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Evidence for a heat shock transcription factor-independent mechanism for heat shock induction of transcription in *Saccharomyces cerevisiae*

(*DDRA2* gene/promoter fusions/heat shock regulatory element binding competition/DNA damage)

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ABSTRACT **Transcription of the *DNA damage-responsive* gene, *DDRA2*, of *Saccharomyces cerevisiae* is activated by heat shock treatment as well as by mutagen/carcinogen exposure. Deletion analysis of upstream noncoding sequence indicated that sequences between approximately −190 and −140 base pairs were necessary for heat shock and DNA damage regulation of transcription. Fusion of this region to a *CYC1-lacZ* reporter gene demonstrated that the sequence between −202 and −165 base pairs was sufficient for basal level and heat shock-induced expression. This DNA sequence was unable to bind heat shock transcription factor as judged by binding competition experiments *in vitro*. These results indicate that yeast possesses a second, heat shock transcription factor-independent mechanism for activating transcription in response to thermal stress.**

The heat shock response is a highly conserved cellular stress response to rapid changes in growth temperature and has been observed in simple bacteria as well as in multicellular organisms including man (1, 2). In *Escherichia coli*, ≈20 proteins are transiently expressed at increased levels following thermal stress (3, 4). Similarly, in eukaryotic cells, heat shock induces discrete sets of proteins. Molecular studies have demonstrated that at least part of the regulation of this response is at the level of transcription (5, 6). In *E. coli* the heat shock genes constitute a regulon that is controlled by a distinct σ factor, σ^{32} (7, 8).

Transcriptional activation of heat shock genes in yeast requires a conserved cis-acting heat shock regulatory element (HSE) that is found upstream of eukaryotic heat shock genes regardless of origin (9, 10). The HSE has been shown to be the binding site for heat shock transcription factor, designated HSF or HSTF, both *in vivo* and *in vitro* (11–13).

We have recently identified a set of genes in *Saccharomyces cerevisiae* that are expressed at high levels in response to DNA-damaging treatments and heat shock (14). In the case of one such DNA damage-responsive (DDR) gene, the *DDRA2* gene, DNA damage or heat shock leads to a 20-fold increase in the level of transcript. DNA sequence and deletion analyses of the upstream regulatory region identified a DNA sequence element that was required for stress regulation of DDRA2. Deletions extending into this region from the 5′ end of the regulatory region abolished DNA damage and heat shock induction of transcription (unpublished data).

Here we demonstrate that a 50-base pair (bp) element identified by deletion studies confers heat shock induction of transcription on a heterologous *CYC1-lacZ* reporter gene. However, the DNA sequence of this region bears no structural resemblance to the classical HSE element of yeast nor does it act as a binding site for HSTF *in vitro*. These observations suggest that the DDRA2 gene of *S. cerevisiae* responds to a distinct HSTF-independent mechanism that regulates transcription in response to thermal stress.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions. Yeast strain M12B (*α trp1-289 ura3-52 gal2*) was transformed with plasmids either by lithium acetate pretreatment (15) or by electroporation (16), and URA$^+$ transformants were selected on SD medium (17) supplemented with tryptophan. Cells were grown to early logarithmic phase in supplemented SD medium at 23°C; one-half of the culture was maintained at 23°C while the other portion was shifted to 42°C for 1 min and further incubated at 37°C (heat shock) for the times indicated in the figure legends. The cultures were cooled briefly on ice and the cells were collected by centrifugation.

Construction of Plasmids. Expression vector pLG669Z (18) was digested with *Sma* I and *Xho* I to delete the UAS1 and UAS2 elements, and *Xho* I linkers were added and ligated. The resulting plasmid, pLGΔ, contained the transcription start sites and TATA elements of *CYC1*. Plasmids pLGA2HCd31 and pLGA2HCd32 contain portions of the *DDRA2* promoter: −446 to −165 bp and −446 to −202 bp from the translation start site, respectively. To construct these plasmids, pBRA2HCd31 and pBRA2HCd32, which contain BAL-31 deletions originating from the *Cla* I restriction site within the *DDRA2* gene, were digested with *Eco*RI and *Bam*HI to excise the upstream fragments, end-filled using the Klenow fragment of DNA polymerase I, appended with *Xho* I linkers, and inserted into the *Xho* I restriction site in pLGΔ. The orientation of the insert was determined from diagnostic restriction digestions.

Centromere-containing promoter fusions were constructed using the pCTΔ (19) and pCZΔ (referred to as pCTdp in ref. 20) vectors. The *Eco*RI–*Xho* I fragments from pLGA2HCd31 and pLGA2HCd32 were cloned into the pCTΔ and pCZΔ plasmids to produce the four plasmids pCTHCd31, pCZHCd31, pCTHCd32, and pCZHCd32. The plasmids pCToligo31/32 and pCZoligo31/32 contain synthetic oligonucleotides corresponding to the *DDRA2* upstream sequence between −212 and −165 bp. The synthetic oligonucleotides were constructed with *Eco*RI and *Xho* I restriction sites at their ends in order to clone these fragments into the polylinker region of the expression vectors following annealing of the complementary strands and treating with polynucleotide kinase as described (21). Plasmid pCTHSE contains three copies of the sequence 5′-AATTCTAGAACGTTCTA-

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; HSTF, heat shock transcription factor; HSE, heat shock regulatory element.
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GAAGCTTCGAGAC-3′ in the plasmid pCTΔ (13). All sequences were confirmed by double-stranded sequencing using Sequenase (22). Synthetic oligonucleotides were prepared using a DuPont generator DNA synthesizer or the Applied Biosystems 391 PCR Mate DNA synthesizer.

Assay of β-Galactosidase. Cells were resuspended in Z buffer containing 0.01% $NaDodSO_4$ and 300 μM phenylmethylsulfonyl fluoride (PMSF) and disrupted by mixing in a Vortex with an equal volume of glass beads (0.5 mm); β-galactosidase activity was then determined (23). Protein concentration was determined using the method of Bradford (24) using bovine serum albumin as standard. The β-galactosidase activity (OD_{420}/min·g of protein) is the average of duplicate samples obtained from two independent transformants.

Gel Retardation and Binding Competition. Crude extracts were prepared as described by Sorger and Pelham (12) from *S. cerevisiae* strain BJ2168 (a *leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2*). Protein concentration was determined as described above.

DNA binding experiments were performed in a total volume of 25 μl using 50 μg of extract, 5 μg of poly(dI-dC)·poly(dI-dC), 20 mM Hepes (pH 8.0), 1 mM $MgCl_2$, 60 mM KCl, 1 mM dithiothreitol, 12% glycerol, and 0.2 ng (10,000 cpm) of DNA probe end-labeled with [α-^{32}P]dCTP using Klenow fragment. Unlabeled competitor DNA was incubated with extract for 15 min at room temperature prior to addition of labeled DNA. Reaction mixtures were incubated for an additional 15 min at room temperature and loaded onto a 4% polyacrylamide gel (acrylamide/bisacrylamide 40:1). Electrophoresis was performed in buffer containing 22.5 mM Tris borate and 0.6 mM EDTA at 200 V for 1.5 hr at room temperature. Gels were dried onto Whatman 3MM paper and placed under Cronex film (DuPont) and an intensifying screen for autoradiography. For the competition experiments the indicated amount of unlabeled competitor was added as described in the figure legends.

RESULTS

Deletion of Stress-Responsive Sequences in the *DDRA2* Upstream Region. The DNA sequence of the upstream region of *DDRA2* and the position of several deletion end points are shown in Fig. 1. Our studies have demonstrated that removal of DNA from the 5′ end of this region to −363 bp (dP) had no effect upon either the basal level of *DDRA2* expression or its inducibility by heat shock or DNA damage stress. Further deletion of the region to −326 bp (d12) caused a slight reduction in the induced level of *DDRA2* transcript. Deletion to −188 bp (d19) caused a substantial increase in the basal level of *DDRA2* transcription, whereas further removal of DNA to −142 bp (d23) resulted in a complete loss of basal and induced *DDRA2* gene expression.

Two 3′ deletions with end points near −180 bp were examined for their effects upon *CYC1-lacZ* expression (Fig. 2) by inserting these control regions into expression vector pLGΔ. Yeast strain M12B was transformed with each of these plasmids (pLGA2HCd31 and pLGA2HCd32) and β-galactosidase was measured under normal growth conditions and after a heat shock stress (Fig. 2). A very low basal level of β-galactosidase was detected in cells containing the pLGΔ vector at 23°C and 37°C. Cells containing plasmid pLGA2HCd32 showed a 4-fold higher basal level of *lacZ* expression at 23°C but no further increase was observed after incubation at 37°C. However, plasmid pLGA2HCd31 produced a nearly 16-fold increase in the level of β-galactosidase expression at 23°C but, more significantly, activity increased >5-fold following incubation at 37°C for 20 min.

In a parallel set of experiments these *DDRA2* upstream fragments were cloned into the centromere-containing promoter fusion vectors, pCTΔ and pCZΔ, at either 128 bp or 19

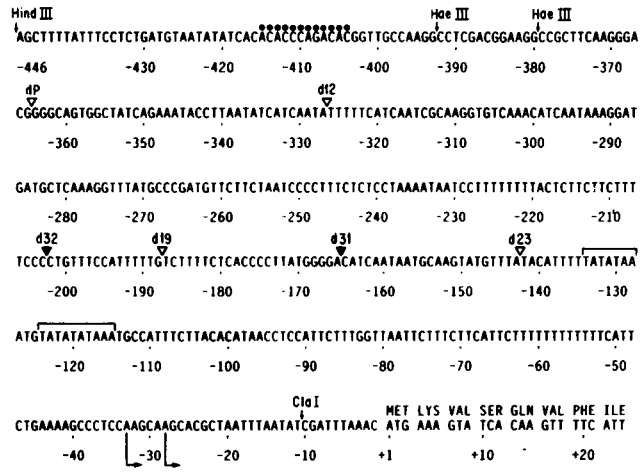
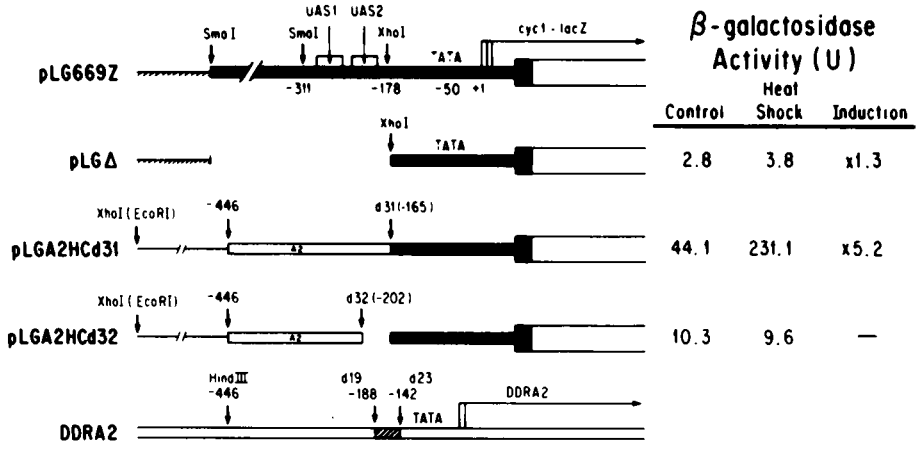


FIG. 1. DNA sequence of the *DDRA2* regulatory region and deletion end points. The nucleotide sequence of upstream regulatory region and eight codons of *DDRA2* protein sequence are shown. Deletion end points are represented by triangles: ▽, 5′ deletions from the *Hin*dIII site (−446 bp); ▼, 3′ deletions from the *Cla* I site (−10 bp). A binding site for the RAP1 (GRFI) regulatory protein is centered at −410 bp and is indicated by ● (unpublished data). The TATA elements are in brackets and the transcript start sites are indicated by arrows.

bp upstream of the major TATA element of *CYC1-lacZ*, respectively (Fig. 3). In the *DDRA2* gene this upstream region is positioned ≈30 bp away from the likely TATA elements (Fig. 1). The pCTHCd32 and pCZHCd32 plasmids expressed relatively low levels of β-galactosidase activity at 23°C and these levels were unaffected by growing cells at 37°C for up to 60 min (Fig. 4). However, cells transformed with plasmid pCTHCd31 or pCZHCd31 showed significantly higher levels of β-galactosidase expression at 23°C and these levels were further induced after heat shock treatment (Fig. 4). These results indicated that the sequences required for heat shock induction were located between −202 and −165 bp from the start of *DDRA2* translation.

Heat Shock Induction Mediated by oligo31/32. A single copy of a synthetic oligonucleotide corresponding to the sequence between the deletion end points (plus an additional 10 bp of sequence), designated oligo31/32, was inserted into the pCTΔ and pCZΔ expression vectors to determine whether this sequence element was sufficient for heat shock regulation of transcription of the *CYC1-lacZ* reporter gene (Fig. 3). As shown in Fig. 4, cells containing the pCToligo31/32 and pCZoligo31/32 plasmids expressed levels of β-galactosidase at 23°C comparable to constructs containing the entire d31 upstream fragment, suggesting that the oligo31/32 oligonucleotide contained activation sequences necessary for basal level expression of β-galactosidase. Following heat shock treatment of these cells, β-galactosidase activity increased >8-fold. This level of induction was somewhat less than that seen with the entire upstream region (12.7-fold to 13.8-fold) but indicated that the oligonucleotide contained sequences responsible for conferring heat shock induction of transcription.

Heat shock induction mediated by oligo31/32 was compared to a promoter fusion plasmid in which three copies of a consensus HSE oligonucleotide were inserted upstream of *CYC1-lacZ* (pCTHSE). As shown in Fig. 4, cells containing plasmid pCTHSE showed a 9.5-fold induction of β-galactosidase activity following heat shock at 37°C. Thus, the oligo31/32 element was as effective as a HSE-containing fragment in conferring thermal inducibility of transcription and functioned when it was located 19 bp or 128 bp upstream of the likely TATA sequences of the *CYC1-lacZ* gene.



β-galactosidase Activity (U)

	Control	Heat Shock	Induction
pLGΔ	2.8	3.8	x1.3
pLGA2HCd31	44.1	231.1	x5.2
pLGA2HCd32	10.3	9.6	—

FIG. 2. Heat shock induction of β-galactosidase activity in *DDRA2-lacZ* promoter fusions. The structures of the parental and promoter fusion vectors are shown. The open box represents *DDRA2* sequences; the filled boxes represent the *CYC1-lacZ* region. Plasmid pLGΔ contains *URA3* sequences (shaded line) immediately upstream of the *CYC1-lacZ* fusion, whereas pBR322 sequences (thin line) are located upstream of the *DDRA2* region in both pLGA2 constructs. β-Galactosidase activity was determined following incubation at 37°C for 20 min.

The oligo31/32 Element Does Not Contain a Binding Site for HSTF. The oligo31/32 element did not show any obvious sequence similarities to the HSE element except for repeats of the TTC half sites (Table 1). It was possible that the oligo31/32 region could contain a binding site for the yeast HSTF since several purified eukaryotic transcription factors bind seemingly unrelated DNA sequences with comparable affinities (26). To determine whether the oligo31/32 element bound HSTF, binding competition was examined using a gel retardation assay, the oligo31/32 element, and either of two HSE-containing double-stranded oligonucleotide probes. In the first set of experiments (Fig. 5), the end-labeled HSE probe was identical to the oligonucleotide in plasmid pCTHSE (Table 1 and *Experimental Procedures*). This HSE-containing oligonucleotide was efficiently retarded (Fig. 5, lane 2) and formation of this complex was inhibited strongly by excess unlabeled HSE oligonucleotide (lanes 3 and 4) but not by excess pUC19 plasmid DNA (lanes 11 and 12). Binding of proteins to the labeled HSE probe was also inhibited strongly by an unlabeled 2.0-kb *Eco*RI restriction fragment from the upstream region of the hsp70 yeast gene, *SSA1* (27), containing a natural heat shock promoter element (lanes 7 and 8). These binding and competition data argue that the retarded complexes detected in polyacrylamide gels were due to binding of yeast HSTF to the HSE probe.

When the unlabeled oligonucleotide, oligo31/32, was used in the competition assay, no competition was detected when the oligo31/32 competitor was present up to a 250-fold molar excess (lanes 5 and 6) or even at 500-fold (data not shown). Similarly, a restriction fragment containing the entire *DDRA2* upstream region did not inhibit binding (lanes 9 and 10).

The HSE oligonucleotide used in the binding experiments shown in Fig. 4 contained multiple overlapping HSTF binding sites that increased the binding affinity for this transcription factor (see below). Consequently, we performed a second series of binding competition experiments using a 26-bp double-stranded oligonucleotide corresponding to the single HSE2 element upstream of the *SSA1* gene of *S. cerevisiae* (28). The sequence of this oligonucleotide, designated HSE2-26, is shown in Table 1. Although there are several HSE elements in the *SSA1* promoter, HSE2 was shown to be most important for heat shock induction of transcription based upon deletion studies (28). Craig and coworkers (25, 28) have demonstrated that a 26-bp oligonucleotide containing the HSE2 element conferred heat shock regulation on a *CYC1-lacZ* fusion *in vivo* and bound HSTF *in vitro*. The HSE2-26 oligonucleotide used in this experiment was identical to the oligonucleotide used by Park and Craig (25) and lacked sequences corresponding to the upstream repression site that partly overlaps HSE2 and is responsible for regulating basal level expression. When the labeled HSE2-26 oligonucleotide was incubated with the yeast extract, a slow migrating complex was detected (Fig. 6) and formation of this complex was inhibited by unlabeled HSE2-26 competitor. That this

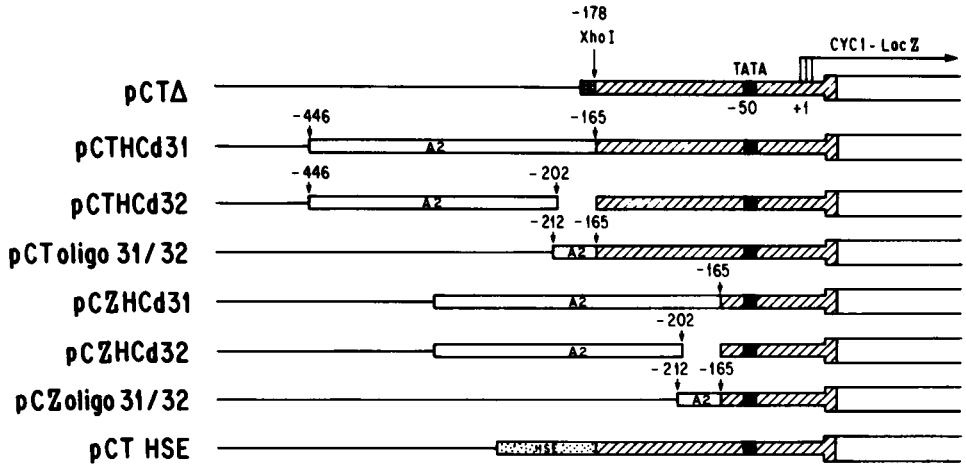


FIG. 3. Structures of *DDRA2-CYC-lacZ* promoter fusions in centromere-containing plasmids. Fusion plasmids were constructed by inserting the indicated fragment or oligonucleotide into the polylinker regions of the CEN4 plasmids pCTΔ and pCZΔ. Numbering in the top line refers to positions in the pCTΔ vector relative to the major transcript start site at +1, whereas numbering in the lower portion of the figure is relative to the translation start site of the *DDRA2* gene. The direction of transcription is indicated by an arrow. The *DDRA2* sequences are represented by open boxes. The dotted bar indicates the synthetic oligonucleotide containing overlapping HSEs. The shaded boxes are *CYC1* sequences. The polylinker region is indicated by crosshatching.

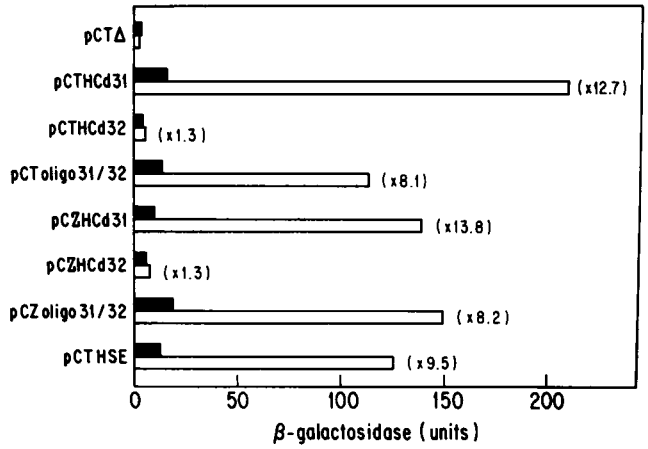


FIG. 4. The oligonucleotide oligo31/32 confers heat shock induction in yeast. Heat shock induction of β-galactosidase activity was determined as described in the text and cells were incubated for 60 min at 37°C. Open bars indicate β-galactosidase activity following heat shock, whereas shaded bars indicate β-galactosidase activity of cells grown at 23°C (control). Induction ratios are given in parentheses.

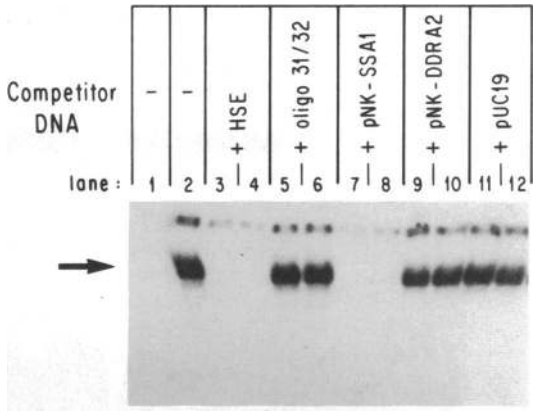


FIG. 5. Binding competition studies of HSE complexes. The 30-bp double-stranded oligonucleotide HSE was radiolabeled and used for DNA binding experiments. Lane 1, labeled HSE oligonucleotide without extract. In lanes 2–12 the HSE probe was incubated with 50 μg of cell extract and unlabeled competitor DNAs as shown. Lane 2, no competitor; lanes 3 and 4, 100- and 250-fold molar excess of HSE oligonucleotide, respectively; lanes 5 and 6, 100- and 250-fold molar excess of oligo31/32, respectively; lanes 7 and 8, 50- and 100-fold molar excess, respectively, of plasmid pNK-SSA1 containing a 2.0-kilobase (kb) *Eco*RI fragment spanning the promoter region of the yeast hsp70 gene *SSA1* (27) cloned into pUC19; lanes 9 and 10, 50- and 100-fold molar excess, respectively, of plasmid pNK-DDRA2 containing a 1.45-kb *Hin*dIII fragment (14) that includes the entire *DDRA2* gene cloned into pUC19; lanes 11 and 12, 80- and 170-fold molar excess of plasmid pUC19, respectively. Protein–HSE complexes are indicated by an arrow. The unbound HSE oligonucleotide (not shown) migrated at the bottom of the gel. The total amount of competitor DNA was maintained at 3.1 μg by addition of pUC19 vector DNA.

complex was due to binding of HSTF to the HSE2-26 element was shown by using the related oligonucleotide HSE2-26M as competitor. This oligonucleotide contains two base substitutions within the HSTF binding site (Table 1) and does not act as a HSE *in vivo* or as a binding site for HSTF *in vitro* (25). As shown in Fig. 6, this oligonucleotide did not inhibit binding to the labeled HSE2-26, even when present at a 1000-fold excess, a result demonstrating that the complexes observed in the gel retardation assay were due to sequence-specific binding of HSTF to these DNAs.

As shown in Fig. 6, unlabeled oligo31/32 did not inhibit HSTF binding to the HSE-26 probe even when this competitor was present at a 1000-fold molar excess. These results demonstrate that the oligo31/32 element was unable to bind HSTF *in vitro* and argue that the ability of this sequence to confer thermal inducibility *in vivo* is not due to binding of HSTF in the *DDRA2* promoter (see *Discussion*).

Binding of HSTF to the HSE2-26 oligonucleotide was competitively inhibited strongly by the HSE oligonucleotide. A 50% reduction in the amount of HSE2-26·HSTF complexes was observed using a 2-fold molar excess of HSE, whereas a 9-fold excess of HSE2-26 was needed to achieve a comparable reduction.

DISCUSSION

Our molecular characterization of the DDR genes, which are transcriptionally activated in *S. cerevisiae* following exposure to DNA-damaging agents, indicated that at least three of these genes (*DDRA2*, *DDR48*, and *UBI4*) were induced by thermal stress. DNA sequence and deletion analysis of the *DDRA2* gene demonstrated that the promoter region did not contain sequences related to the consensus HSE identified in the promoter regions of heat shock genes from several eukaryotes.

Fusions of the *DDRA2* promoter to the *CYC1-lacZ* reporter gene indicated that sequences between −202 and −165 bp from the start of *DDRA2* translation were necessary for heat shock induction of transcription. A 51-bp oligonucleotide corresponding to this sequence was synthesized and inserted into the pCTΔ and pCZΔ expression vectors. This oligonucleotide, designated oligo31/32, stimulated basal expression of *CYC1-lacZ* and conferred a level of heat shock induction that was comparable to that of plasmid pCTHSE, containing multiple copies of the heat shock consensus element. These results demonstrate that the oligo31/32 fragment contained one or more elements that activate transcription following heat stress.

Using a gel retardation assay we investigated whether the oligo31/32 oligonucleotide contained a binding site for HSTF. DNA sequence of this region revealed no similarity to the well-characterized HSEs found in the upstream region of heat shock genes. Nevertheless, it was possible that oligo31/

Table 1. Oligonucleotides used for promoter fusion and gel retardation studies

Oligonucleotide	Sequence	Source or ref.
oligo31/32*	aaTTCTTTTCCCCTGTTTCCATTTTTGTCTTTTCTCACCCCTTATGGGGAC	This work
HSE†	aaTTCTAGAACGTTCTAGAAGCTTCGAGAc	13
HSE2-26‡	tcgaTTTTCCAGAACGTTCCATCGGC	25
HSE2-26M§	tcgaTTTTCCAAAACGTTTCATCGGC	25
	Heat shock element consensus: CnnGAAnnTTCnnG	

Lowercase letters in the sequence identify nucleotides added for cloning into pCT and pCZ vectors.
*Complementary strand contained sequence tcga at 5′ end.
†This HSE sequence is identical to that described by Wiederrecht *et al.* (13) except for the different linker nucleotides at the termini.
‡The 5′ nucleotides tcga were present in the oligonucleotide used by Park and Craig (25).
§Base substitutions are underlined.

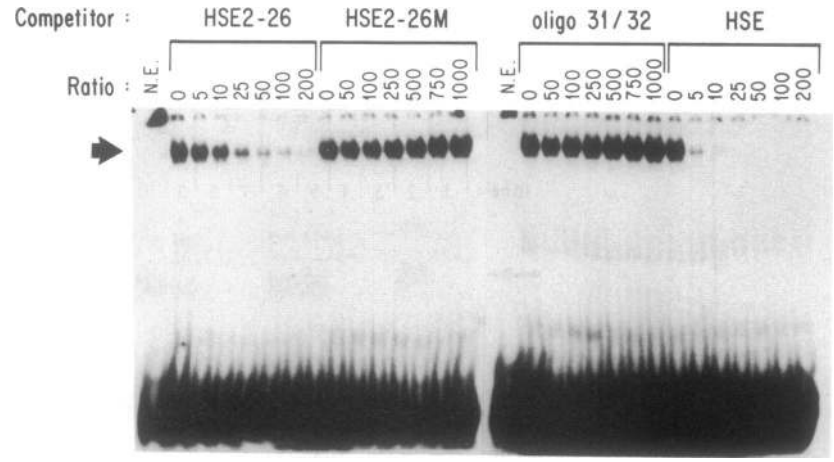


FIG. 6. HSTF binding competition studies with HSE2-26. The 26-bp double-stranded oligonucleotide HSE2-26 of *SSA1* was radiolabeled and used as a probe in gel retardation assays. The number above each lane corresponds to the molar ratio of competitor to the HSE2-26 probe. The sequences of the HSE2-26, HSE2-26M, oligo31/32, and HSE oligonucleotide are given in Table 1. N.E., no extract. The arrow indicates the position of HSTF–HSE2-26 complexes. The total amount of DNA in each reaction was maintained at 400 ng by addition of pUC19 DNA.

32 contained a binding site for HSTF that was distinct from the HSE consensus. Recently it has been shown that another transcription factor, HAP1, regulates transcription of the cytochrome *c* genes, *CYC1* and *CYC7*, by binding with comparable affinity to distinct and unrelated promoter elements of these genes (29). As shown in Fig. 5, the oligo31/32 oligonucleotide failed to inhibit binding of HSTF to the HSE-containing oligonucleotide even at a 250-fold molar excess. Similarly, a *Hin*dIII fragment containing the entire *DDRA2* upstream region did not inhibit binding. Binding of HSTF to the synthetic HSE-containing oligonucleotide was inhibited effectively by an *Eco*RI restriction fragment containing a natural HSE from the *SSA1* promoter. Furthermore, the oligo31/32 failed to inhibit binding of HSTF to the HSE2-26 element even at a 1000-fold molar excess (Fig. 6). Although we cannot exclude the possibility that HSTF binds extremely weakly to oligo31/32, such weak binding is unlikely to account for the heat shock inducibility of the *CYC1-lacZ* reporter gene.

In the experiment shown in Figs. 5 and 6, the HSTF-containing fractions were prepared from nonstressed cells. We have also found that oligo31/32 cannot competitively inhibit binding of the more highly phosphorylated form of HSTF from heat shocked cells to the HSE oligonucleotide (data not shown). Taken together these results suggest that transcription of the *DDRA2* gene in *S. cerevisiae* is activated by a HSTF-independent mechanism in response to heat stress.

In *E. coli*, the heat shock response requires the positive regulatory protein σ^{32}, the product of the *rpoH* gene. Recently another σ factor, σ^{E}, has been identified that is required for transcription of the *rpoH* and *htrA* (*degP*) genes at temperatures above 42°C (30, 31). Thus, in *E. coli* there are at least two distinct transcriptional regulators responsible for gene expression at elevated temperatures. By analogy with *E. coli* and based upon the results presented in this work, we propose that yeast cells and possibly other eukaryotes encode additional transcription activator proteins, distinct from HSTF, that regulate expression of different sets of heat stress responsive genes. We have recently identified a 15-bp subelement of oligo31/32 that is responsible for the heat shock regulation of transcription of *DDRA2*. A homologous element, found in the upstream region of the polyubiquitin gene of yeast, *UBI4*, is able to confer thermal induction of a *CYC1-lacZ* reporter gene (N.K., unpublished results). The role of this regulatory element in transcription of other stress-regulated genes must be examined.

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