MOLECULAR AND CELLULAR BIOLOGY, Jan. 1993, p. 248–256
0270-7306/93/010248-09$02.00/0

Vol. 13, No. 1

Identification of *cis* and *trans* Components of a Novel Heat Shock Stress Regulatory Pathway in *Saccharomyces cerevisiae*

NAOKO KOBAYASHI† AND KEVIN MCENTEE*

Department of Biological Chemistry and Laboratory of Biomedical and Environmental Sciences, University of California Los Angeles School of Medicine, 900 Veteran Avenue, Los Angeles, California 90024

Received 23 June 1992/Returned for modification 2 September 1992/Accepted 17 September 1992

The stress-responsive *DDR2* gene (previously called *DDRA2*) of *Saccharomyces cerevisiae* is transcribed at elevated levels following stress caused by heat shock or DNA damage. Previously, we identified a 51-bp promoter fragment, oligo31/32, which conferred heat shock inducibility on the heterologous *CYC1-lacZ* reporter gene in *S. cerevisiae* (N. Kobayashi and K. McEntee, Proc. Natl. Acad. Sci. USA 87:6550–6554, 1990). Using a series of synthetic oligonucleotides, we have identified a pentanucleotide, CCCCT (C_4T), as an essential component of this stress response sequence. This element is not a binding site for the well-characterized heat shock transcription factor which recognizes a distinct *cis*-acting heat shock element in the promoters of many heat shock genes. Here we demonstrate the ability of oligonucleotides containing the C_4T sequence to confer heat shock inducibility on the reporter gene and show that the presence of two such elements produces more than additive effects on induction. Gel retardation experiments have been used to demonstrate specific complex formation between C_4T-containing fragments and one or more yeast proteins. Formation of these complexes was not competed by fragments containing mutations in the C_4T sequence nor by heat shock element-containing competitor DNAs. Fragments containing the C_4T element bound to a single 140-kDa polypeptide, distinct from heat shock transcription factors in yeast crude extracts. These experiments identify key *cis*- and *trans*-acting components of a novel heat shock stress response pathway in *S. cerevisiae*.

In eukaryotes, many of the fundamental features of gene activation by heat shock appear to have been conserved. Heat shock genes from organisms as diverse as *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and vertebrates contain a specific DNA sequence, the heat shock element (HSE), which serves as a binding site for the heat shock transcription factor (HSF). The consensus HSE was initially identified as a palindromic sequence, CnnGAAnnTTCnnG, where n is any nucleotide (14). More recently, however, the HSE has been shown to consist of repeats of the 5-bp module nGAAn arranged in alternating orientations (1, 15, 31). HSFs have been purified from *S. cerevisiae*, *D. melanogaster*, and human cells (4, 22, 27, 30), and their structural genes have been cloned from *S. cerevisiae* and *D. melanogaster* (2, 23, 26). Recently, multiple HSF genes have been isolated from tomato, human, and mouse cells (17–20). The mechanism by which HSF activates transcription likely involves structural changes and/or modification of the HSF polypeptide, but the detailed features appear to be fundamentally different in yeasts and in higher eukaryotes. In *S. cerevisiae*, HSF binds constitutively to the HSE and appears to undergo phosphorylation immediately following thermal stress. It has been proposed that the degree of phosphorylation correlates with transcriptional activation of HSF (21, 23). In contrast, HSE-binding activity is present only after heat shock or other stresses in HeLa cells and *Drosophila* tissue culture cells, and activation does not require protein synthesis, suggesting that conversion of HSF from the non-DNA-binding to the DNA-binding configuration is crucial for transcriptional activation of heat shock genes (29, 32).

We recently demonstrated that a 51-bp sequence (oligo31/32) of the *DDR2* upstream region contained a *cis*-acting regulatory element which conferred heat shock inducibility on the reporter gene, *CYC1-lacZ* (6). The level of β-galactosidase induction by thermal stress was comparable to that produced by multiple HSEs inserted upstream of the reporter gene in analogous promoter fusion constructs. However, oligo31/32 does not share significant sequence homology with HSE, except for a repeated TTC trinucleotide. Furthermore, quantitative competitive gel retardation assays demonstrated that oligo31/32 does not compete with binding of HSF to HSE. These results suggested that a distinct stress response pathway was responsible for heat shock induction of *DDR2* expression.

In this report, we further define the thermal stress upstream activating sequence (UAS) and show that it contains the pentanucleotide CCCCT (C_4T). This new thermal stress-responsive element has been designated TRS. The C_4T element is repeated within oligo31/32, and two copies of the TRS were shown to activate transcription synergistically upon heat shock treatment. In vitro, fragments containing the TRS form specific complexes with one or more yeast proteins, and these proteins do not bind DNA fragments containing alterations in the C_4T sequence. Our results demonstrate that the major protein which recognizes the TRS-containing fragments is a polypeptide having an apparent molecular mass of 140 kDa. These results suggest that a novel transcription factor distinct from HSF regulates ex-

* Corresponding author.
† Present address: Department of Microbiology and Molecular Genetics, University of California Los Angeles, Los Angeles, CA 90024.

TABLE 1. Oligonucleotides used for *DDR2* promoter analysis

Oligonucleotide	Length (bp)	Sequence[a]
Oligo31/32	51	aaTTCTTTTCCCCTGTTTCCATTTTTGTCTTTTCTCACCCCTTATGGGGAC
A	34	aaTTCTTTTCCCCTGTTTCCATTTTTGTCTTTTC
B	31	aattcTGTCTTTTCTCACCCCTTATGGGGAC
C	14	aattcTGTCTTTTC
D	20	tcgagACCCCTTATGGGGAC
H	12	aattcTCCCCTc
I	14	aattcGTTTCCATc
J	13	aattcACCCCTTc
K	14	aattcATGGGGTCc
HSE	30	aaTTCTAGAACGTTCTAGAAGCTTCGAGAc
HSE-26	26	tcgaTTTTCCAGAACGTTCCATCGGC

[a] Lowercase letters in the sequences identify nucleotides added for cloning into pCZΔ expression vector.

pression of *DDR2*, and possibly other genes of *S. cerevisiae*, in response to heat shock and other stresses.

MATERIALS AND METHODS

Strains, growth conditions, β-galactosidase assays, and extract preparation. *Saccharomyces cerevisiae* M12B (α *trp 1-289 ura3-52 gal2*) was used for transformation with recombinant plasmids and determination of β-galactosidase activity as previously described (6). For preparation of proteins for Southwestern and gel retardation experiments, yeast whole-cell extracts were prepared from strain BJ2168 (**a** *leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2*) according to published procedures (22). To prepare crude protein extracts from heat shock-treated cultures of strain BJ2168, cells were grown in 1 liter of YPD medium at room temperature to late log phase, 500 ml of fresh YPD medium prewarmed to 61°C was added to rapidly raise the temperature to 37°C, and incubation was continued at 37°C for an additional 30 min. Cells were harvested by centrifugation, and extracts were prepared as described elsewhere (6).

Construction of plasmids. Cloning of double-stranded synthetic oligonucleotides into expression vector pCZΔ (8) was performed as described elsewhere (6). Oligonucleotides used in this study (Table 1) were synthesized on a DuPont Generator or Applied Biosystems 391 PCR Mate DNA synthesizer. All synthetic oligonucleotides except oligonucleotide D were constructed with *Eco*RI and *Xho*I restriction sites at 5′ and 3′ ends, respectively, and cloned into pCZΔ, which had been digested with *Eco*RI and *Xho*I. Oligonucleotide D, containing *Xho*I sites at both ends, was cloned into vectors pCZΔ and pCZ-A, which had both been restricted with *Xho*I and dephosphorylated at their 5′ ends by using bacterial alkaline phosphatase. The sequences of the promoter fusions were confirmed by double-stranded dideoxynucleotide sequencing using Sequenase version 2.0 (U.S. Biochemical), and compression at GC regions was resolved by using 7-deaza-dGTP (Pharmacia).

Gel retardation assay. Competition gel retardation analysis was performed as previously described (6). The binding reaction mixture contained 65 μg of yeast whole-cell extract, 1 μg of poly(dI-dC) · poly(dI-dC), 20 mM HEPES-KOH (*N*-2-hydroxyethylpiperazine-*N*, *N*′-ethanesulfonic acid; pH 8) 1 mM $MgCl_2$, 60 mM KCl, 1 mM diethiothreitol (DTT), 0.1% Nonidet P-40, 12% glycerol, and 0.2 ng (10,000 cpm) of DNA probe in a total volume of 25 μl.

DNA probes. The two complementary synthetic oligonucleotides were annealed and isolated from polyacrylamide gels as described previously (5, 9). The purified duplex fragments were phosphorylated by using T4 polynucleotide kinase in buffer containing 50 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, and 0.5 mg of bovine serum albumin per ml. Concatemers were prepared by incubating the fragment with T4 DNA ligase in the same buffer overnight at 16°C. The DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated in absolute ethanol at −20°C. Concatemers with mean lengths of 6 to 10 repeats were produced in this way. The multimeric DNAs were radiolabeled by end-filling with the Klenow fragment of *Escherichia coli* DNA polymerase I and [α-^{32}P]dATP (6,000 Ci/mmol) and then were separated from unincorporated nucleotides by gel filtration through a G-50 Sephadex column as described elsewhere (9). DNA probes prepared in this way had specific activities in the range of 7×10^7 to 25×10^7 cpm/μg of DNA.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, transfer, and DNA probing of immobilized proteins (Southwestern assay). Yeast whole-cell extracts were prepared from unstressed and heat shock-treated cells as described previously (22). Extracts (250 μg) were incubated for 5 min at 95°C with an equal volume of loading buffer (2% SDS, 20% glycerol, 60 mM Tris-HCl [pH 6.8], 200 mM DTT, 0.001% bromophenol blue) and loaded onto an SDS–5% polyacrylamide gel (40:1 acrylamide:bisacrylamide), and the proteins were fractionated by electrophoresis. Amersham rainbow markers were used as molecular weight standards. Proteins were electrophoretically transferred onto a nitrocellulose membrane in buffer containing 25 mM Tris–190 mM glycine (pH 8.3) in a Bio-Rad TRANSBLOT apparatus. The probing of nitrocellulose-bound proteins with radiolabeled concatenated oligonucleotides was done by the method of Vinson et al. (24), with slight modifications. All subsequent procedures were carried out at 4°C. Filters were rinsed in binding buffer (25 mM HEPES [pH 8], 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT) for 5 min, denatured in 6 M guanidine hydrochloride in binding buffer for 10 min, and then renatured in a series of washes (5 min each) in five successive 1:1 dilutions of the guanidine hydrochloride solution in binding buffer. The filters were washed twice (5 min each) with binding buffer alone and blocked by incubation in binding buffer containing 5% nonfat dry milk (Carnation) for 30 min. The probe was added to the binding buffer containing 2.5% dry milk at a final specific activity of 1×10^6 to 2×10^6 cpm/ml. Filters were incubated with end-labeled probe for approximately 2 to 3 h, washed with four changes of binding buffer containing 0.25% dry milk for 20 to 30 min, dried in air, and placed under film (DuPont Cronex) overnight at −70°C with an intensifying screen for autoradiography.

Expression of HSF in *E. coli* and preparation of bacterial extracts. *E. coli* AR68 containing either the HSF expression plasmid pOTSNco12-8* or its derivative p2650 (26) were grown in Luria broth containing 100 μg of ampicillin per ml at 30°C to an A_{595} of 0.6. The culture was quickly transferred to a 43°C water bath and shaken for 25 min. The temperature was reduced to 37°C, and the cultures were incubated for an additional 60 min. The cells were pelleted, resuspended in extraction buffer (50 mM Tris-Cl [pH 7.5], 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride) and quickly frozen in dry ice-ethanol. The frozen cells were rapidly thawed, lysozyme was added to a final concentration of 0.5 mg/ml, and the suspension was incubated on ice for 15 min. Following lysis, NaCl was added to a concentration of 1 M and the lysate was incubated on ice for 15 min and then centrifuged for 30 min in a Microfuge. The cleared lysate was dialyzed against extraction buffer and used for Southwestern experiments as described by Weiderrecht et al. (26).

RESULTS

Oligo31/21 contains two elements which confer heat shock induction. In order to further delineate the sequences responsible for heat stress induction in the *DDR2* promoter, double-stranded oligonucleotides corresponding to different portions of oligo31/32 were cloned into promoter fusion vector pCZΔ (see Materials and Methods) and the β-galactosidase activities of the transformants were measured before and after heat shock treatment.

The analysis of several 3′ deletions of the *DDR2* promoter cloned into the *CYC1-lacZ* reporter vector demonstrated that a sequence which was necessary and sufficient for transcriptional activation by heat shock was located between −212 (d32) and −165 (d31) relative to the start of *DDR2* translation. The results obtained from both 5′ and 3′ deletion analyses further argued that the important regulatory element(s) was located between −187 (d19) and −165 (d31) (6). Oligonucleotide B (Table 1 and Fig. 1A) encoding the sequence between these end points was cloned in the *CYC1-lacZ* promoter fusion vector pCZΔ to yield plasmid pCZ-B. The level of β-galactosidase activity was induced 4.9-fold when yeast cells containing pCZ-B were exposed to 37°C for 60 min (Fig. 1B).

Interestingly, oligonucleotide A, corresponding to sequences between −212 and −180 (Fig. 1A), also conferred heat shock-inducible expression of β-galactosidase activity (Fig. 1B), and the amount of β-galactosidase activity in cells containing plasmids pCZ-A following heat shock treatment (20 U) was comparable to that in cells containing plasmid pCZ-B (21 U). The induction ratio for pCZ-A (2.4-fold) was lower than that for the pCZ-B plasmid (4.9-fold) because of the higher basal-level expression in cells containing plasmid pCZ-A. This result suggested either that the overlapping 8-bp region of oligonucleotide A and oligonucleotide B contained the regulatory element or that two or more heat shock-inducible regulatory elements were present within oligo31/32.

The sequence of oligonucleotide B was further divided into oligonucleotide C (9 bp) and oligonucleotide D (15 bp) (Fig. 1A). Oligonucleotide C, containing sequences corresponding to the overlapping region between oligonucleotides A and B, did not contain measurable UAS activity and was unable to confer heat shock-inducible expression when placed upstream of the *CYC1-lacZ* reporter gene (Fig. 1B). Cells containing plasmid pCZ-D showed a threefold heat shock induction of β-galactosidase activity, indicating that oligonucleotide D, as well as oligonucleotide A, contained elements which responded to thermal stress. The induction ratio of β-galactosidase activity determined for cells containing plasmid pCZ-D (3.0-fold) was slightly lower than that seen in cells containing plasmid pCZ-B. However, in cells transformed with plasmid pCZ-DD (containing two direct repeats of oligonucleotide D), β-galactosidase activity increased to 77 U (9.8-fold induction) after heat shock treatment, more than six times the level measured in cells containing the pCZ-D plasmid. Taken together, these results argued that the 15-bp fragment D contained UAS activity that conferred heat shock-inducible expression to the reporter gene and that this UAS functioned synergistically when another copy of this element was placed in direct repeat.

The transcription-activating sequence located within oligonucleotide D also functioned in a reverse orientation. Plasmids pCZ-D^R and pCZ-D^RD^R contained one and two copies, respectively, of oligonucleotide D in the reverse orientation (Fig. 1A). Cells transformed with pCZ-D^R and pCZ-D^RD^R showed 3.1- and 10.7-fold induction of β-galactosidase activity, respectively, following heat shock treatment (Fig. 1B). These induction ratios and the induced levels of β-galactosidase activity were comparable to those found for the pCZ-D and pCZ-DD plasmids, respectively.

Oligonucleotides A and D were inserted into vector pCZΔ to prepare plasmid pCZ-AD. The relative order and orientation of the two fragments in pCZ-AD were identical to those of the original oligo31/32 (Fig. 1A). Cells containing plasmid pCZ-AD expressed 76 U (12.7-fold induction) of β-galactosidase activity after heat shock treatment (Fig. 1B). This level of induction was significantly greater than the sum of the activities produced by oligonucleotides A and D inserted individually into the pCZΔ vector.

The above results indicated that both oligonucleotides A and D contained UAS activity but that heat shock induction measured in cells containing either plasmid pCZ-A or pCZ-D was significantly lower than induction in cells containing plasmid pCZoligo31/32. By placing both oligonucleotide A and oligonucleotide D in one promoter, a heat shock induction ratio similar to that of cells containing pCZoligo31/32 was obtained. Therefore, heat shock induction of β-galactosidase expression conferred by the oligo31/32 in promoter fusion vector pCZΔ appeared to be due to the contribution of two separate activating elements, one located in region A and the other in region D of oligo31/32.

A 7-bp half-site within fragment D is sufficient for heat shock induction of transcription. Analysis of the sequence of oligo31/32 revealed that it contained two types of short repeated elements which were located within oligonucleotides A and D (Fig. 2A). Regions J and K′ are palindromic sequences comprising fragment D. Moreover, the H element within fragment A contains a sequence homologous to regions J and K′. Additionally, sequence I, which is located within fragment A, and sequence K, which is located within fragment D, are palindromic sequences which share homology at six of eight nucleotides. To test whether these repeated sequences function as upstream elements which confer heat shock inducibility, double-stranded synthetic oligonucleotides encoding each repeat were cloned into the pCZΔ vector. As shown in Fig. 2B, cells containing plasmid pCZ-J showed a 3.2-fold induction of β-galactosidase activity following heat shock treatment, which was similar to the induction ratio observed with cells containing the pCZ-D plasmid. Cells containing plasmid pCZ-I and cells containing plasmid pCZ-K repeatedly showed little induction of β-gal-

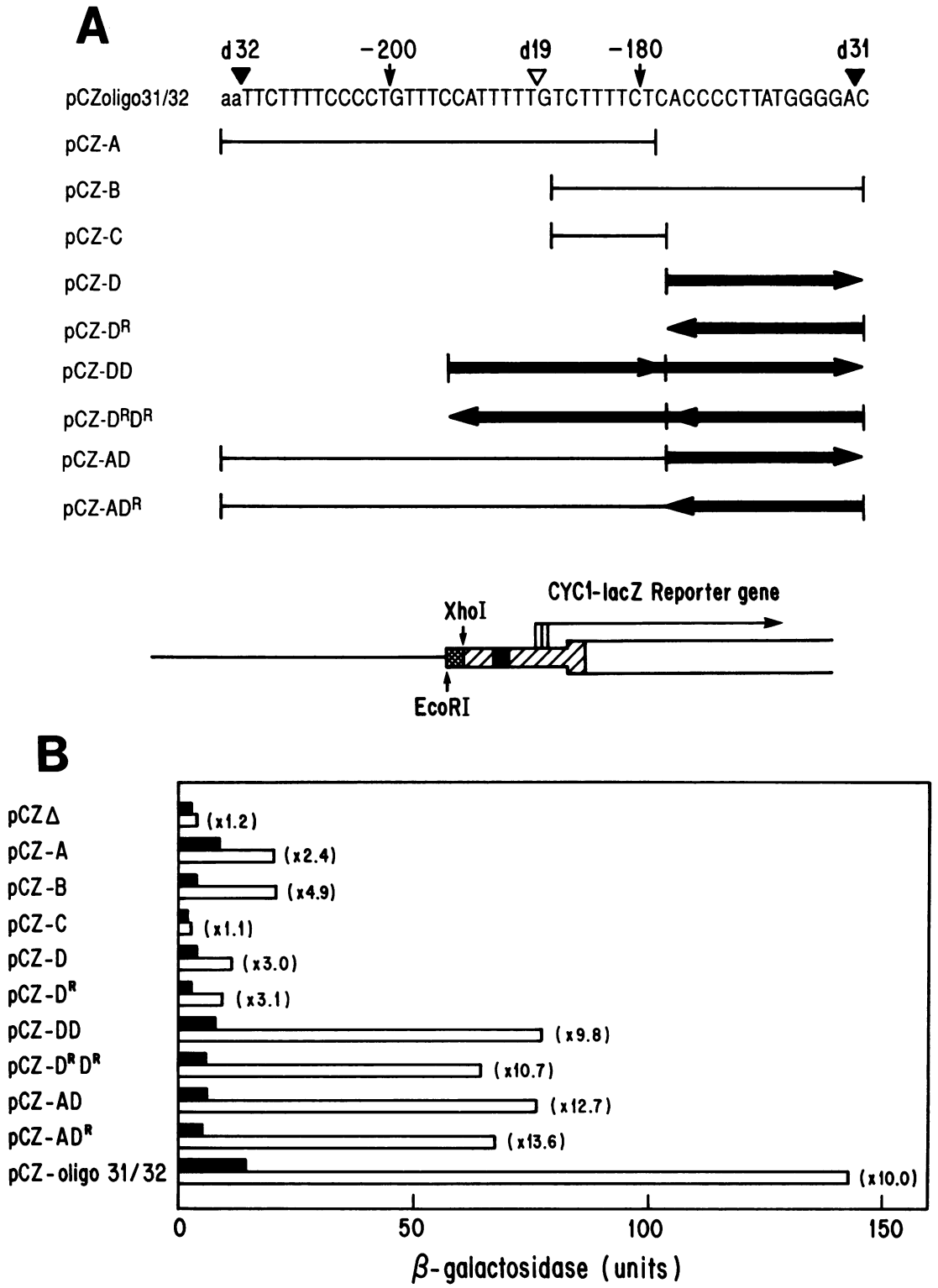

FIG. 1. Heat shock induction of *CYC1-lacZ* promoter fusions containing oligonucleotides from the oligo31/32 region of the *DDR2* promoter. (A) The nucleotide sequence of oligo31/32 is presented on the top line along with the deletion endpoint of 5′ deletion d19 (open triangle) and the deletion endpoint of 3′ deletion d31 (filled triangle). The fragments cloned into the *CYC1-lacZ* promoter fusion vector pCZΔ are indicated below. Heavy arrows correspond to the oligonucleotide D fragment, and the direction of the arrow indicates the orientation of the fragment in the vector; i.e., in the pCZ-D construct oligonucleotide D is in the same orientation as it is in oligo31/32, whereas in pCZ-D^R the oligonucleotide is in the opposite orientation. At the bottom is a representation of the pCZΔ fusion vector showing *Eco*RI and *Xho*I restriction sites within the polylinker (6). The striped box represents *CYC1* sequences, and the filled box corresponds to the TATA element. (B) Derivatives of the pCZΔ plasmid containing oligonucleotides corresponding to *DDR2* upstream regulatory sequences were transformed into yeast strain M12B. The β-galactosidase activity was determined for transformants grown at 23°C (control [filled bars]) and for transformants subjected to heat shock at 37°C for 60 min (open bars). Induction ratios are given in parentheses. The β-galactosidase activities reported are the average values of two or more samples obtained from each of at least two independent transformants.

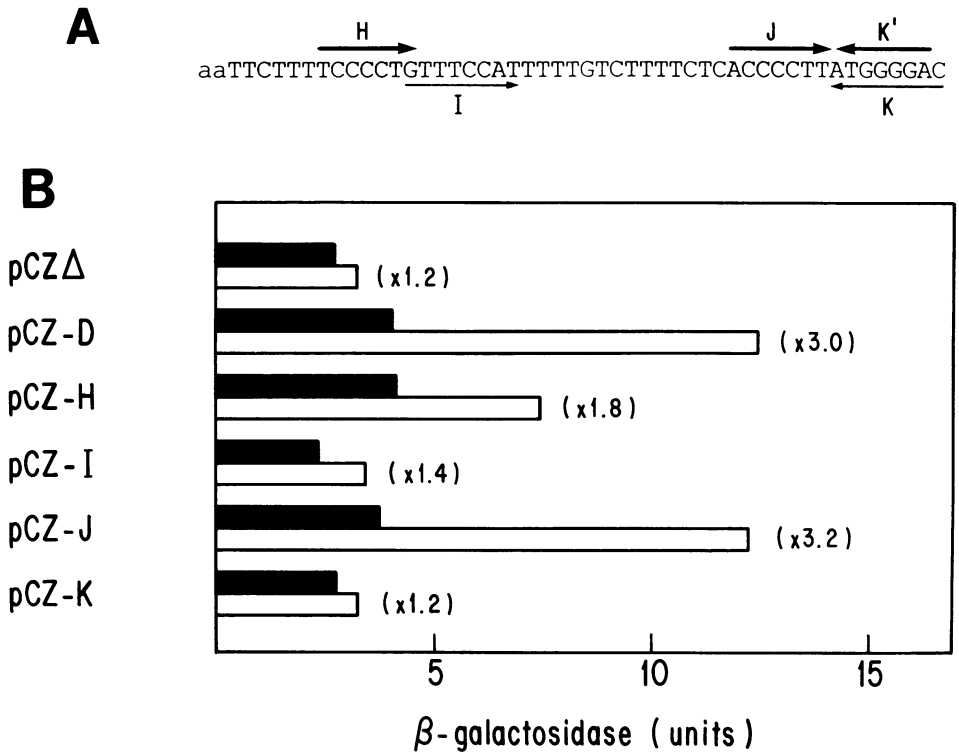

FIG. 2. Short sequence elements within oligo31/32 confer heat shock regulation to a *CYC1-lacZ* promoter fusion. (A) Computer analysis of oligo31/32 DNA was used to identify repeats (shown by arrows). Sequences presented by thick arrows (J, K', and H) share homology at five of six nucleotides. Sequences I and K (thin arrows) contain the motif 5'GTnnCCAT3' (where n stands for any nucleotide). (B) Double-stranded synthetic oligonucleotides corresponding to the repeated elements within oligo31/32 (H, I, J, and K) were cloned into the *CYC1-lacZ* reporter plasmid pCZΔ and tested for heat shock-inducible expression of β-galactosidase. Filled bars represent β-galactosidase activities of control cells grown at 23°C. Open bars represent β-galactosidase activities of cells incubated at 37°C for 60 min. Induction ratios are given in parentheses. β-Galactosidase activities are averages of two or more samples obtained from two independent transformants.

actosidase activity; these induced enzyme levels were significantly lower than those observed in cells containing the pCZ-J plasmid (12 U) and were comparable to levels obtained with pCZΔ. Although cells containing the pCZ-H plasmid showed a modest increase in β-galactosidase activity following heat shock (1.8-fold), we observed that multiple insertions of the H oligonucleotide resulted in high levels of β-galactosidase induction which were comparable to those produced by cells containing the complete oligo31/32 fragment (data not shown). These results indicated that the H fragment likely contained a stress-regulatory element.

These results demonstrated that oligonucleotides H and J, each containing only 7 bp of *DDR2* sequence, were sufficient to confer heat shock-inducible expression to the heterologous promoter. Oligonucleotides J and H share a pentanucleotide sequence, CCCCT (C_4T), which appears to be an essential component of the heat shock-inducible UAS. We designate this new element containing the C_4T sequence a thermal stress-responsive sequence or TRS.

Mutations in the C_4T sequence abolish heat stress-inducible expression of β-galactosidase. A derivative of oligonucleotide A in which the C_4T sequence was changed to A_4G was prepared. The oligonucleotide containing this alteration, designated oligonucleotide AM (Fig. 3), was ligated into the pCZΔ expression vector. Similarly, oligonucleotide BM, in which the C_4T element within the B fragment was changed to A_4G, was prepared, and this oligonucleotide was also inserted into the pCZΔ expression vector. As shown in Fig. 3, there was little or no increase in β-galactosidase activity after heat shock treatment of cells containing either plasmid pCZ-AM or plasmid pCZ-BM. These results further argue that the C_4T element is required for heat shock stress induction of the *CYC1-lacZ* reporter gene and, by inference, of the *DDR2* gene itself.

Specific binding of yeast proteins to promoter fragments containing the C_4T motif. The specific binding of one or more yeast proteins to oligonucleotides spanning the oligo31/32 region was investigated by using a gel retardation assay (see Materials and Methods). As shown in Fig. 4, labeled oligonucleotide B formed a complex when incubated with yeast protein extracts, and this complex was specific as judged by competition with excess unlabeled oligonucleotide B. Moreover, complexes were subjected to competition by adding unlabeled oligo31/32 to the reaction but not by adding a 500-fold molar excess of oligonucleotide BM, which lacked the C_4T sequence. These complexes were also subject to competition by excess unlabeled fragment A but not by fragment AM lacking the TRS element. These results indicated that these complexes are specific and require the C_4T element present in fragments A and B. Figure 4 also shows that complexes with oligonucleotide B were formed efficiently in the presence of a 500-fold molar excess of the HSE2-26 oligonucleotide, corresponding to a functional heat shock element within the promoter of the *SSA1* gene (6, 13). Thus, formation of a specific protein-DNA complex with fragment B was not due to binding of HSF. This latter result is consistent with our earlier demonstration that oligo31/32

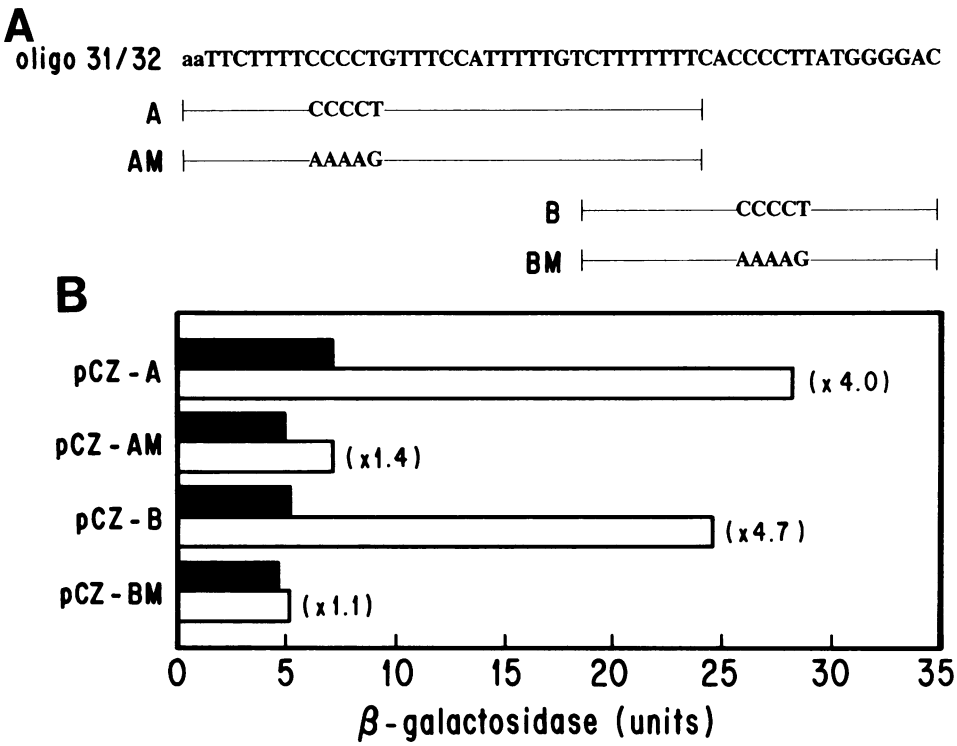

FIG. 3. Alterations of the C_4T motif abolish β-galactosidase induction by heat shock. (A) A derivative of oligonucleotide A was synthesized in which the C_4T pentanucleotide was changed to A_4G, and it was designated oligonucleotide AM. Similarly, the C_4T motif was changed to A_4G in oligonucleotide B. This oligonucleotide was designated oligonucleotide BM. Each was cloned into expression vector pCZΔ as described in Materials and Methods. (B) β-Galactosidase activity measured in strains containing plasmids pCZ-AM and pCZ-BM are indicated for both control (filled bars) and heat shock-treated cells (open bars). The β-galactosidase activities are averages of two experiments performed with two separate transformants.

does not contain a binding site for the "classical" HSF protein (6).

Oligonucleotide D contains a binding site for a 140-kDa protein. Additional evidence that the 15-bp oligonucleotide D contains a specific binding site for yeast proteins was obtained by probing cellular proteins which had been immobilized on nitrocellulose with radiolabeled fragment D. Yeast soluble proteins were separated by electrophoresis in SDS-polyacrylamide gels, transferred to nitrocellulose membranes, renatured in situ, and incubated with radiolabeled, concantenated, double-stranded oligonucleotides (see Materials and Methods). With oligo31/32 as a probe, a single protein band was detected in extracts, and this protein migrated at the position of a polypeptide having an apparent molecular mass of 140 kDa (Fig. 5, lanes 1 and 2). Radiolabeled oligonucleotide D also bound to a protein with identical mobility in polyacrylamide gels (Fig. 5, lanes 3 and 4). This binding activity was present in extracts prepared from both unstressed and heat shock-treated cells.

When the proteins were incubated with a labeled HSE probe, a single protein was detected, and this protein migrated at a position expected of a 150-kDa polypeptide (Fig. 5, lanes 5 and 6). This result was consistent with the previously reported molecular weight of HSF determined by SDS-polyacrylamide gel electrophoresis (22). In order to show that the oligo31/32-binding protein was distinct from HSF, the protein blot was incubated with both labeled HSE and labeled fragment D probes. As can be seen in Fig. 5 (lanes 7 and 8), two binding activities which migrated close to one another in SDS-polyacrylamide gels were detected following autoradiography. On the basis of the relative intensity and mobility of the binding proteins, the upper band corresponds to HSF, while the lower band corresponds to the TRS-binding protein. We estimate that these proteins differ in size by 5 to 10 kDa.

The HSF protein expressed in *E. coli* binds the HSE but not oligo31/32. The Southwestern assay results, together with our previously reported competitive gel retardation data (6), argue that the classical HSF does not bind oligo31/32 and that a distinct protein binds to oligo31/32 in yeast cell extracts. We have further demonstrated that oligo31/32 does not contain an HSF-binding site by expressing this protein in *E. coli* and examining binding by the Southwestern technique. As shown previously (26), full-length HSF protein, as well as the carboxyl-terminal deletion mutant (p2650), expressed in strain AR68 were able to bind to an HSE probe on a Southwestern blot (Fig. 6, lanes 3 and 4). For both the wild-type and deletion proteins, multiple bands were seen either because of protein breakdown or because of premature termination of translation of the HSF protein. In both cases, however, bands corresponding to the full-length translation product were detected. However, incubation of the same extracts with labeled oligo31/32 showed a different (and unexpected) result. This probe identified a single binding activity migrating at the position of a 70-kDa polypeptide. This band does not correspond to any of the HSF fragments and, in fact, was detected in extracts of the parental AR68 strain lacking the cloned HSF gene (Fig. 6, lane 5) as well as in extracts of uninduced cells containing the HSF gene (Fig. 6, lane 6). Thus, this binding activity is

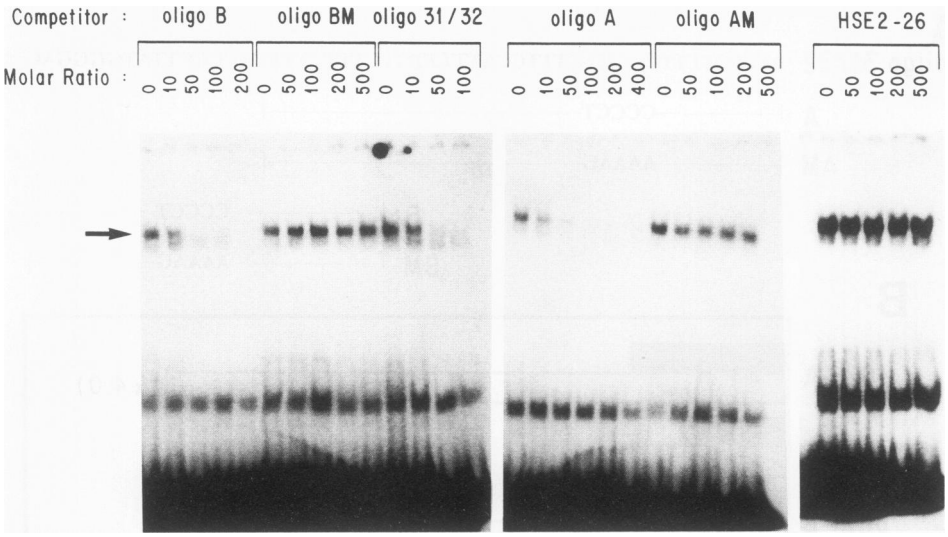

FIG. 4. Specific binding of yeast proteins to oligonucleotides containing the C_4T motif. Labeled oligonucleotide B was prepared by end filling with Klenow fragment as described in Materials and Methods and incubated for 15 min at room temperature with a crude extract prepared from untreated cells as described elsewhere (6). For competition experiments, the indicated amount of unlabeled competitor DNA (expressed as a molar ratio) was incubated with yeast whole-cell extract for 15 min at room temperature prior to addition of the labeled fragment. The arrow indicates the position of the specific protein-DNA complex. The faster-migrating band was not subject to competition and was composed of nonspecific DNA-binding proteins. The sequences of the oligonucleotides are given in Table 1 and Fig. 3. The unbound labeled oligonucleotide probe migrated at the bottom of the gels.

an *E. coli* protein. We do not know whether the C_4T motif is recognized by this bacterial factor or whether the factor recognizes a distinct sequence element within oligo31/32. These results, however, further demonstrate that *S. cerevisiae* HSF does not recognize sequences within the oligo31/32 promoter element and that the 140-kDa protein is a novel binding factor distinct from HSF.

DISCUSSION

In this report, we have described *cis* and *trans* components of a novel heat stress regulatory pathway in *S. cerevisiae*. The identification of these elements resulted from a molecular analysis of the yeast *DDR2* gene. Transcription of this gene increases more than 20-fold following brief heat shock or exposure to a variety of DNA-damaging agents (eg, UV irradiation, alkylating agents, etc.). Previously, we demonstrated that a 51-bp oligonucleotide corresponding to a region upstream of the TATA elements in the *DDR2* promoter contained all the sequences that were necessary and sufficient for heat shock induction of a *CYC1-lacZ* reporter gene (6). We also demonstrated that oligo31/32 did not bind the well-characterized heat shock transcription factor (HSF) as judged by competitive gel retardation experiments using labeled HSE-containing DNAs. By further dissecting oligo31/32, we have shown that this oligonucleotide contains two elements which are capable of conferring heat shock induction when placed in front of the *CYC1-lacZ* reporter. Oligonucleotide A and nonoverlapping oligonucleotide D each conferred approximately two- to threefold inducibility when placed upstream of the *CYC1-lacZ* reporter. Combining the two fragments produced a more than 12-fold increase in expression after heat shock. Significantly, insertion of two copies of oligonucleotide D in either orientation led to a 9- to 11-fold increase in β-galactosidase expression after heat shock treatment. These results demonstrate the presence of two transcriptional regulatory elements in oligo31/32, each of which can function independently but which evoke a more than additive response when combined upstream of the TATA box. This behavior suggests that interaction between these two elements is important for optimal induction of *DDR2* transcription following heat shock stress.

Sequence inspection of oligonucleotides A and D revealed

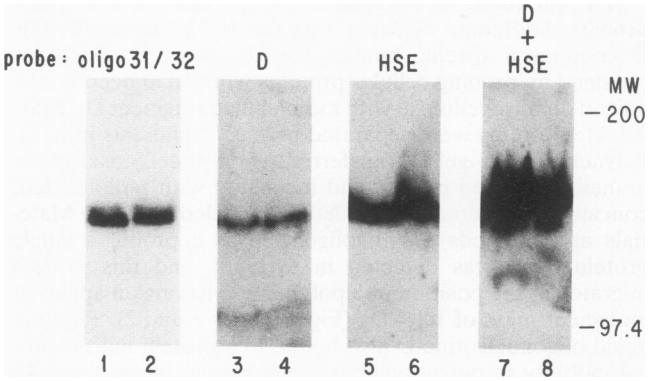

FIG. 5. Specific DNA binding by a protein in whole-cell extract of *S. cerevisiae*. Yeast whole-cell extracts were fractionated by electrophoresis in a 5% polyacrylamide gel containing SDS, transferred to nitrocellulose, renatured after guanidine hydrochloride treatment, and hybridized with radiolabeled concatenated oligonucleotide. Lanes: 1 and 2, hybridization with oligo31/32; 3 and 4, hybridization with oligonucleotide D; 5 and 6, hybridization with HSE; 7 and 8, hybridization with both oligonucleotide D and HSE. The sequences of oligonucleotides used as probes are listed in Table 1. Oligonucleotide HSE contains multiple overlapping HSEs. Lanes 1, 3, 5, and 7 contained 250 μg of protein prepared from control cells, and lanes 2, 4, 6, and 8 contained 250 μg of extract prepared from heat shock-treated cells (see Materials and Methods). The positions of molecular weight (MW) standards are indicated.

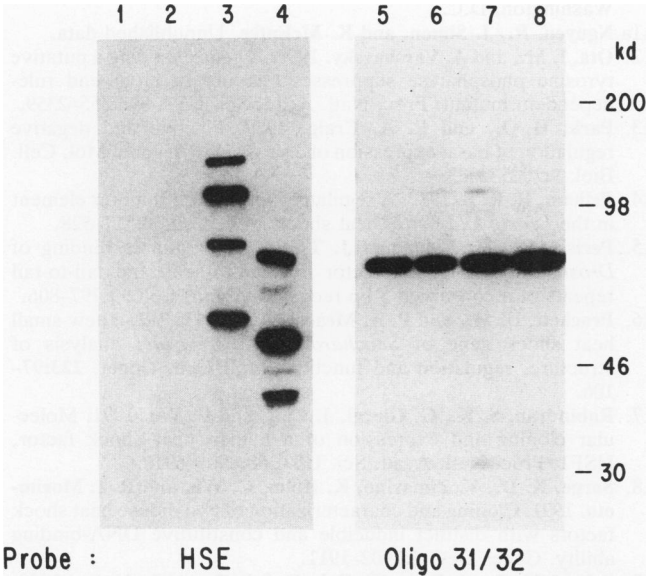

FIG. 6. HSF expressed in *E. coli* binds HSE but not oligo31/32. Expression of HSF was induced in cells by incubating them at 43°C, and extracts were prepared and fractionated through a 7.5% polyacrylamide gel as described in Materials and Methods. Following transfer to nitrocellulose and renaturation, the filters were hybridized with a labeled HSE probe (lanes 1 through 4) or with labeled oligo31/32 (lanes 5 through 8). The positions of molecular mass standards in kilodaltons (kd) are indicated at right. Lanes: 1 and 5, *E. coli* AR68 without HSF clone; 2 and 6, uninduced strain AR68 containing plasmid pOTSNco12-8*, corresponding to the full-length HSF clone (26); 3 and 7, strain AR68 containing plasmid pOTSNco12-8* induced at 43°C; 4 and 8, strain AR68 containing plasmid p2650 encoding a truncated HSF and induced at 43°C.

two types of repeat elements that were candidates for the stress response element. By preparing smaller oligonucleotides and inserting them in front of *CYC1-lacZ*, we determined that only those oligonucleotides containing the pentanucleotide sequence C_4T could evoke a stress response. Moreover, by introducing base changes within the C_4T element we abolished the ability of these oligonucleotides to confer heat shock induction on the reporter gene.

The gel retardation experiments shown in Fig. 4 demonstrated the presence of one or more proteins in *S. cerevisiae* which bind to oligonucleotides A and B. These complexes likely represent functionally important interactions, since they are not bound by oligonucleotides in which the C_4T motif has been mutationally altered (oligonucleotides AM and BM) and which do not confer heat shock inducibility in vivo. Additionally, we have observed that excess oligonucleotide A competes with oligonucleotide B for binding, demonstrating that the proteins responsible for complex formation recognize the same sequence element(s) within each of these DNA fragments. The failure of a 500-fold molar excess of the HSE-containing oligonucleotide, HSE2-26, to compete for retardation of oligonucleotide B argues strongly that the proteins which recognize the C_4T element are distinct from HSF. This conclusion is further strengthened by the demonstration that HSF expressed in *E. coli* binds an HSE-containing DNA fragment but not one containing the C_4T sequences (Fig. 6). This novel stress regulatory element containing this pentanucleotide sequence has been designated TRS, for thermal stress response sequence.

Southwestern blotting was used to identify a yeast protein which recognizes the C_4T motif in oligo31/32 as well as in oligonucleotide D. Our data indicate that the binding activity migrates as a polypeptide with an apparent molecular mass of approximately 140 kDa. This protein was found to migrate slightly faster than HSF in our gel system and was present in extracts prepared from both stressed and unstressed cells. Furthermore, our results demonstrate that there is neither an increase in the amount nor an obvious alteration in the mobility of the TRS-binding factor in heat shock-treated cells. However, additional experiments with the purified TRS-binding factor will be required to determine whether heat shock or other stress treatments alter the conformation or modify the structure of this protein. We have designated this protein the thermal stress transcription factor or TSF. Recently, we have observed that sequence changes outside of the C_4T element can modulate its interaction with TSF (11a). These results suggest that flanking sequences may play a role in mediating a heat or stress response.

The identification of a new stress-regulatory pathway in *S. cerevisiae* raises several interesting and important new questions. What is the relationship between the HSF and TSF proteins? Do other eukaryotes possess this pathway for stress regulation of transcription? How many other stress-regulated genes in *S. cerevisiae* are controlled by TRS elements? The first two questions will be answered when the complete sequence of the cloned TSF gene is determined. These experiments are in progress. As for the last question, we have already observed that the stress-regulated yeast *UBI4* gene contains a promoter sequence related to the TRS elements of *DDR2*, and at least one of these sequences appears to participate in heat shock induction of *UBI4* transcription (20a). It is also worth noting that two copies of the TRS element are located in the promoter of the stress-regulated *HSP12* gene (16), and two sequences closely related to the TRS element have been found in the promoter of the stress-regulated cytosolic catalase T gene (*CTT1*) of *S. cerevisiae*. Furthermore, deletion studies implicate one of these elements in stress regulation of *CTT1* expression (28). Recently, Ota and Varshavsky (12) identified sequences in the 5′ flanking region of the yeast *PTP2* gene (encoding a heat shock-inducible phosphotyrosine phosphatase) which closely resemble the heat stress-regulatory region of *DDR2*. In this region are located two C_4T elements. It will be important to determine whether these sequence elements are functionally important in the regulation of these different stress-inducible genes.

Our original deletion studies of the *DDR2* promoter demonstrated that sequences within the oligo31/32 region were needed for *DDR2* transcription induction by DNA-damaging agents as well as by heat shock (6). Although it is almost certain that the TRS elements and TSF protein are required for induction by DNA-damaging agents, our recent molecular studies suggest that additional promoter sequences are needed for regulation by chemical mutagens and/or carcinogens. We are currently investigating the nature of these sequences and their possible interactions with TRS and determining what additional factor(s) binds to these promoter elements.

In *E. coli*, σ^{32} regulates transcription of 17 or more genes in response to heat shock (for a review, see reference 11). Recently, a second transcription factor, σ^E, has been purified; σ^E controls expression of the *rpoH* gene (encoding σ^{32}) at elevated temperatures and at least one additional stress-regulated gene (3, 25). It has been proposed that σ^E activates gene expression in response to potentially lethal temperatures (>42°C) and plays a role in establishing a thermotoler-

ant condition for cells (3). The TRS/TSF pathway may function analogously to establish thermotolerance in yeast. Alternatively, the TRS/TSF pathway may control functions which are needed to repair or mitigate certain types of damage common to both heat-shocked and mutagen- and/or carcinogen-treated cells. Interestingly, there is considerable overlap between heat shock and DNA damage (or replication blockage) regulation in *E. coli*, in which expression of as many as seven heat shock genes, including *dnaK* and *groEL*, occurs in response to conditions of replication arrest or DNA damage (7). In *S. cerevisiae*, expression of the major heat shock genes is not significantly influenced by DNA-damaging treatments, indicating a greater separation of these two regulatory pathways (10). Genetic analysis of the TRS/TSF system should help to identify the role of this stress response pathway in *S. cerevisiae*.

ACKNOWLEDGMENTS

We thank Akihiko Okuda and Carl S. Parker for the HSF expression plasmids. We are grateful to Janet Treger and John Simon for thoughtful discussions and for communicating their results prior to publication. We thank Francine Scott and Larry Tabata for their assistance in preparing the manuscript.

This work was supported by a research grant from the National Institutes of Health (GM38456) to K.M. and by the Director of the Office of Energy Research, contract no. DE-FC03-87-ER60615 (operated for the U.S. Department of Energy by the University of California). K.M. is a recipient of a Faculty Research Award from the American Cancer Society.

REFERENCES

1. **Amin, J., J. Ananthan, and R. Voellmy.** 1988. Key features of heat shock regulatory elements. Mol. Cell. Biol. **8**:3761–3769.
2. **Clos, J., J. T. Westwood, P. B. Becker, S. Wilson, K. Lambert, and C. Wu.** 1990. Molecular cloning and expression of a hexameric *Drosophila* heat shock factor subject to negative regulation. Cell **63**:1085–1097.
3. **Erickson, J. W., and L. A. Gross.** 1989. Identification of the σ^E subunit of *Escherichia coli* polymerase: a second alternate σ factor involved in high-temperature gene expression. Genes Dev. **3**:1462–1471.
4. **Goldenberg, C. J., Y. Luo, M. Fenna, R. Baler, R. Weinmann, and R. Voellmy.** 1988. Purified human factor activates heat shock promoter in a HeLa cell-free transcription system. J. Biol. Chem. **263**:19734–19739.
5. **Kadonaga, J. T., and R. Tjian.** 1986. Affinity purification of sequence specific DNA binding proteins. Proc. Natl. Acad. Sci. USA **83**:5889–5893.
6. **Kobayashi, N., and K. McEntee.** 1990. Evidence for a heat shock transcription factor-independent mechanism for heat shock induction of transcription in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **87**:6550–6554.
7. **Krueger, J. H., and G. C. Walker.** 1984. GroEL and dnaK genes of *Escherichia coli* are induced by UV irradiation and nalidixic acid in an *htpR*$^+$-dependent fashion. Proc. Natl. Acad. Sci. USA **81**:1499–1503.
8. **Lue, N. F., and R. D. Kornberg.** 1987. Accurate initiation at RNA polymerase II promoters in extracts from *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **84**:8839–8843.
9. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **McClanahan, T., and K. McEntee.** 1982. DNA damage and heat shock dually regulate genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6**:90–96.
11. **Neidhardt, F. C., and R. A. Van Bogelen.** 1987. Heat shock response, p. 1334–1345. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.

11a. **Nguyen, R., J. Simon, and K. McEntee.** Unpublished data.

12. **Ota, I. M., and A. Varshavsky.** 1992. A gene encoding a putative tyrosine phosphatase suppresses lethality of an N-end rule-dependent mutant. Proc. Natl. Acad. Sci. USA **89**:2355–2359.
13. **Park, H.-O., and E. A. Craig.** 1989. Positive and negative regulation of basal expression of a yeast HSP70 gene. Mol. Cell. Biol. **9**:2025–2033.
14. **Pelham, H. R. B.** 1982. A regulatory upstream promoter element in the *Drosophila* hsp70 heat shock gene. Cell **30**:517–528.
15. **Perisic, O., H. Xiao, and J. T. Lis.** 1989. Stable binding of *Drosophila* heat shock factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition unit. Cell **59**:797–806.
16. **Prackett, U. M., and P. A. Meacock.** 1990. HSP12, a new small heat shock gene of *Saccharomyces cerevisiae*: analysis of structure, regulation and function. Mol. Gen. Genet. **223**:97–106.
17. **Rabindran, S. K., G. Giorgi, J. Clos, and C. Wu.** 1991. Molecular cloning and expression of a human heat shock factor, HSF1. Proc. Natl. Acad. Sci. USA **88**:6906–6910.
18. **Sarge, K. D., V. Zimarino, K. Holm, C. Wu, and R. I. Morimoto.** 1991. Cloning and characterization of two mouse heat shock factors with distinct inducible and constitutive DNA-binding ability. Genes Dev. **5**:1902–1911.
19. **Scharf, K.-D., S. Rose, W. Zoh, F. Schoff, and L. Nover.** 1990. Three tomato genes code for heat stress transcription factors with a region of remarkable homology to the DNA-binding domain of the yeast HSF. EMBO J. **9**:4495–4501.
20. **Schuetz, T. J., G. J. Gallo, L. Sheldon, P. Tempst, and R. E. Kingston.** 1991. Isolation of cDNA for HSF2: evidence for two heat shock factor genes in humans. Proc. Natl. Acad. Sci. USA **88**:6911–6915.

20a. **Simon, J., and K. McEntee.** Unpublished data.

21. **Sorger, P., M. Lewis, and H. R. B. Pelham.** 1987. Heat shock factor is regulated differently in yeast and HeLa cells. Nature (London) **329**:81–84.
22. **Sorger, P., and H. R. B. Pelham.** 1987. Purification and characterization of a heat shock element binding protein from yeast. EMBO J. **6**:3035–3041.
23. **Sorger, P., and H. R. B. Pelham.** 1988. Yeast heat shock factor is an essential DNA binding protein that exhibits temperature-dependent phosphorylation. Cell **39**:855–864.
24. **Vinson, C. R., K. L. LaMarco, P. F. Johnson, W. H. Landschulz, and S. L. McKnight.** 1988. In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. Genes Dev. **2**:801–806.
25. **Wang, Q., and J. Kaguni.** 1989. A novel sigma factor is involved in expression of the *rpoH* gene of *Escherichia coli*. J. Bacteriol. **171**:4248–4253.
26. **Weiderrecht, G., D. Seto, and C. Parker.** 1988. Isolation of the gene encoding the *S. cerevisiae* heat shock transcription factor. Cell **54**:841–853.
27. **Weiderrecht, G., D. Shuey, W. Kibbe, and C. Parker.** 1987. The *Saccharomyces* and *Drosophila* heat shock transcription factors are identical in size and DNA binding properties. Cell **48**:507–515.
28. **Wieser, R., G. Adam, A. Wagner, C. Schuller, G. Marchler, H. Ruis, Z. Krawiec, and T. Bilinski.** 1991. Heat shock factor-independent heat control of transcription of the CTT1 gene encoding the cytosolic catalase T of *Saccharomyces cerevisiae*. J. Biol. Chem. **266**:12406–12411.
29. **Wu, C.** 1984. Two protein-binding sites in chromatin implicated in the activation of heat-shock genes. Nature (London) **309**:229–234.
30. **Wu, C., S. Wilson, B. Walker, I. Dawid, T. Paisley, V. Zimarino, and H. Ueda.** 1987. Purification and properties of *Drosophila* heat shock activator protein. Science **238**:1247–1253.
31. **Xiao, H., and J. Lis.** 1988. Germline transformation used to define key features of heat shock response elements. Science **239**:1139–1142.
32. **Zimarino, V., and C. Wu.** 1987. Induction of sequence-specific binding of heat shock activator protein without protein synthesis. Nature (London) **327**:727–730.