLER, C., J. L. BREWSTER, M. R. ALEXANDER, M. C. GUSTIN and H. RUIS, 1994 The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of *Saccharomyces cerevisiae CTT1* gene. EMBO J. **13:** 4382–4389.

SHERMAN, F., and J. W. STEWART, 1971 Genetics and biosynthesis of cytochrome c. Ann. Rev. Genet. **5:** 257–296.

SHU, Y., and R. L. HALLBERG, 1995 *SCS1,* a multicopy suppressor of *hsp60-ts* mutant alleles, does not encode a mitochondrially-targeted protein. Mol. Cell. Biol. **15:** 5618–5626.

SNEDDEN, A. A., P. T. W. COHEN and M. J. R. STARK, 1990 *S. cerevisiae* protein phosphatase 2A performs an essential cellular function and is encoded by two genes. EMBO J. **9:** 4339–4346.

STRUHL, K, 1985 Naturally occurring poly(dA-dT) sequences are upstream promoter elements for constitutive transcription in yeast. Proc. Natl. Acad. Sci. USA **82:** 8419–8423.

SUTTON, A., D. IMMANUEL and K. T. ARNDT, 1991 The SIT4 protein phosphatase functions late in G_1 for progression into S phase. Mol. Cell. Biol. **11:** 2133–2148.

VAN ZYL, W., W. HUANG, A. A. SNEDDON, M. STARK, S. CAMIER *et al.,* 1992 Inactivation of the protein phosphatase 2A regulatory subunit A results in morphological and transcriptional defects in *Saccharomyces cerevisiae.* Mol. Cell. Biol. **12:** 4946–4959.

WATERLAND, R. A., A. BASU, B. CHANCE and R. O. POYTON, 1991 The isoforms of yeast cytochrome c oxidase subunit V alter the *in vivo* kinetic properties of the holoenzyme. J. Biol. Chem. **266:** 4180–4186.

WERNER-WASHBURNE, M., E. BRUAN, G. R. JOHNSTON and R. A. SINGER, 1993 Stationary phase in yeast. Microbiol. Rev. **57:** 383–401.

WRIGHT, C. F., and R. S. ZITOMER, 1984 A positive regulatory site and a negative regulatory site control the expression of the *Saccharomyces cerevisiae CYC7* gene. Mol. Cell. Biol. **4:** 2023–2030.

ZITOMER, R. S., J. W. SELLERS, D. W. MCCARTER, G. A. HASTINGS, P. WICK *et al.,* 1987 Elements involved in oxygen regulation of the *Saccharomyces cerevisiae CYC7* gene. Mol. Cell. Biol. **7:** 2212–2220.

ZOLNIEROWICZ, S., C. CSORTOS, J. BONDER, A. VERIN, M. C. MUMBY *et al.,* 1994 Diversity of regulatory B-subunits of protein phosphatase 2A: identification of a novel isoform highly expressed in brain. Biochemistry **33:** 11858–11867.

Communicating editor: M. JOHNSTON