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# Functional Analysis of the Stress Response Element and Its Role in the Multistress Response of *Saccharomyces cerevisiae*

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**The *DDR2* gene of *Saccharomyces cerevisiae* is a multistress response gene whose transcription is rapidly and strongly induced by a diverse array of xenobiotic agents, and environmental and physiological conditions. The multistress response of this gene requires the pentanucleotide, 5′CCCCT, ($C_4T$; STRE (STress Response Element)) and the zinc-finger transcription factors, Msn2p and Msn4p. A 51bp oligonucleotide (oligo 31/32) containing two STREs from the *DDR2* promoter region was previously shown to direct heat shock activation of a *lacZ* reporter gene. In this work we demonstrate that the same element conferred a complete multistress response to an *E. coli galK* reporter gene introduced into yeast cells. A variant oligonucleotide in which both the STRE spacing and neighboring sequences were altered responded to the same spectrum of stresses, while substitution of nucleotides within the pentanucleotide completely abolished the multistress response. These results directly demonstrate that STREs are not only necessary but are sufficient for mediating a transcriptional response to a surprisingly diverse set of environmental and physiological conditions.** 

Cells alter their patterns of gene expression in response to environmental challenges or stresses. Frequently, these changes result from transient modifications in the rate of transcription initiation for specific sets of genes. These rapid transcriptional activation events are thought to allow the cell to adapt to the stress conditions or survive the environmental insult more efficiently through the expression of gene products that mitigate cellular damage caused by adverse environmental conditions.

An interesting and biologically significant aspect of stress-induced gene expression is the demonstration that a surprisingly diverse array of environmental and/or physiological conditions activate transcription of certain sets of genes. For example, the *DDR2, HSP12, TPS2, DDR48* and *UB14* genes are transcribed at high levels following exposure of cells to DNA damaging agents (methyl methane sulfonate, (MMS)) and are also transcriptionally responsive to heat shock and other cellular stresses (1-5). The *CTT1* gene of *S. cerevisiae* encoding a cytosolic catalase, is induced by several environmental conditions including heat shock, osmotic shock, nitrogen starvation and oxidative damage (1,6).

Our molecular analysis of the *DDR2* promoter and studies performed by others on the *CTT1* promoter have identified a pentanucleotide element, 5′CCCCT [$C_4T$; also named STress Response Element (STRE)], required for increased transcription following heat shock and other stresses (6-8). While it was shown that a single copy of the $C_4T$ element was sufficient for a modest increase in reporter activity following heat shock, two or more copies of this pentanucleotide provided a greater than additive effect on stress-induced gene expression (8). This latter observation is consistent with a positive physical interaction between transcription factors bound at these sites. Recently, several additional yeast genes were shown to be regulated via STREs. Promoter deletion studies of the *HSP12, TPS2* and *GSY2* genes have shown a requirement for STREs in mediating their transcription induction following heat shock, osmotic shock, post-diauxic shift growth and nitrogen starvation (9-11). The promoter regions of these genes contain multiple STREs in either orientation relative to the TATA box. This latter finding agrees with our previous demonstration that the STRE element functioned to confer heat shock inducibility

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to a *lacZ* reporter gene irrespective of its orientation (5′CCCCT or 5′AGGGG; 8).

In an expression library screen for proteins that bind $C_4T$-containing DNA, we identified the Zn-finger transcription factor, Msn2p, as the primary STRE-specific binding activity in yeast extracts and demonstrated that Msn2p together with its structural homolog, Msn4p, account for stress induced transcription of the *DDR2, CTT1, HSP12* and *TPS2* genes (3). Martinez-Pastor and coworkers have also provided compelling evidence for a direct role of these proteins in the multistress transcriptional response in yeast (2).

In this report we present a functional study of the STRE and its role in the yeast multistress response. Our results extend the list of environmental and physiological conditions that activate gene expression through this unique regulatory element. Moreover, we have constructed mutational variants of the STRE and show that base changes within the STRE completely abolish its function, while its efficacy as a regulatory element was only slightly reduced by changes in neighboring nucleotide sequences and interelement spacing. These results directly demonstrate that the STRE is by itself sufficient for transcriptional activation of gene expression in response to an unusually diverse set of environmental and physiological stresses.

## MATERIALS AND METHODS

### *Strains, Growth Medium, and Chemicals*

Yeast strains used in this study were: RZ49-4 (*MATa trp1 gal1Δ ade1-100 leu2-3,112*), YLA45 (*MATa trp1 gal1Δ ade1-100 leu2-3,112* / YCpR2-DDR2; this work) and M12B (*Matα trp1-289 ura3-52 gal2;* 2). Strains containing plasmids were grown in SC trp− medium (12). Cells were grown in YPD (12) for yeast transformations. STMD medium (13) was used for nitrogen starvation experiments.

### *Stress Treatments*

Preliminary *galK* reporter assays were performed to determine the optimal induction conditions (data not shown). The general protocol for growth and treatment of the cells was as follows: Fresh cultures of the yeast strains were diluted into SC trp− medium and grown logarithmically at 30 °C to a cell density of approximately $7 \times 10^6$ cells/ml ($OD_{595} \sim 0.2$), unless otherwise noted. Cultures were divided into two aliquots, one of which served as control and the other was treated as detailed below. Following exposure to the particular stress condition, cells were collected by centrifugation and soluble protein extracts and total RNA were prepared (see below). The specific conditions for each stress were:

*Methyl methanesulfonate (MMS).* MMS was added to a final concentration of 0.1% for Northern analysis and 0.07% for *galK* assays and cells were treated for 60 min.

*Hydrogen peroxide ($H_2O_2$).* $H_2O_2$ was added to a final concentration of 300 $\mu$M for 60 min.

*Post-diauxic shift (PDS).* For RNA measurements, PDS conditions were determined by monitoring optical density of the culture and identifying the change in growth rate of the culture that occurs during the shift from logarithmic to respiratory growth. For strain RZ49-4 and its derivatives this shift occurs at a density of $\sim 7 \times 10^7$ cells/ml under our experimental conditions and was reached approximately 12-14 h after the control sample was harvested. For galactokinase measurements, the PDS samples were collected 22-24 hr after the control samples were harvested. Control cultures were harvested at a density of $\sim 7 \times 10^6$ cells/ml.

*Heat shock (HS).* Cultures were grown overnight at 23 °C and were divided into two aliquots. One aliquot was shifted to 37 °C for 20 minutes, while the control culture was incubated for an equivalent time at 23 °C.

*Nonfermentative carbon sources.* Cultures were grown in SC trp− medium containing either glucose (2%) or a mixture of lactate (2%) and glycerol (3%) as the carbon source and harvested at a density of $\sim 7 \times 10^6$ cells/ml.

*Nitrogen starvation (NS).* Cultures were grown overnight in YPD to a cell density of $\sim 7 \times 10^6$ cells/ml, collected by centrifugation, washed with an equal volume of distilled water, and resuspended in an equal volume of STMD. Cultures were grown for an additional 3 hours for Northern analysis and 7 hours for galactokinase measurements. Control cultures were suspended in STMD medium and harvested immediately.

*Osmotic shock.* NaCl was added to a final concentration of 0.3 M. Cells were incubated for 60 min for Northern analysis. For galactokinase measurement, cells were exposed to 0.45 M NaCl for 120 min.

*4-Nitroquinoline-1-oxide (NQO).* NQO was added to a final concentration of 1.5 $\mu$g/ml and cells were treated for 90 min.

*Hydroxyurea (HU).* Solid hydroxyurea was added to a final concentration of 100 mM for Northern analysis and 200 mM for galactokinase induction measurements. Cells were treated for 120 minutes.

*Cadmium chloride ($CdCl_2$).* $CdCl_2$ was added to a final concentration of 200 $\mu$M for Northern analysis and 400 $\mu$M for galactokinase measurements. Cells were treated for 30 minutes or 60 minutes, respectively.

*Paraquat (PQ).* PQ was added to a final concentration of 100 mM and cells were treated for 60 min.

### *RNA Preparation and Northern Hybridization*

Total RNA was prepared from yeast cultures as previously described (14). Denatured RNA (50 $\mu$g) was fractionated by electrophoresis in formaldehyde-agarose gels and transferred to nylon membranes. RNA blots were UV-crosslinked and hybridized with [$\alpha$-$^{32}$P]-dCTP radiolabeled probes in 500 mM sodium phosphate, pH 7.2, 7% sodium dodecyl sulfate (SDS), 10 mg/ml BSA and 1 mM EDTA at 65°C overnight. After washing twice in 2× SSC (1× SSC is 300 mM sodium chloride, 30 mM sodium citrate, pH 7.0)/0.1% SDS, and twice in 0.1× SSC/0.1% SDS at 65°C, the Northern blots were exposed to X-ray film and quantitated using an Ambis radio-imager system (Scanalytics). The *DDR2* probe used for hybridization was the 1.45 kb *Hind* III fragment from plasmid pBRA2 (1). The *ACT1* hybridization probe was a PCR fragment corresponding to nucleotides +1 to +714 (15) of this gene. Individual M12B yeast colonies were used for preparation of the PCR template.

### *Construction of galK Fusion Plasmids*

Complementary oligonucleotides corresponding to the $C_4T$-containing region of the *DDR2* gene were synthesized using an Applied Biosystems 391 DNA synthesizer. The oligonucleotides were synthesized with either *BamH*I or *Xho* I restriction sites at their 5′ termini to facilitate subsequent cloning. Complementary oligonucleotides were annealed and cloned into the *BamH* I and *Xho* I sites of plasmid YCpR2 (16) containing the yeast *CYC1* promoter region fused to the *galK* gene of *E. coli.* The sequences of the parental oligonucleotides are: oligo 31/32, 5′CTTCTTTTCCCCTGTTTCCATTTTTGTCTTTTCTCACCCCTTATGGGGACC3′; oligo 27M, 5′CTTCTTTTAAAAG-

GTTTCCATTTTTGTCTTTTCTCAAAAAGTATGGGGACC3′; oligo 24, 5′CGAGACCCCTTATCTAGAGACCCCTTAC3′. To construct the Δ1 plasmid, the YCpR2 plasmid was cut with restriction enzymes *BamH* I and *Xho* I, end-filled using Klenow fragment and recircularized. All constructs were sequenced using Sequenase (US Biochemical) according to the manufacturer's protocols to confirm the structures of the inserts.

Plasmid YCpR2-DDR2 contains the entire upstream region of *DDR2* from the *Hind* III site to the first ATG codon of the *DDR2* coding sequence fused in frame to the *galK* gene. Primers flanking this region and containing either terminal Xho I or *EcoR* I restriction sites were synthesized, used to amplify the corresponding DNA fragments by PCR and cloned into the *EcoR* I and *Xho* I sites of plasmid YCpR2. The in-frame junction between the *DDR2* and *galK* sequences in the resulting plasmid was confirmed by DNA sequence analysis.

### *Extract Preparation and galK Assay*

Galactokinase assays were performed essentially as described by Rymond et al. (16) with minor modifications. Cultures were harvested by centrifugation and pellets were resuspended in galK buffer (20mM Hepes, pH 7.5, 1 mM DTT, 300 μg/ml BSA). One-half volume of cold, sterile, acid washed glass beads was added and cell suspensions were vortexed 6 times for 15 seconds and incubated on ice between pulses. Supernatants were removed, the glass beads washed with galK buffer and the supernatants were combined. Protein concentrations were determined and the extracts diluted to ~3 mg protein/ml. Extract (20 μl) was added to 80 μl of reaction mixture [5 mM $MgCl_2$, 125 mM Tris, pH 8.0, 1.25 mM DTT, 4 NaF, 2 mM ATP, 0.2 μC $^{14}$C-galactose (20 μC/ml, 2 μC/μmole; Dupont NEN)] which had been preincubated for 1 minute at 32°C. At times (0 and 15 min) after addition of extract, aliquots (20 μl) were removed, added to 5 μl unlabeled 100 mM galactose on ice to stop the reaction, and samples were then spotted onto DE81 filters (2.4 cm, Whatman). After washing in 0.1% galactose, the filters were dried and the radioactivity retained on the filters was measured by liquid scintillation counting. The assay was linear with respect to time and extract concentration. All values are expressed in *galK* units (pmoles of galactose-1-phosphate formed/min/mg protein) and are the average of two independent assays.

## RESULTS

### *Transcription of the DDR2 Gene Is Induced by a Variety of Environmental Stresses and Metabolic Conditions*

*DDR2* transcript levels were examined by Northern hybridization of RNA isolated from cultures of yeast strain YLA45 exposed to several different chemical agents, environmental and physiological stresses. The results shown in Figure 1 confirm previous studies demonstrating that heat shock, DNA damaging agents (MMS and NQO) and inhibitors of DNA replication (HU) effectively induce

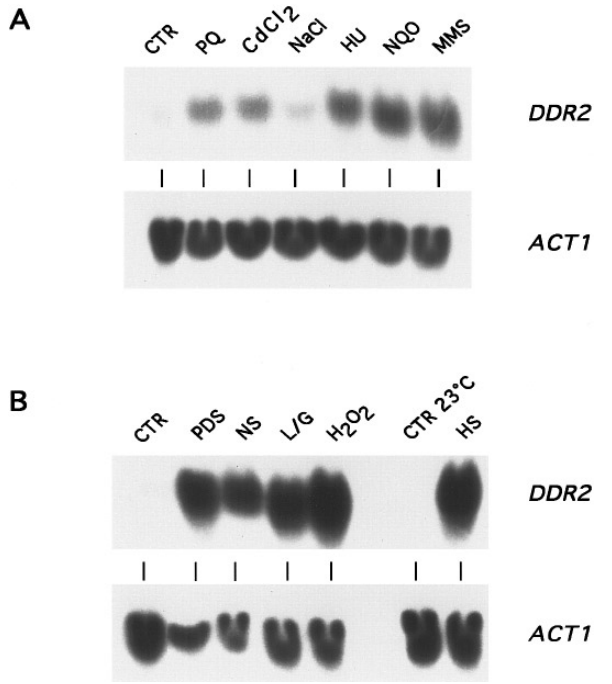


**FIG. 1.** *DDR2* mRNA expression following stress induction. Yeast strain YLA45 (containing plasmid YCpR2-DDR2) was treated with the various stresses (See Materials and Methods for details of stress treatment.) and total RNA was extracted. 50 μg of total RNA was electrophoresed on a 1.0% formaldehyde agarose gel, transferred to a nylon membrane, then probed with *DDR2*. The blots were stripped and reprobed with *ACT1*. Panel A: Lane 1, control 30 °C; lane 2, paraquat; lane 3, cadmium chloride; lane 4, sodium chloride; lane 5, hydroxyurea; lane 6, NQO; lane 7, MMS. Panel B: Lane 1, control, 30 °C; lane 2, post-diauxic shift growth; lane 3, nitrogen starvation; lane 4, lactate/glycerol; lane 5, hydrogen peroxide; lane 6, control, 23 °C; lane 7, heat shock.

*DDR2* transcript accumulation (5, 17). In addition *DDR2* transcript levels increased markedly following starvation for nitrogen, during post-diauxic shift growth, growth on nonfermentable carbon sources, and exposure to hydrogen peroxide. Treatments with paraquat or the heavy metal, cadmium, produced modest increases in *DDR2* transcript levels. While only a slight *DDR2* transcript response was observed following exposure to 0.3 M NaCl (Fig. 1), a more significant osmotic induction of *DDR2* transcripts was observed at higher salt concentrations in this yeast strain (0.7 M NaCl; data not shown). This latter result may reflect strain differences for osmotic induction since *DDR2* transcripts were highly induced in other strain backgrounds at the lower salt concentration (7). These results illustrate that the *DDR2* gene is poised to respond to a remarkably diverse array of environmental and physiological stresses.

### *The $C_4T$ Element Mediates the Multistress Response of the DDR2 Gene*

To more carefully investigate the role of the $C_4T$ elements in the multistress response, a portion of the

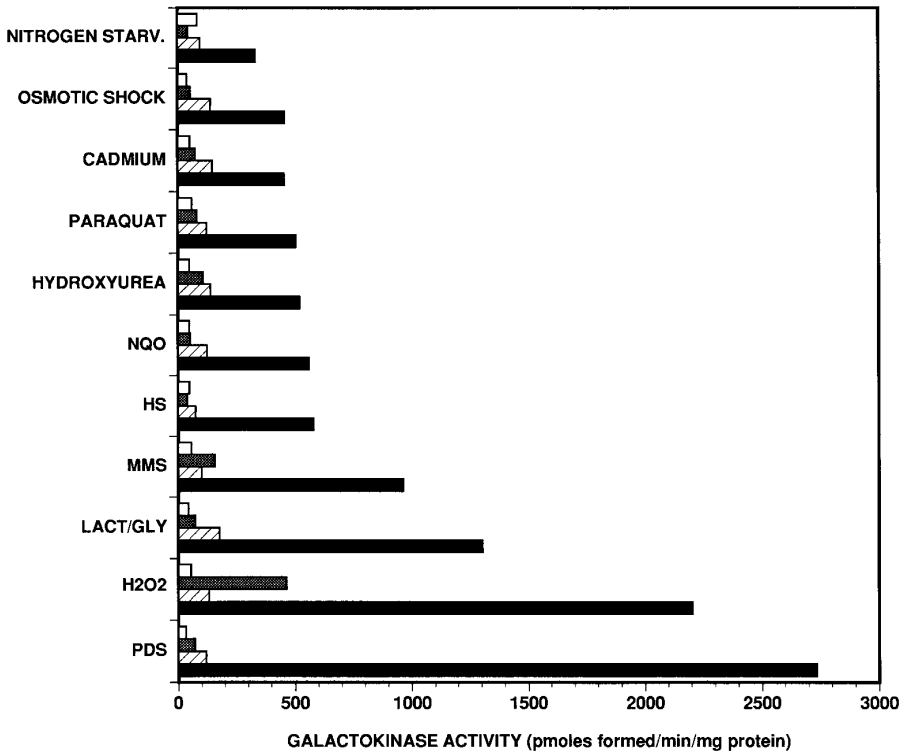


**FIG. 2.** Plasmid encoded galactokinase activity following stress treatment. Cells containing either the native *DDR2* $C_4T$ sequence (YCpR2-31/32) or the mutant $A_4G$ sequence (YCpR2-27M) were treated with various stresses and *galK* activity measured as described in Materials and Methods. YCpR2-27M, control, open bar; YCpR2-27M, stress, stippled bar; YCpR2-31/32, control, striped bar; YCpR2-31/32, stress, black bar.

*DDR2* upstream region (oligo 31/32) was fused to the *E. coli galK* reporter gene. The *galK* gene has been used as a reporter gene for other promoter studies in yeast (18-20) and encodes the bacterial galactokinase that can be quantitatively assayed in yeast extracts. The yeast galactokinase deficient strain, RZ49-4, was transformed with plasmid YCpR2-31/32 and enzyme levels were measured in response to each stress shown to induce chromosomal *DDR2* transcript levels (Figure 2). As expected from earlier studies (8) brief heat shock treatment caused a significant increase (8-fold) in reporter gene expression. Stresses that led to the greatest increases ($>$ 7-fold) in galactokinase activity were, in addition to heat shock, exposure to MMS and hydrogen peroxide, growth in a nonfermentative carbon source and post-diauxic shift growth. These results parallel RNA induction levels measured by Northern analysis (Figure 1). Significant but smaller increases in galactokinase activity (approximately 3 to 5-fold) were observed following exposure to paraquat, cadmium, NQO, hydroxyurea or sodium chloride (Figure 2) with enzyme levels paralleling *DDR2* transcript levels (Figure 1; 5, 17). Nitrogen starvation had a modest effect upon *galK* enzyme activity, whereas transcript levels increased significantly following removal of a nitrogen source from the medium. The difference in these results may be due to post transcriptional effects on *galK* enzyme synthesis resulting from the starvation conditions.

### *$C_4T$ Mutations Block a Multistress Response*

Galactokinase activity was also measured in yeast strain RZ49-4 containing plasmid YCpR2-27M following identical stress treatments. Oligonucleotide 27M was synthesized with both $C_4T$ elements of oligo 31/32 replaced by the transversion sequence 5′AAAAG ($A_4G$; see Materials and methods). Previously, we showed that this sequence alteration of the $C_4T$ element prevented heat shock induction of a *lacZ* reporter (8). The RZ49-4 cells containing plasmid YCpR2-27M showed little or no induction of galactokinase activity in response to multiple stresses as shown in Figure 2 indicating that stress induction of *galK* activity was mediated via the STRE. One exception, hydrogen peroxide treatment, gave a small but reproducible increase in galactokinase activity directed by plasmid YCpR2-27M. However, this level was less than 20% that of $C_4T$-containing plasmid YCpR2-oligo 31/32. Similar results were obtained using extracts prepared from a strain containing the plasmid YCpR2-$\Delta$1 that lacks a $C_4T$ insert (data not shown) indicating that uncharacterized plasmid sequences were likely responsible for this

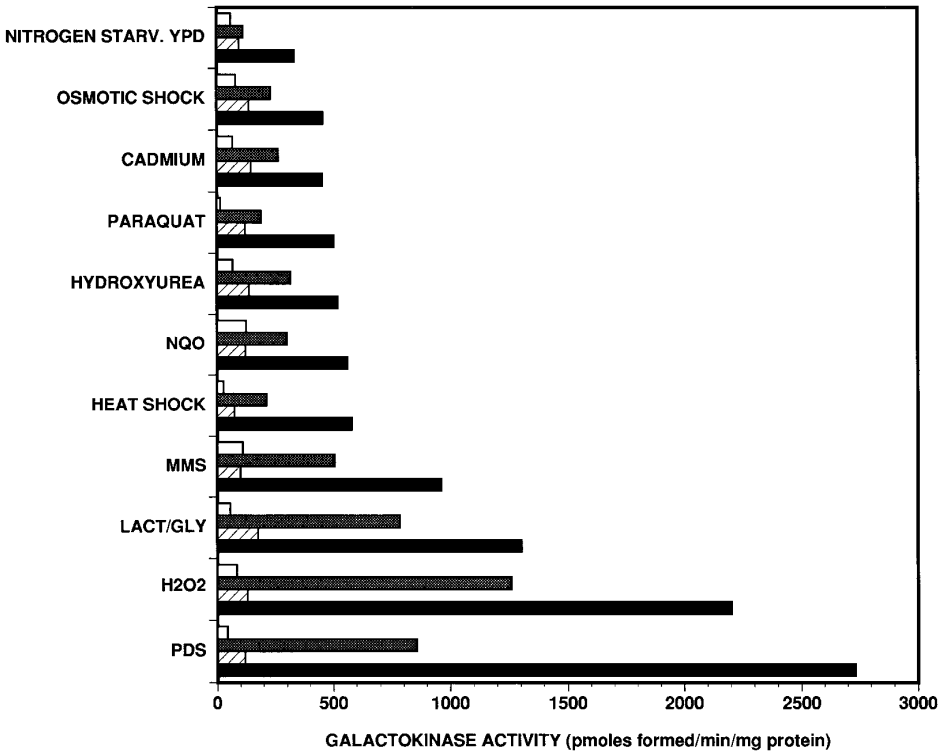


**FIG. 3.** Effects of STRE spacing and nucleotide context on *galK* activity induced by stress. Cells containing either the native *DDR2* $C_4T$ sequence (YCpR2-31/32) or the $C_4T$ sequence with altered spacing and altered flanking sequences (YCpR2-24) were treated with various stresses and *galK* activity measured as described in Materials and Methods. YCpR2-24, control, open bar; YCpR2-24, stress, stippled bar; YCpR2-31/32, control, striped bar; YCpR2-31/32, stress, black bar.

modest increase in *galK* enzyme levels following hydrogen peroxide exposure. Nevertheless, the results shown in Figure 2 demonstrate that the $C_4T$ pentanucleotide is an essential element for the response of the *DDR2* gene to more than a dozen diverse environmental and physiological stresses.

### *A Multistress Response Is Observed after Changes in the Spacing between and Sequences Surrounding the $C_4T$ Elements*

While the results shown in Figure 2 demonstrate that the $C_4T$ elements are required for the multistress response, it was important to determine whether these elements by themselves were sufficient for directing stress-induced gene expression. To address this question, we synthesized an oligonucleotide in which the $C_4T$ elements were flanked by DNA sequences that were unrelated to the native sequence in the *DDR2* promoter. Additionally, the spacing between the $C_4T$ elements in this oligo (oligo 24) was deliberately altered. Despite these flanking sequence and spacing alterations, oligo 24 conferred a significant multistress response when inserted upstream of the *galK* reporter gene (Figure 3). Significantly, the response pattern of the oligo 24 construct to different stress conditions was qualitatively similar to that of oligo 31/32. The stresses producing the greatest induction of *galK* activity in strain RZ49-4/YCpR2-24 were post-diauxic shift growth and hydrogen peroxide treatment which were also the most effective inducers of *galK* expression in cells containing the YCpR2-31/32 plasmid. Although the absolute induced levels of galactokinase activity were lower in cells containing plasmid YCpR2-24 compared to those containing the YCpR2-31/32 plasmid, the relative stress-induced increases in *galK* expression were very similar (Table 1). Taken together, our results argue that the $C_4T$ pentanucleotide is sufficient for transcription induction by a wide and diverse assortment of environmental and metabolic stresses.

## DISCUSSION

The number of distinct stresses or conditions known to activate *DDR2* transcription via the STRE promoter element now totals thirteen and includes DNA damaging treatments (MMS and NQO), arrest of DNA synthesis (HU), respiratory inhibition (PQ), heavy metals (cadmium), growth in nonfermentable carbon sources (lactate/glycerol) as well as post diauxic growth, heat shock, osmotic shock, starvation for nitrogen and exposures to hydrogen peroxide, low pH and sorbate (6-

**TABLE 1**

Comparison of the Induction Mediated by Oligo 31/32 and Oligo 24 Following Different Stresses

| Stress | Fold induction[a] | |
|---|---|---|
| | Oligo 31/32 | Oligo 24 |
| Nitrogen starvation | 3.5 | 1.9 |
| Osmotic shock | 3.3 | 2.9 |
| Cadmium chloride | 3.1 | 4.0 |
| Paraquat | 4.2 | 12.5 |
| Hydroxyurea | 3.8 | 4.7 |
| NQO | 4.6 | 2.4 |
| Heat shock | 7.8 | 8.1 |
| MMS | 9.7 | 4.7 |
| Lactate/glycerol | 7.4 | 14.5 |
| Hydrogen peroxide | 17.0 | 15.1 |
| PDS | 23.2 | 17.4 |

[a] Fold induction values are calculated as the ratio of galactokinase activity (induced extracts)/galactokinase activity (control extracts).

8). The remarkable number and diversity of stresses capable of activating *DDR2* expression suggest that STRE-regulated genes are likely to perform general protective roles following environmental and physiological insults. Other genes such as *CTT1* (cytosolic catalase) and *TPS2* (trehalose phosphate phosphatase) also show a multistress response pattern and their biochemical functions likely contribute to enhancing cell survival. *TPS2* is needed for the synthesis of trehalose, a carbohydrate that accumulates following exposure to heat shock and other stresses and is proposed to act as a general intracellular protectant of both membrane and protein structural integrity during environmental adversity (21). For those STRE-regulated genes whose biochemical functions are unknown (such as *DDR2*), it is reasonable to suggest that their gene products also perform general physiological roles in mitigating cell damage, modulating cell metabolism or enhancing recovery from stress.

This report confirms the requirement for $C_4T$ elements in the multistress response of *Saccharomyces cerevisiae* and further demonstrates that this pentanucleotide by itself is sufficient to confer complete multistress regulation on a *galK* reporter gene. Oligo 24 efficiently mediated reporter gene expression in a manner qualitatively similar to the native *DDR2* upstream element (oligo 31/32) indicating that the STRE functioned independently of nucleotide sequence context and was relatively insensitive to changes in spacing between elements. These results argue that the STRE is the only sequence required for multistress control of transcription of the *DDR2* gene and, by inference, other stress-regulated yeast genes. It is worth noting that neither spacing nor sequence context is conserved among the $C_4T$ elements found in the promoters of *DDR2, CTT1, TPS2, HSP12* and *GSY2* genes. In these multistress response genes, STRE spacing varies from 10 bp in the *CTT1* promoter to 175 bp in the *GSY2* promoter (6, 10).

The best characterized stress regulatory element in eukaryotes is the heat shock element (HSE) located upstream in the promoters of the heat shock protein (HSP) genes. While it has been shown that several HSP genes are transcriptionally activated by stresses other than heat shock (eg. anoxia, amino acid analogs, DNA damage; 22), it appears that at least in yeast these responses are not mediated through the HSE. Reporter gene studies by Kirk and Piper (23) have demonstrated that the yeast HSE responds strongly to heat shock and does not respond to several other stresses that increase HSP gene transcription *in vivo.* Thus, it is likely that the multistress character of HSP gene expression is due to one or more additional promoter elements. In this regard it is worth noting that several HSP genes contain STREs in their promoter regions, and our studies have provided evidence for a role of these STREs in stress control of HSP gene expression (Treger and McEntee, unpublished results).

The results presented in this paper underscore the unique character of the STRE in its ability to direct transcription in response to a broad range of physiological conditions and environmental treatments. We have used the fact that the STRE functions independently of nucleotide context and inter-element spacing to design a pattern search of the entire yeast genome for other STRE-controlled genes. In this way we have now identified several new stress response genes that are members of this important regulon (Schmitt and McEntee, unpublished data).

## ACKNOWLEDGMENTS

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