Heat Shock Element Architecture Is an Important Determinant in the Temperature and Transactivation Domain Requirements for Heat Shock Transcription Factor

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The baker’s yeast Saccharomyces cerevisiae possesses a single gene encoding heat shock transcription factor (HSF), which is required for the activation of genes that participate in stress protection as well as normal growth and viability. Yeast HSF (yHSF) contains two distinct transcriptional activation regions located at the amino and carboxyl termini. Activation of the yeast metallothionein gene, CUP1, depends on a nonconsensus heat shock element (HSE), occurs at higher temperatures than other heat shock-responsive genes, and is highly dependent on the carboxyl-terminal transactivation domain (CTA) of yHSF. The results described here show that the noncanonical (or gapped) spacing of GAA units in the CUP1 HSE (HSE1) functions to limit the magnitude of CUP1 transcriptional activation in response to heat and oxidative stress. The spacing in HSE1 modulates the dependence for transcriptional activation by both stresses on the yHSF CTA. Furthermore, a previously uncharacterized HSE in the CUP1 promoter, HSE2, modulates the magnitude of the transcriptional activation of CUP1, via HSE1, in response to stress. In vitro DNase I footprinting experiments suggest that the occupation of HSE2 by yHSF strongly influences the manner in which yHSF occupies HSE1. Limited proteolysis assays show that HSF adopts a distinct protease-sensitive conformation when bound to the CUP1 HSE1, providing evidence that the HSE influences DNA-bound HSF conformation. Together, these results suggest that CUP1 regulation is distinct from that of other classic heat shock genes through the interaction of yHSF with two nonconsensus HSEs. Consistent with this view, we have identified other gene targets of yHSF containing HSEs with sequence and spacing features similar to those of CUP1 HSE1 and show a correlation between the spacing of the GAA units and the relative dependence on the yHSF CTA.

All organisms possess a highly conserved response to elevated temperatures and to a variety of chemical and physiological stresses commonly designated as the heat shock response (38). In eukaryotic cells this response involves the rapid activation of a transcription factor known as heat shock transcription factor (HSF) (70). Once activated, HSF induces the expression of genes whose products ensure the survival of the cell during stressful conditions by providing defense against general protein damage. These heat shock proteins (Hsps) also play essential roles in the synthesis, transport, translocation, proteolysis, and proper folding of proteins under both normal and stressful conditions (38). Although the heat shock response is conserved among eukaryotes, both the number and overall sequence of HSFs vary widely among different species. Yeasts (Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Kluyveromyces lactis) and Drosophila melanogaster appear to have a single HSF gene, whereas most vertebrates and higher plants possess multiple HSF genes: at least three HSF genes have been isolated from the human, mouse, chicken, and tomato genomes (15, 23, 28, 41, 44, 50, 51, 53, 60, 69). Despite sequence divergence, all members of the HSF family have two highly conserved features: a helix-turn-helix DNA binding domain and coiled-coil hydrophobic repeat domains which mediate the trimerization of HSF (26, 45, 66).

A key step in the induction of heat shock gene transcription is the interaction of HSF with a short, highly conserved cis-acting DNA sequence, the heat shock element (HSE) found in the promoters of HSF-responsive genes. All HSEs contain multiple copies of the repeating 5-bp sequence $5^{'-nGAAn-3^{'}}$ (where n is any nucleotide) arranged in alternating orientation (2, 71). The number of pentameric units in an HSE can vary; while a minimum of three is thought to be required for heat-inducible expression, some HSEs harbor eight contiguous inverted repeats (19). Furthermore, the degree of homology of each pentamer unit to the consensus nGAA motif can vary, as can the nature of the initial pentamer, beginning with either GAA or its complement TTC, with the latter displaying significantly higher levels of biological activity in yeast cells and the capability to bind two HSF trimers instead of one (4). A functional HSE can tolerate a 5-bp insertion between repeating units, provided that the spacing and orientation of the pentameric elements are maintained (2). The binding of HSF to DNA has been shown to be highly cooperative, and deviations from the nGAA motif sequence may be tolerated in vivo because multiple HSEs foster cooperative interactions between multiple HSE trimers (4, 65, 72). These variations in the sequence of the binding site can influence the affinity of HSF for the HSE(s) of a particular heat shock gene, thereby influencing the level of transcriptional activation, and ultimately fine-tune the nature of the heat shock gene response.

The existence of multiple HSF species in higher eukaryotes suggests that HSF isoforms may have specialized functions that can be triggered by distinct stimuli or may activate specific target genes. For example, in human K562 erythroleukemia cells, HSF2 responds to hemin treatment and is constitutively
active in mouse embryonal carcinoma cells and at the blastocyst stage during embryogenesis and spermatogenesis (46, 55). These observations are consistent with HSFs functioning as a regulator of heat shock gene expression during development and differentiation, such as its potential regulation of the hsp70.2 gene during spermatogenesis (33, 49). Human HSF1 responds to thermal stress and other stresses at the level of trimerization, phosphorylation and DNA binding to activate transcription of Hsp genes (16, 48, 74). Consistent with the possibility that distinct mammalian HSF isoforms activate different target genes, mouse HSF1 (mHSF1) utilizes a higher degree of cooperativity in DNA binding and demonstrates a preference for HSEs containing four to five pentamers, while mHSF2 has a binding preference for HSEs containing only two to three pentamers (30). This notion is further supported by a recent functional analysis of human HSF1 and HSF2 expressed in yeast, which showed that HSF1 bound with highest affinity to and activated transcription most potently from the SSA43 promoter, which has an extended array of pentameric elements in the HSE (35). On the other hand, HSF2 bound with highest affinity to and activated transcription most potently from the yeast metallothionein gene, CUP1, which has only three pentamers in HSE1 and has a gap between the last two pentamers and an A-to-G substitution (GAG) in the last pentameric unit (35).

Yeast cells utilize the single essential HSF to activate the expression of a wide variety of genes in response to heat and other stresses and to coordinate the expression of genes required for growth under normal physiological conditions. The DNA binding domain of yeast HSF (yHSF) may be more conformationally flexible than HSF1 or HSF2 from higher eukaryotes (20) and allow a wide range of distinct interactions of the DNA binding domain with HSEs. The observation that a single amino acid substitution in the DNA binding domain of yHSF alters the specificity of HSF on different promoters is consistent with this idea (54). A feature distinguishing yHSF from HSFs of higher eukaryotes is the presence of two transcriptional activation domains which respond differentially to heat shock (42, 56). Studies of a synthetic HSE-lacZ reporter gene suggested that the yHSF amino-terminal activation domain mediates a transient response to elevated temperatures, while the carboxyl-terminal activation domain (CTA) is required to regulate both a transient and a sustained response (56). Both activation domains are restrained under normal growth conditions by intramolecular interactions with the DNA binding domain, the trimerization domain, and a conserved element, denoted CE2 (5, 14, 28, 42, 56). The presence of two activation domains in yHSF may provide additional levels of regulation or selectivity in gene activation. Previous studies have established that the CUP1 gene is transcriptionally activated by yHSF via heat and oxidative stress (36, 63). Interestingly, expression of CUP1 in response to heat shock and oxidative stress exhibits a strong requirement for the CTA of HSF, activation of CUP1 by yHSF differs from that of typical heat shock genes in that the robust activation of CUP1 requires a temperature of 39 rather than 37°C (63).

The CUP1 promoter HSE is thought to be atypical in that it contains only one HSE (HSE1) composed of three pentameric units. A compilation of HSEs from many organisms demonstrated that for promoters that contain an HSE composed of three pentameric units, additional flanking HSEs are present (4, 43). Furthermore, HSE1 deviates significantly from consensus HSEs in that there is a gap between the second and third pentamers; however, the gap preserves both the space and the orientation between these two repeats. Since yHSF-dependent activation of CUP1 and the SSA genes is distinct, we have carried out a detailed analysis of CUP1 gene expression to understand how yHSF regulates the activation of genes via distinct HSEs and with distinct transactivation domain requirements. We present evidence for a second nonconsensus HSE in the CUP1 promoter, HSE2, which serves to modulate the transcriptional activation of CUP1 in response to both heat and oxidative stress. Furthermore, we demonstrate that the nature of HSE1 plays a crucial role in the dependence on the yHSF CTA for CUP1 activation by heat stress. The expression of two additional yeast genes which contain a gapped HSE is also strongly dependent on the yHSF CTA. Chymotrypsin sensitivity strongly suggests that the arrangement of pentameric units in the CUP1 HSE1 affects the conformation of DNA-bound yHSF and suggests that at least part of the distinct features of CUP1 activation by yHSF may be due to the generation of specific yHSF structures by the HSE. Therefore, this work demonstrates that yeast cells activate and fine-tune the expression of a wide variety of target genes via a single HSE isoform, in part by virtue of the nature of the yHSF binding sites and distinct transactivation domain requirements.

MATERIALS AND METHODS

Strains and growth conditions. S. cerevisiae MCY1093, a gift from Marian Carlson, was used as the wild-type parental strain throughout this study and is designated DTY123. Strain PS145 (a gift from Hillary Nelson) contains a deletion of the endogenous yHSF gene (60). The HSF(1-583) strain, DTY179, has been previously described (63). Cell culture conditions for inducing CUP1 expression by heat shock and oxidative stress using menadione treatment were as previously described (36, 63). All CUP1-lacZ fusion plasmids are URA3 based, and all strains were grown in synthetic complete medium minus uracil unless otherwise specified. Strain DTY123skn7 is isogenic to DTY123 and carries a hasp-URAS-hisG (1) disrupted SKN7 gene (10). The SKN7 gene was disrupted following previous protocols (11) by transforming DTY123 with an SKN7/lacG-URA3-hisG fragment that was released from plasmid pBS:SKN7/URA3 by XbaI digestion. The chromosomal arrangement of this disrupted skn7 allele was confirmed by both PCR and the increased sensitivity of the skn7 disruption strain to peroxan and hydroperoxide (37).

Plasmids. All plasmids are numbered according to the 5′ and 3′ termini of the CUP1 insert; numbering is relative to the start site of CUP1 transcription. Plasmids containing mutations in the CUP1 HSE that are used for RNA analyses of gene expression are denoted with “m” to distinguish them from plasmids containing mutations in the CUP1 HSE that are used for DNA binding analyses, which are denoted with “M.” For analyzing regions of the CUP1 promoter important for CUP1 activation by HSF, restriction enzyme-generated fragments of the CUP1 promoter containing different 5′ upstream termini but all extending through the 12th codon of CUP1 (BspH site at +105 from the transcription start site) were ligated into the lacZ fusion vector YEp357R (39). Plasmid pYeCUP1-397 was generated by using a BspH-BspHI fragment from plasmid pGEXEx (63). Plasmids pYeCUP1-393, pYeCUP1-241, and pYeCUP1-167 were generated by using BamHI-, EcoRV-, and XbaI-BspHI CUP1 fragments, respectively, from plasmid pYeP336 (12). Mutant CUP1 promoter plasmids pYeCUP1HSE1P, pYeCUP1HSE2p, and pYeCUP1ACEn were generated by using a Chameleon double-stranded site-directed mutagenesis kit (Stratagene, La Jolla, Calif.), plasmid pYeCUP1-393, and the following oligonucleotides: CUP1Acem (5′-GCGATGCGTCTTTTCCCGTGAACCGTTCCAGC-3′), CUP1HSE2m (5′-ATGCGTCTTTTTCGCTAAACCGTTTCAGCAAAAAAGACTACC-3′), and pBluescript SK+ (5′-GGATCCTCTTTTCCTTTCAAGAAGATGGGAAAGATGGCAATC-3′), and pBluescript SK- (5′-GGATCCTCTTTTCCTTTCAAGAAGATGGGAAAGATGGCAATC-3′), and

Plasmids pCUP1Acem (5′-GCGATGCGTCTTTTCCCGTGAACCGTTCCAGC-3′), CUP1HSE2m (5′-ATGCGTCTTTTTCGCTAAACCGTTTCAGCAAAAAAGACTACC-3′), CUP1Acem (5′-GGATCCTCTTTTCCTTTCAAGAAGATGGGAAAGATGGCAATC-3′), and pBluescript SK+ (5′-GGATCCTCTTTTCCTTTCAAGAAGATGGGAAAGATGGCAATC-3′), and pBluescript SK- (5′-GGATCCTCTTTTCCTTTCAAGAAGATGGGAAAGATGGCAATC-3′).
upstream activation sequences (UAS) was used testing the CUC1-lacZ fusion plasmid pCM64, a gift from Charles Mochele. Plasmid pBS:SK7::URA3 was constructed as follows. The hisG-URA3-hisG cassette (1) was removed from plasmid pNC515 by BglII-BamHI digestion, filled in, and ligated into plasmid pBS:SK7 that had been digested with Styl-MscI. The Styl-MscI digestion removes nucleotides that code for approximately 480 of the 622 amino acids of SK7 from plasmid pBS:SK7. Digestion of pBS:SK7::URA3 with XhoI-XhoI produces a fragment of 109 bp that was subsequently ligated to the SK7 sequence flanking each end. To facilitate the purification of full-length yHSF from Escherichia coli, plasmid pET3d-HSF-His6, which contains a six-His tag added to the carboxy terminus of the HSF open reading frame cloned into pET3d, was constructed. The fragment of pET3d-HSF-His6 utilized for mass-spectrometric analysis contained T7 RNA polymerase and for RNase protection assays. Plasmids pSKACT1 and pSKaZC, for determining CUP1-lacZ and ACT1 mRNA levels, were described elsewhere (32). Plasmid pSSS3A was constructed by ligating a 159-bp EcoRI-HindIII fragment from the SS3A gene into the EcoRI-Smal sites of pBluescript KS+. Plasmid pSKCUP1 was constructed by inserting a 149-bp EcoRI-BamHI fragment from the CUP1 gene into the same sites of pBluescript KS+. pKHS6C82 was constructed by ligating a PCR product containing a 115-bp fragment of the HSP82 gene to which EcoRI-BamHI sites were introduced into the same sites of pBluescript KS+. PhosphorImager analysis. The amount of yHSF-DNA complexes and free probe remaining after limited proteolysis was quantitated by PhosphorImager analysis of the dried gels.

**Expression and purification of yHSF from E. coli.** Full-length yHSF was expressed and purified by standard protocols (3), with minor modifications. Six liters of LB, freshly transformed E. coli BL21(DE3)pLysS cells containing plasmid pET3d-HSF-His6 was grown in Superbroth (Dignose Diagnostics, Beltsville, Md.) at 37°C to an A600 of 0.8. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM, and induction was carried out at 25°C for approximately 6 h. Cells were harvested, and cell pellets were frozen in liquid nitrogen and stored at −80°C. All subsequent steps in this purification were carried out at 4°C. Cell pellets were thawed in the presence of protease inhibitor cocktail (3); cells were resuspended in approximately 100 ml of breaking buffer (50 mM sodium phosphate [pH 7.5], 10% glycerol) and broken by one passage through a French pressure cell (SLM Aminco, Champaign, Ill.) at 16,000 lb/in². The cell extract was centrifuged for 30 min at 30,000 × g, the pH of the supernatant was adjusted to approximately 8, and incubation continued for 30 min with gentle mixing at 4°C in batch, using 3 ml of packed Ni-nitrilotriacetic acid (NTA) resin (Qiagen, Chatsworth, Calif.) per 50 ml of extract. The resin was washed twice by centrifugation at 500 rpm in a RT6000B swinging-bucket centrifuge (Sorval, Wilmington, Del.) at 4°C with wash buffer 1, which was replaced by breaking buffer except that it contained 500 mM NaCl and 5 mM imidazole. The resin was then washed once with wash buffer 2, which was identical to wash buffer 1 except that it contained 10 mM imidazole. The resin was pooled, and elution of yHSF was effected by two successive incubations with 6 ml of elution buffer (identical to wash buffer 1 except that it contained 200 mM imidazole) for 15 min with gentle mixing. The eluted sample was aliquoted, frozen by using liquid nitrogen, and stored at −80°C. HSF was further purified by gel filtration chromatography on a Superose column (10/300) for use in the binding assay, procedures for calibration and FPLC (fast protein liquid chromatography) purification using the Superose 6 column (31, 59, 68) and the chromatography buffer (68) have been described elsewhere. Briefly, the Ni-NTA resin eluate was thawed and adjusted to 0.1 mM EDTA-0.1 mM EGTA-0.1% NP-40 immediately prior to injection of 0.2 ml onto the Superose column. The column chromatography buffer was modified by the addition of 0.1% NP-40, the column was run at 0.3 ml/min, and 0.5-ml fractions were taken. After binding of the centri-fuged cell lysate to the Ni-NTA resin, the protease inhibitor mix was replaced by the single protease inhibitor Pefabloc (Boehringer Mannheim). Pefabloc was used in all buffers for all remaining steps. HSF eluted at approximately 12 ml (fractions 37 to 45), immediately before the position where the thyroglobulin standard (660 kDa) elutes. The purified yHSF was stable at 0°C for several weeks.

**Protein extraction and immunoblotting.** Whole-cell protein extracts for immunoblotting were prepared exactly as described previously (35) by glass bead extraction using sodium dodecyl sulfate (SDS) harvest buffer (50 mM SDS, 10 mM Tris-HCl [pH 7.4]). 1 mM EDTA containing protease inhibitors. Protein concentration was determined by the Bradford assay (Bio-Rad). Extracts were resolved by SDS-PAGE (10% gel), transferred to nitrocellulose, and immunoblotted under standard conditions. Immunoblotting was carried out with reagents and protocols from Amersham, using anti-yHSF polyclonal antiserum (a gift of P. Sorger), Hsc82/Hsp82 polyclonal antibody (a gift from S. Lindquist), Su33/Sas4p polyclonal antibody (a gift from E. Craig), and monoclonal antibodies against mouse glyceraldehyde 3-phosphate dehydrogenase (GA3PDH; Molecular Probes, Eugene, Oreg.). Proteins of interest were detected by using the Renaissance chemiluminescence detection system (NEN Life Sciences, Boston, Mass.). Band intensity was estimated using NIH Image version v1.61.
motermotor fragments but essentially eliminated the activation of
CUP1 by HSF in response to both heat shock and oxidative stress (pYEpCUP1-163 [Fig. 1]). Analysis of the 3′ CUP1 pro-
motermoter promoter region showed that deletions of the CUP1 transcribed region to +9 from the start site of transcription had no significant effect on the magnitude of the transcriptional activation of CUP1 in response to heat shock or oxidative stress (data not shown).

Based on the observation that DNA sequences upstream of −241 are not required for heat shock induction of CUP1, we investigated whether the CUP1 HSE1 alone was sufficient to function as a heat-inducible UAS. The HSEs from the SSA1, SSA3, and SSA4 genes were previously shown to be sufficient to function as heat-inducible UAs (6, 73). DNA sequences encompassing the CUP1 HSEs from −168 to −141 or −168 to −116 from the transcription start site were unable to activate heat-induced transcription when fused to the yeast CYC1 basal promoter, while the SSA4 HSE strongly activated heat-induced transcription in this context (data not shown). Longer fragments of the wild-type CUP1 promoter (−393 to −91, −183 to −79, −393 to −1, or −168 to −1 from transcription start) were also unable to activate transcription in this context, implicating a requirement for specific CUP1 basal promoter elements for yHSF-mediated activation of CUP1. Therefore, the CUP1-lacZ fusions used throughout this study contain the CUP1 HSE and basal promoter fused to lacZ.

In contrast to the HSEs found within the SSA1 and SSA3 promoters, the CUP1 HSE1 contains only three pentameric units, with a gap between the second and third units (Fig. 2A). However, DNase I footprinting and methylation interference analyses have shown that the HSF trimer interacts with all three pentameric sites (2, 63). Since a 200-fold molar excess of an oligonucleotide containing sequences adjacent to HSE1 (−141 to −107) competes for the binding of yHSF in crude extracts with a probe containing −241 to +37 of the CUP1 promoter (54), we investigated whether a second nonconsen-
sus HSE, HSE2 (Fig. 2A), might function in CUP1 transcriptional activation. The two HSEs are similar in that both contain only three GAA units and both start with TTC.

CUP1 HSE architecture and arrangement modulates transcriptional potency. To determine whether the gap in the CUP1 HSE1 or the putative HSE2 plays a role in the regula-
tion of CUP1 transcription in response to heat shock, expression from CUP1 promoters with mutationally altered HSE1 and HSE2 elements (summarized in Fig. 2A) was analyzed by RNase protection. Two strategies were adopted. First, the spacing in HSE1 was altered to match the consensus HSEs such as those found in the SSA1 and SSA3 promoters. Second, the putative HSE2 was mutagenized within each pentamer at positions known to be essential for HSF binding to consensus HSEs (19). Conversion of the gapped CUP1 HSE1 to an HSE with four GAA repeats, designated HSE1 “perfect” (HSE1P/ 2), resulted in 3.9- and 3.6-fold increases in CUP1 activation in response to both heat shock and oxidative stress (data not shown), respectively, compared to the wild type (Fig. 2B). Mutagenesis of HSE2 (HSE1/2m [Fig. 2]) resulted in a 3.5-fold increase in the transcriptional activation of CUP1 in response to heat shock and oxidative stress (data not shown) compared to the wild type (Fig. 2B). This hyperactivation depended on the functional integrity of HSE1, as demonstrated by the inactivity of the double mutant HSE1m/2m (Fig. 2B). Combination of the two mutations (HSE1P/2m) did not result in any significant difference in expression as compared to the CUP1 HSE1P/2 promoter (Fig. 2B). Mutation of HSE1 alone (HSE1m/
2 (Fig. 2A) also resulted in a \textit{CUP1-lacZ} fusion that was transcriptionally inactive to both heat shock and oxidative stress (Fig. 2B and data not shown), confirming our previous results demonstrating the requirement for HSE1 in the stress induction of \textit{CUP1} \cite{36, 63}. These results with either double mutation (HSE1P/2m and HSE1m/2m) demonstrate that modulation of the transcriptional activation of \textit{CUP1} through HSE2 is highly dependent on the nature of HSE1.

Based on the results obtained with the HSE1P \textit{CUP1-lacZ} fusion, we synthesized oligonucleotides spanning the HSE1P mutation to determine whether HSE1P could confer heat shock-inducible expression to the \textit{CYC1} basal promoter. An oligonucleotide containing the HSE1P mutation and spanning from −168 to −116 or from −168 to −141 of the \textit{CUP1} promoter potently activated the \textit{CYC1-lacZ} reporter in response to heat shock (data not shown). Therefore, the requirement for the basal promoter region in the yHSF-mediated transcriptional activation of \textit{CUP1} in response to heat shock can be dispensed with by using a canonical HSE but not the \textit{CUP1} HSE1.

The activation of \textit{CUP1} by heat shock and oxidative stress has been previously shown to be independent of the Cu ion-dependent transcription factor, Ace1p \cite{54, 63}. However, a high-affinity Ace1p binding site overlaps the TTC and partially overlaps the second GAA unit in HSE2 (Fig. 2A), and the HSE2 mutation converts the GAA to AAA, disrupting one nucleotide in this Ace1p site. We therefore analyzed transcriptional activation from a \textit{CUP1-lacZ} fusion which destroys the high-affinity Ace1p site that overlaps the HSE2 sequence but does not mutate HSE2 (Fig. 2A, Ace1m). Activation of the Ace1m \textit{CUP1-lacZ} fusion in response to heat stress was indistinguishable from the wild-type promoter (data not shown). Therefore, the increased transcriptional activation observed for the HSE1/2m \textit{CUP1-lacZ} fusion gene is independent of activation by Ace1p.

Another stress-responsive transcription factor found in
S. cerevisiae. Skn7p, possesses significant homology to the DNA binding domain of yHSF (9) and binds to a GAA-containing sequence of the TRX2 promoter (37). Therefore, we investigated whether the increased transcriptional response of the HSE1P/2 and HSE1/2m CUP1-lacZ promoters might be due to Skn7p-mediated activation. The heat shock responses of both wild-type and mutant CUP1-lacZ fusions in a skn7 disruption strain were indistinguishable from that of the wild-type SKN7 strain (data not shown). Taken together, these results demonstrate that the modulation of CUP1 expression in response to heat shock is mediated by HSF, HSE1, and HSE2.

The architecture of CUP1 HSEs imparts specificity to the mode of activation of CUP1. Transcriptional activation of CUP1 by yHSF differs from that of SSA3 in that CUP1 activation requires an optimal heat shock temperature of 39 rather than 37°C (63). Furthermore, CUP1 expression in response to heat shock is highly dependent on the CTA of yHSF, whereas the SSA1 and SSA3 promoters are much less dependent on this domain. To determine if HSE1 and HSE2 are determinants in these features of CUP1 transcriptional activation, we compared expression from the wild-type and mutational altered CUP1-lacZ fusion genes at 37 and 39°C (Fig. 3A). Consistent with previous results (63), activation of CUP1 at 37°C was only 25% of that observed at 39°C (Fig. 3A). Interestingly, the generation of either HSE1P or HSE2M did not alter the temperature induction profile of CUP1; that is, expression of both derivatives was maximal at 39°C. Both mutations, however, change the efficacy of transcription at 37°C. The HSE1/2m and HSE1P/2 CUP1 derivatives give rise to a level of heat shock-inducible transcription at 37°C that is comparable to that observed for the wild-type fusion at 39°C (Fig. 3A). Therefore, both HSE2 and the gap between pentamers 2 and 3 in HSE1 act to limit the expression of CUP1 at a temperature where many other HSF-responsive genes are near maximal expression.

Isogenic wild-type HSF and HSF(1-583) cells harboring wild-type and mutant CUP1-lacZ fusions were analyzed to ascertain whether HSE1 or HSE2 plays a role in the dependence of CUP1 transcriptional activation on the yHSF CTA. As shown in Fig. 3B and consistent with previous analyses (36, 63), heat shock activation of the wild-type CUP1-lacZ reporter in the HSF(1-583) strain is greatly reduced (approximately 70%) compared to a strain with wild-type HSF. In contrast, heat shock activation of the HSE1P CUP1-lacZ reporter in the HSF(1-583) strain is reduced only 43% compared to a strain with wild-type HSF. These results suggest that the gapped HSE1 plays a critical role in determining the degree of dependence of CUP1 expression in response to heat shock on the HSF CTA. Transcription from the HSE1P/2 CUP1 promoter derivative was hyperactivated in the HSF(1-583) strain, exhibiting approximately threefold greater activation than the wild-type promoter in the wild-type HSF strain (Fig. 3B). In contrast to expression in a wild-type HSF strain, the HSE1/2m reporter expression is greatly reduced in the HSF(1-583) strain, with an induction approximately equal to that observed for the wild-type reporter in the HSF(1-583) strain (Fig. 3B). This finding suggests that the transcriptional activation observed for the HSE1/2m promoter under stress conditions is dependent on the interaction of yHSF with HSE1. Similar results were obtained in response to oxidative stress using the wild-type, HSE1/2m, and CUP1/2 reporter plasmids in the HSF(1-583) strain and wild-type HSF strain (data not shown). The data for the HSF(1-583) strain suggest that the HSE1P promoter increases the ability of the amino-terminal activation domain of HSF to activate CUP1 expression. Since the HSF(1-583) protein completely lacks a CTA, the results in Fig. 3B suggest that the gapped HSE1 plays a critical role in determining the contribution of the amino-terminal activation domain of HSF to the magnitude of CUP1 expression in response to both heat shock and oxidative stress.

To ascertain whether the correlation between a gapped HSE architecture and higher dependence on the yHSF CTA is a general phenomenon, other Hsp gene promoters with similarly organized HSEs were examined. Inspection of HSEs found in the promoters of HSP82, HSC82, and CUP1 suggests that nonconsensus HSEs may be commonly used for transcriptional responses to heat shock. Previous analysis of the HSE1 from the HSC82 and HSP82 promoters (8, 18, 24, 25) showed that, like the CUP1 HSE1, they are composed of only three pentamer units containing a gap between pentamers 2 and 3, with all three sites properly oriented and spaced (Fig. 3C). Based on these observations, we measured the heat-induced levels of endogenous CUP1, HSC82, and HSP82 mRNAs to determine if these genes exhibit a strong dependence on the yHSF CTA. Heat shock-induced expression of the CUP1 and HSC82 genes in the HSF(1-583) strain are most affected, with heat shock transcription being only 23 and 24%, respectively, of that observed in an isogenic wild-type HSF strain. Expression of HSP82 in the HSF(1-583) strain is only 37% of that in the wild-type HSF strain, while heat shock expression of SSA3 in the HSF(1-583) strain was least affected by the loss of the yHSF CTA (56% of the level in wild-type strain). This result with SSA3 is identical to that observed with the HSE1P/2 reporter in Fig. 3B, where 56% of the steady-state expression level in the HSF(1-583) background was retained compared to that present in the HSF wild-type strain. To determine whether there are significant physiological consequences for the reduction in the magnitude of transcriptional activation for the HSP82, HSC82, and SSA3 genes in the HSF(1-583) strain, the levels of these proteins in control and heat-shocked cells were determined by immunoblotting. Consistent with the steady-state RNA measurements for the HSP82 and HSC82 genes demonstrating a strong requirement for the HSF CTA in heat-induced expression of these two genes, protein levels of both Hsp90 isoforms were severely diminished (80 to 90%) in HSF(1-583) cells (Fig. 3D). In contrast, the levels of Ssa3/Ssa4 proteins were only slightly reduced (10 to 20%) in HSF(1-583) cells (Fig. 3D). These results strongly suggest that heat shock-induced expression from promoters containing contiguous HSEs is less dependent on the yHSF CTA than expression from promoters with gapped HSEs.

yHSF binding to HSE2 modulates interactions at HSE1. The data described here implicate the presence of a second CUP1 HSE1, HSE2, in the modulation of CUP1 transcription that is dependent on both yHSF and the nonconsensus HSE1. To ascertain whether yHSF interacts directly with HSE2 and whether this interaction might modulate the occupancy of HSE1, in vitro DNA binding studies were carried out. Since yeast cells express two proteins (10, 21) bearing homology to the yHSF DNA binding domain that appear to play no role in CUP1 regulation but which may confound in vitro DNA binding studies, full-length yHSF was expressed in and purified from E. coli. To facilitate the purification of yHSF for DNA binding studies, we constructed a yHSF allele in which a His$_6$ tag was placed at the carboxyl terminus of the coding region. This HSF-His$_6$ protein fully complemented the viability defect associated with disruption of the single endogenous yHSF gene at both 30 and 37°C (data not shown). yHSF was obtained after sequential purification on Ni-NTA agarose and FPLC Superose-6 chromatography (31, 59, 68). Purified yHSF migrated on a Coomassie blue-stained SDS-polyacrylamide gel at approximately 150 kDa and comigrated with HSF present in whole-cell yeast extracts from non-heat-shocked cells, as detected by
FIG. 3. **CUP1** promoter HSE mutations alter the temperature response and obviate the requirement for the yHSF CTA. (A) The HSE1P and HSE2m **CUP1-lacZ** fusions lower the temperature threshold for transcriptional activation of **CUP1**. The steady-state levels of **CUP1-lacZ** mRNA from the wild-type, HSE1P, and HSE2m fusions were analyzed before (control [C]; 27°C) and after heat shock (HS) at either 37 or 39°C for 20 min. **ACT1** and **CUP1-lacZ** mRNA levels were assayed and quantitated as described for Fig. 1. Below the data are schematic representations of the **CUP1-lacZ** promoter derivatives assayed in this RNase protection experiment and normalized expression levels of **CUP1-lacZ** mRNA. Details of the mutations in the **CUP1** HSEs are given in Fig. 2. (B) The HSE1P but not the HSE2m **CUP1-lacZ** fusion reduces dependency of **CUP1** transcriptional activation on the yHSF CTA. Experiments were carried out as described for panel A except that the **CUP1-lacZ** fusions were also assayed in a yeast strain containing the HSF(1-583) allele. (C) **HSC82** and **HSP82** possess similar GAA unit arrangements in HSE1, with a gap between units 2 and 3; **SSA3** contains a contiguous array of 5-bp units. (D) Deletion of the HSF carboxyl-terminal activation domain results in severe reduction in Hsp82/Hsc82 protein levels, while Ssa3/Ssa4 protein levels are only moderately affected, as determined by Western blot analysis of Hsp82/Hsc82, Ssa3/Ssa4, and Pgk1 protein levels in yeast strains containing either wild-type HSF or HSF(1-583). Yeast cells were heat shocked (HS) at 39°C for 1 h, and extracts were prepared by glass bead disruption as described in Materials and Methods. Samples were subjected to SDS-PAGE and immunoblotted with polyclonal antisera to Hsp82/Hsc82, Ssa3/Ssa4, and Pgk1. Pgk1 levels were used for normalizing sample loads.
Western blot analysis (Fig. 4A and B). Furthermore, purified yHSF specifically bound to the CUP1 promoter in a manner similar to yHSF present in crude cell extracts from non-heat-shocked cells (Fig. 4C). The amount of yHSF from crude cell extracts binding to CUP1 DNA is lower than that in the recombinant yHSF samples due to the low abundance of endogenous yHSF in the cell extracts used in the binding reaction.

The dissociation constants for yHSF-CUP1 promoter complexes were determined by quantitative EMSAs. As shown in Fig. 5, yHSF interacts with the CUP1 promoter fragment encompassing HSE1 and HSE2 with a $K_{d,app}$ of $(3.7 \pm 0.5) \times 10^{-9}$ M. Since the CUP1 HSE1P2 derivative gave rise to increased expression in response to stress and this expression was less dependent on the yHSF CTA, we determined whether these effects were due to an increase in binding affinity of yHSF for the CUP1 HSE1P1 promoter. yHSF bound to the CUP1 HSE1P2 probe with a $K_{d,app}$ of $(3.4 \pm 0.9) \times 10^{-9}$ M, demonstrating that yHSF does not have a significantly higher affinity for the CUP1 HSE1P1 compared to the wild-type promoter. The apparent affinity of yHSF for the CUP1 HSE2M probe, $(4.0 \pm 0.2) \times 10^{-9}$ M, was not significantly different from that for either the CUP1 HSEW or CUP1 HSE1P.

Consequently, no difference was observed in the apparent Hill coefficients obtained for the three CUP1 promoter sequences (approximately 1.5), suggesting a lack of differences in yHSF binding cooperativity to these three CUP1 promoter derivatives. This Hill coefficient is highly reproducible, and the intermediate value for the apparent Hill coefficient of between 1 and 2 suggests that one yHSF trimer may bind stably, and a second may bind only weakly or partially, to the CUP1 HSE1. Since the SSA3 and CUP1 promoters also exhibit marked differences in heat shock-inducible gene expression as a function of their HSEs, we explored whether yHSF exhibits different binding affinities for these two promoters. In three independent experiments, differences in neither affinity nor binding cooperativity were observed (data not shown). Therefore, it does not appear that the differences in heat shock-responsive expression between the CUP1 HSE1P1, CUP1 HSE2M, and SSA3 promoters and the CUP1 HSEWT promoter are due to differences in the affinity of yHSF for the HSEs or in binding cooperativity. Rather, differences may be due to binding site context-dependent alterations in bound HSF or interactions with other factors.
I footprinting was carried out with the CUP1 HSE2M probe (Fig. 6B). The complete lack of protection observed over HSE2, even at high yHSF concentrations, demonstrates the specificity of the interaction of yHSF with HSE2 (compare −134 through −120 in Fig. 6A and B). The 5' boundary of the protected region over HSE1 is identical to that of the HSEWT probe (−172 [Fig. 6]). However, in contrast to the wild-type CUP1 probe, there are no alterations in the protection over HSE1 as more yHSF is added (compare −148 through −155 in Fig. 6A and B). Furthermore, the major cleavage site at −143 (Fig. 6B) and the residues immediately upstream of this site, TCG (bottom strand, −144 to −146) are no longer protected (compare Fig. 6A and B). Therefore, yHSF bound at HSE2 may facilitate the binding of yHSF to the third pentameric unit (GAG) of HSE1.

HSF adopts distinct conformations when bound to consensus and atypical HSEs. So far, our results show that differences in the transcriptional activity of the CUP1 promoter derivatives cannot be attributed to any changes in either the binding affinity or the cooperativity with which yHSF binds these DNA sequences. To more directly assess whether the differences in transcriptional activation from the CUP1 promoter derivatives are due to changes in the conformation of DNA-bound HSF, protease sensitivity assays were carried out with purified yHSF bound to the CUP1 HSEWT, HSE1P, and HSE2M DNA fragments. The proteolytic clipping band shift assay (52) utilizes limited proteolysis of DNA-bound protein and has been used to probe the structure of transcription factor-DNA complexes (22, 52, 64). HSF-CUP1 HSE complexes were subjected to limited proteolysis by incubation with increasing concentrations of chymotrypsin, and the resulting complexes were separated on EMSA gels (Fig. 7A). The differences in sensitivity to digestion of the HSF-HSEWT and HSF-HSE1P complexes were striking. The HSF-HSE1P complex was routinely more sensitive to digestion than either the HSF-HSEWT or HSF-HSE2M complex (compare lanes 3, 8, and 13 in Fig. 7A). There is approximately an order of magnitude difference in the amount of chymotrypsin required to obtain similar levels of proteolytic sensitivity for the HSEWT and HSE1P. The sensitivities of the HSF-HSEWT and HSF-HSE2M complexes were nearly indistinguishable, suggesting that the major determinant in the chymotrypsin sensitivity of the DNA-bound yHSF is HSE1. Addition of chymostatin to the binding reactions prior to chymotrypsin resulted in complexes that were completely resistant to chymotrypsin digestion (Fig. 7A; compare lanes 5, 10, and 15 with lanes 1, 6, and 11). There were no obvious differences in the pattern of products generated by chymotrypsin digestion of yHSF bound to the HSEWT, HSE1P, or HSE2M (Fig. 7A and data not shown).

The data in Fig. 7B shows that the difference in chymotrypsin sensitivity between the HSF-HSE1P and HSF-HSEWT and HSF-HSE2M can also be observed in the rate of proteolysis of these complexes. After 12 min of chymotrypsin digestion, the HSF-HSEWT and HSF-HSE2M complexes are almost completely degraded whereas approximately 25% of the HSF-HSE1P complex remains (Fig. 7B). Thus, the HSF-HSE1P complex adopts a conformation distinct from the HSF-HSEWT and HSF-HSE2M complexes which can be demonstrated as differences in both the concentration and rate of limited digestion with chymotrypsin (Fig. 7).
Higher eukaryotic cells possess multiple distinct HSF isoforms, encoded by different genes. This diversity is further increased through differential splicing, responses to distinct stresses, and preferences for binding to distinct arrangements of HSEs (70). In contrast, the S. cerevisiae HSF is encoded by a single, essential gene and binds to some HSEs constitutively, while binding to other HSEs is induced in response to an environmental or pharmacological stimulus (24, 27, 58). Furthermore, yHSF differentially activates gene expression through the use of separate amino- or carboxy-terminal transactivation domains or by responding to distinct stressors (36, 42, 56, 63). Therefore, yHSF may represent a composite of the functions carried out by individual HSF isoforms in higher eukaryotes. The observation that human HSF isoforms are differentially functional when expressed in yeast cells lacking the endogenous HSF gene further underscores this notion (35).

To explore the mechanisms underlying the differential use of yHSF transactivation domains for target gene activation, HSF-dependent activation of the CUP1 gene was investigated in detail. CUP1 represents a heretofore atypical HSF-dependent gene in that it contains a nonconsensus HSE in its promoter, requires heat shock at 39°C for robust activation, as opposed to 37°C for other HSF target genes, responds to superoxide radical generators for HSF-mediated activation, and exhibits a strong requirement for the yHSF CTA (36, 63). Here, we have demonstrated that the CUP1 promoter harbors two HSEs, neither of which resembles those found in typical HSF-responsive genes, such as SSA1 or SSA3, in their fundamental architecture. Consistent with the known interaction of HSF with HSEs as homotrimeric proteins, both the CUP1 HSE1 and HSE2 harbor three repeats of the pentameric element. Furthermore, the separation of CUP1 HSE1 and HSE2 by a single helical turn provides a mechanism for potential interactions between DNA-bound HSF trimers on the same face of the DNA helix. Although the CUP1 HSE1 contains three pentamers, the distance of one helical turn between pentamers 2 and 3 allows occupancy of major grooves on the DNA with a distinct geometry compared to contiguous pentamers such as those found in SSA1 and SSA3. Indeed, the generation of a CUP1 HSE1 derivative which mimics those found in SSA1 and SSA3 (CUP1 HSE1P) results in stress-responsive transcriptional activation characteristics which more closely resemble these genes in terms of the temperature optimum, their reduced dependence on the yHSF CTA, and ability to activate a heterologous CYC1 basal promoter. One possible mechanism by which the CUP1 HSE1P might enhance CUP1 expression is by affecting the affinity of yHSF for DNA. However, our results suggest no significant difference in the apparent affinity of yHSF for the CUP1 promoter fragment containing HSE1P compared to HSE1WT.

Previous experiments have demonstrated that HSF-dependent activation of CUP1 in response to heat and oxidative stress is absolutely dependent on HSE1 (36, 63). Here, we have shown that although neither HSE1 nor HSE2 functions to activate heat-inducible expression in the context of a fusion to the yeast CYC1 core promoter, HSE2 plays an important role...
in modulating CUP1 expression through HSE1. The inability of HSE2 alone to function as an activating HSE, even in the context of the CUP1 promoter, may in part be due to its low affinity for yHSF, a consequence of the altered spacing between each of the three pentamers (2, 30, 71). This architecture of HSE2 may also affect structural changes in the CUP1 promoter DNA upon binding of yHSF. DNase I treatment of yHSF-CUP1 promoter DNA complexes results in hypersensitive sites within and adjacent to HSE2, suggesting that the binding of yHSF to the CUP1 DNA induces conformational changes in the DNA. Hypersensitive sites have been observed in DNase I treatment of mHSF1- and mHSF2-HSE complexes (31). Although HSE2 is incapable of autonomously driving yHSF-dependent activation of CUP1 transcription and is bound by yHSF with low affinity, the occupation of HSE2 has dramatic effects on stress-dependent activation of CUP1 transcription and the manner in which HSE1 is bound by yHSF. The generation of a form of HSE2 incapable of binding yHSF renders CUP1 heat-inducible transcription hyperactivated at 37 and 39°C, in a manner similar to the conversion of CUP1 HSE1 to HSE1P. Furthermore, consistent with potential interactions between yHSF trimers bound both at HSE1 and HSE2, DNase I footprinting assays demonstrate that the occupation of HSE2 alters the manner in which yHSF is bound at HSE1. Studies of the Drosophila hsp70 promoter have demonstrated the presence of a high- and a low-affinity HSE, the latter of which plays a critical role in the heat-inducible transcriptional response (65). It is interesting that an HSE found in the human prion-erelukin 1-β gene, which consists of only two pentameric units, fails to serve as a heat shock-inducible element but restraints expression from the promoter in response to heat shock joinedly administered with the inducer, lipopolysaccharide (13). It is thought that this may provide a mechanism to temper the inflammatory response. Furthermore, Westwood et al. have demonstrated the binding of Drosophila HSF to chromosomal loci that far exceed the predicted number of heat shock-inducible genes (67). These observations, taken together with the data described here, suggest that in addition to their role as gene-specific positive transcriptional regulatory elements, HSEs might modulate both HSF activity and the activity of distinct cis-acting promoter elements. Similar context-dependent activation or repression has been observed with the retinoic acid receptor bound to its cognate DNA response element (34).

The organization of the CUP1 HSE1 is very similar to that of the HSE1 in the HSP82 and HSC82 genes. We found that the heat shock induction of these three genes is highly dependent on the yHSF CTA. Like CUP1, HSP82 and HSC82 have multiple HSEs; however, others have shown that only the HSE1 of HSP82 and HSC82 is constitutively occupied in vivo (18). Girardina and Lis have shown that there is a change in the in vivo footprint of the HSP82 HSEs following heat shock (24). The changes in HSF-DNA binding upon heat shock were seen mainly on the low-affinity HSEs, HSE2 and HSE3 of HSP82. The binding of yHSF to these weaker HSEs in the HSP82 promoter was transient, and these sites were largely unoccupied once cells progressed through a recovery stage and into the non-heat-shocked stage. It may be that a similar situation occurs on the CUP1 promoter with HSE1 representing the constitutively occupied HSE with HSE2 occupied only upon stress induction. This is consistent with in vitro DNA binding studies performed in this report showing that HSE2 is a low-affinity site. Our DNase I footprinting assays demonstrate that the occupation of HSE2 alters the manner in which yHSF is bound at HSE1 and perhaps in vivo occupancy of HSE2 following stress induction tempers the transcriptional response of CUP1. The Hill coefficient for the HSE2M was approximately 1.5, suggesting that a second trimer may be only weakly bound to HSE1. The occupancy of HSE2 upon stress induction may also act to stabilize yHSF bound to HSE1. Future in vivo footprinting experiments will address these possibilities.

How do the specific architecture of HSE1 and the presence of HSE2 impart unique HSF-dependent regulatory characteristics to CUP1? One mechanism may be that HSF binds to the CUP1 HSE1 with less cooperativity than for a large contiguous HSE, thereby leading to a tempering of CUP1 expression. mHSF1 and mHSF2 differ in the potential for cooperative interactions with HSEs; mHSF1 binds cooperatively to extended HSEs much like that found in the SSA3 gene, and mHSF2 has a binding preference for HSEs harboring two or three pentamers like that in the CUP1 promoter (30, 31). Since the DNA binding domain of yHSF may be more conformationally flexible than that of mHSF1 or mHSF2 (20), perhaps

**FIG. 7.** yHSF binds to the CUP1 HSEWT, HSE1P, and HSE2M with distinct conformations. (A) The partial proteolysis with chymotrypsin of the yHSF-HSEWT complex is compared with that of the yHSF-HSE1P and yHSF-HSE2M complexes. Binding reactions containing the indicated probe were carried out exactly as described for Fig. 5 except that all lanes contained 1.5 × 10⁻⁸ M yHSF. Following the standard binding assay, chymotrypsin was added to all reactions except those in lanes 1, 6, and 11. The yHSF-HSE complexes in lanes 2 through 4, 7 through 9, and 12 through 14 were digested with 0.01, 0.1, and 1 ng chymotrypsin, respectively. Binding reaction mixtures were incubated for 10 min following chymotrypsin addition before loading onto EMSA gels. The chymotrypsin inhibitor chymostatin (1 μg) was added to the samples in lanes 5, 10, and 15 immediately prior to chymotrypsin (1 ng) addition. Bound HSF-HSE complexes (B) and free probe (F) are indicated. (B) Graphical representation of the results from time course assays of partial proteolysis of yHSF-HSE complexes. Experimental conditions were as for panel A except that chymotrypsin digestion (1 ng) was carried out for the indicated time followed by chymostatin addition to terminate the digestion prior to loading onto EMSA gels. The bound yHSF-HSE complexes (B) and free probe (F) were quantitated with a PhosphorImager and plotted. The data represent the averages of two separate experiments.
yHSF extracts binding site context information to influence the level of cooperativity used to bind a given promoter. However, our results suggest that yHSF binds to the CUP1 HSE1WT, HSE1P, and HSE2M with nearly identical levels of apparent cooperativity. It is also possible that when bound to HSE1, HSF adopts a conformation that alters its interactions with the basal transcription machinery in the core promoter of the CUP1 gene. Indeed, our results which demonstrate that a consensus HSE from SSA3, or the CUP1 HSE1P but not the CUP1 HSE1WT element, can confer heat-inducible expression to the CYC1 basal promoter are consistent with a requirement for the adaptation of distinct HSF conformations on the different HSEs. Consistent with this idea, substitution of the Gcn4 leucine zipper for the yHSF trimerization domain has recently demonstrated that the oligomeric state of the DNA-bound HSF-Gcn4 chimeric protein depends on the number and orientation of pentameric units within the HSE (17). Therefore, it is possible that the HSE structure can similarly influence the overall yHSF conformation. The in vitro DNA binding, in vivo gene expression, and protease sensitivity assay results described here are consistent with a change in the conformation of DNA-bound yHSF. The digestion of yHSF-HSE complexes with chymotrypsin shows that a yHSF surface is more readily accessible to the protease in the yHSF-HSEWT complex than it is in the yHSF-HSE1P complex. The ability of DNA to induce conformational changes in transcription factors has been previously proposed for the nuclear hormone receptors (61), the yeast pheromone/receptor transcription factor (64), and nuclear factor NF-xB (p50), (22). Therefore, it is possible that HSE structure can similarly influence the conformation of yHSF, and perhaps the extended linker region in yHSF facilitates such changes. What might be the mechanisms by which HSEs with contiguous pentamers exhibit a reduced dependence on the yHSF carboxyl-terminal activation domain compared to CUP1? Since the CTA is known to harbor an additional coiled-coil domain (14), it is conceivable that this region (HSF584-833) is responsible for intermolecular interactions that serve to stabilize yHSF trimers on the CUP1 promoter or to augment interactions between yHSF trimers bound at HSE1 and HSE2. It is also possible that yHSF receives context information from the HSE that specifies which functional surfaces of yHSF will be presented to the transcriptional machinery or to other, non-DNA binding regulatory factors. The chymotrypsin sensitivity data suggest that a gapped HSE such as the CUP1 HSE1 might induce a conformation of HSF which more efficiently utilizes the C-terminal rather than the N-terminal transactivation domain. The more canonical HSE such as HSE1P might result in more of the HSF N-terminal than C-terminal transactivation domain being presented to the transcriptional machinery. A similar mechanism has been proposed to dictate whether the glucocorticoid receptor functions through its response element as a transcriptional activator or repressor (61). As we show here, the architecture of HSEs in the CUP1, HSC82, HSP82, and HSP70 family of genes plays an important role in the features of the heat shock transcriptional response. Perhaps the sequences of these promoter HSEs have evolved to facilitate differential use of the yHSF transactivation domains and thus impart distinct characteristics to the heat shock transcriptional response. Although HSFs are regulated at many levels in response to stress, these studies demonstrate that promoter context represents a further level for regulation of transcription during the stress response.

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REFERENCES

1. Alani, E., L. Cao, and N. Kleckner. 1987. A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. Genetics 116:541–545.
2. Amin, J., J. Ananthan, and R. Voelmlry. 1998. Key features of heat shock regulatory elements. Mol. Cell. Biol. 8:3761–3769.
3. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
4. Bonner, J. J., C. Ballou, and D. L. Fackenthal. 1994. Interactions between DNA-bound trimers of the yeast heat shock factor. Mol. Cell. Biol. 14:501–508.
5. Bonner, J. J., S. Heyward, and D. L. Fackenthal. 1992. Temperature-dependent regulation of a heterologous transcription activation domain fused to yeast heat shock transcription factor. Mol. Cell. Biol. 12:1021–1030.
6. Boorstein, W. R., and E. A. Craig. 1990. Structure and regulation of the SSA4 HSP70 gene of Saccharomyces cerevisiae. J. Biol. Chem. 265:18912–18921.
7. Boorstein, W. R., and E. A. Craig. 1990. Transcriptional regulation of SSA4, an HSP70 gene from Saccharomyces cerevisiae. Mol. Cell. Biol. 10:3262–3267.
8. Borkovich, K. A., F. W. Farrelly, D. B. Finkelstein, J. Taulien, and S. Lindquist. 1989. hsp104 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. Mol. Cell. Biol. 9:3919–3930.
9. Brown, J. L., H. Bussey, and R. C. Stewart. 1994. Yeast Skn7p functions in a eukaryotic two-component regulatory pathway. EMBO J. 13:5186–5194.
10. Brown, J. L., S. North, and H. Bussey. 1993. SKN7, a yeast multicopy suppressor of a mutation affecting cell wall β-glucan assembly, encodes a product with domains homologous to prokaryotic two-component regulators and to heat shock transcription factors. J. Bacteriol. 175:6098–6105.
11. Butler, G., and D. J. Thiele. 1991. ACE2, an activator of yeast metallothio- nein expression which is homologous to SWI5. Mol. Cell. Biol. 11:476–485.
12. Butt, T. R., E. Sternberg, J. Herd, and S. T. Crooke. 1984. Cloning and expression of a yeast copper metallothionein gene. Gene 27:23–33.
13. Caihll, C. M., W. R. Waterman, X. Yie, P. E. Auuron, and S. K. Calderwood. 1996. Transcriptional repression of the pointierleukin 1β gene by heat shock factor 1. J. Biol. Chem. 271:24874–24879.
14. Caihll, C. M., N. A. Barløy, O. W. Liaard, and B. K. Jakobsen. 1993. Identification of the C-terminal activator domain in yeast heat shock factor: independent control of transient and sustained transcriptional activity. EMBO J. 12:5007–5018.
15. Caihll, J. L., 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.
16. Cotto, J. J., M. Kline, and R. L. Morimoto. 1996. Activation of heat shock factor 1 DNA binding precedes stress-induced serine phosphorylation. Evidence for a multistep pathway of regulation. J. Biol. Chem. 271:3355–3358.
17. Drees, B. L., E. K. Grotkop, and H. C. M. Nelson. 1997. The GCN4 leucine zipper can functionally substitute for the heat shock transcription factor’s trimerization domain. J. Mol. Biol. 272:61–74.
18. Erkine, A. M., C. C. Adams, M. Gao, and D. S. Gross. 1995. Multiple protein-DNA interactions over the yeast HSC82 heat shock gene promoter. Nucleic Acids Res. 23:1822–1829.
19. Fernandes, M., H. H. Xiao, and J. T. Lis. 1994. Fine structure analyses of the Drosophila and Saccharomyces heat shock factor heat shock element inter- actions. Nucleic Acids Res. 22:167–173.
20. Fink, K. E., L. Gonzalez, Jr., C. J. Harrison, and H. C. Nelson. 1994. Yeast heat shock transcription factor contains a flexible linker between the DNA-binding and trimerization domains. Implications for DNA binding by trim- eric proteins. J. Biol. Chem. 269:12475–12481.
21. Fujita, A., Y. Kikuchi, S. Kuhara, Y. Misumi, S. Matsumoto, and H. Koba- yashi. 1980. Domains of the S. cerevisiae protein of yeast heat shock gene homologous to MYC oncogenes of yeast heat shock transcription factor. Gene 85:321–326.
22. Fujita, T., G. Nolan, S. Ghosh, and D. Baltimore. 1992. Independent modes of activation by the p50 and p65 subunits of NF-κB. Genes Dev. 6:775–787.
23. Gallo, G. J., H. Prentice, and R. E. Kingston. 1993. Heat shock factor is required for growth at normal temperatures in the fission yeast Schizosac-
Chapter 1: Introduction to Heat Shock Proteins

1.1 Heat Shock Proteins in Eukaryotic Cells

Heat shock proteins (HSPs) are a family of proteins that are upregulated in response to various cellular stresses, such as heat, oxidative stress, and DNA damage. They are crucial for cellular survival as they assist in the refolding of denatured proteins, the degradation of misfolded proteins, and the protection of critical cellular functions. The expression of HSPs is regulated by heat shock factor (HSF), a transcription factor that undergoes a series of post-translational modifications, including tyrosine phosphorylation and trimerization, to enable it to bind to heat shock responsive elements (HSEs)

1.2 Evolution of Heat Shock Proteins

HSPs are conserved across a wide range of species, from bacteria to humans, indicating their essential role in cellular stress response and survival. The understanding of HSPs and their regulation has been facilitated by the identification of heat shock transcription factors (HSFs) in yeast and subsequent studies in higher eukaryotes.

1.3 The Role of Heat Shock Proteins in Disease

HSPs play a critical role in various diseases, including neurodegenerative disorders, cancer, and age-related conditions. They are involved in facilitating protein folding and degradation, maintaining metabolic homeostasis, and protecting against cellular stress.

Chapter 2: Heat Shock Factor (HSF)

2.1 Structure and Function of HSF

HSF is a dimeric transcription factor that exists in an inactive monomeric form in the cytoplasm under normal conditions. Upon cell stress, HSF undergoes tyrosine phosphorylation and trimerization, enabling it to bind to HSEs in the DNA. Each HSF monomer contains an N-terminal activation domain, a DNA-binding domain, and a C-terminal repression domain. The activation domain mediates dimer-dimer interactions, while the DNA-binding domain binds to HSEs.

2.2 Regulators of HSF Function

Various factors, including DNA damage, oxidative stress, and temperature changes, can activate HSF. The phosphorylation of HSF at tyrosine residues is a critical step in its activation. The trimerization of HSF is facilitated by the presence of HSEs.

Chapter 3: Heat Shock Responsive Elements (HSEs)

3.1 Identification and Characteristics of HSEs

HSEs are short DNA sequences that are crucial for the transcriptional activation of HSP genes. They are typically 5 bp long and contain a central core sequence that is highly conserved across species. HSEs can be found in the promoter regions of HSP genes and are essential for the transcriptional activation of these genes under stress conditions.

3.2 Functional Analysis of HSEs

The functional analysis of HSEs has revealed that the strength of HSEs can vary, and this variation can be attributed to differences in the context of the surrounding DNA sequences. These studies have also suggested that HSEs can function in a cooperative manner, indicating that the binding of multiple HSF trimers is necessary for maximal activation.

Chapter 4: Regulation of Heat Shock Protein Expression

4.1 Post-Translational Regulation of HSF

The regulation of HSF is a complex process that involves multiple post-translational modifications. In addition to tyrosine phosphorylation and trimerization, HSF can also undergo ubiquitination, acetylation, and SUMOylation, which can modulate its stability and activity.

4.2 Transcriptional Regulation of HSP Expression

The transcriptional regulation of HSP expression is largely mediated by HSF. HSF binding to HSEs in the promoter regions of HSP genes initiates the transcription of these genes. The regulation of HSF binding to HSEs is influenced by various factors, including the presence of binding partners and the context of the surrounding DNA sequences.

Chapter 5: Interaction of HSF with Other Transcription Factors

5.1 Interaction of HSF with Other Transcription Factors

HSF can interact with other transcription factors to influence the transcription of specific genes. These interactions can occur between HSF and factors involved in the regulation of stress response genes, such as NF-kB and AP-1, or between HSF and factors involved in the regulation of cell cycle, such as E2F.

5.2 Interactions with Non-Transcription Factors

HSF can also interact with non-transcription factors, such as histones, chromatin remodeling complexes, and other transcription factors, to influence the transcription of genes. These interactions can occur at various stages of the transcriptional machinery, from DNA binding to RNA polymerase binding.

Chapter 6: HSPs in Disease

6.1 Role of HSPs in Neurodegenerative Diseases

HSPs play a critical role in neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases. They are involved in the degradation of misfolded proteins, the maintenance of protein homeostasis, and the protection of neuronal cells from stress.

6.2 Role of HSPs in Anti-Aging

HSPs are involved in the maintenance of cellular homeostasis and the protection against stress, which is essential for aging. The upregulation of HSPs can delay the onset of aging and improve cellular function.

6.3 Role of HSPs in Cancer

HSPs are involved in various aspects of cancer, including the promotion of cell survival, the inhibition of apoptosis, and the regulation of cell cycle progression. Understanding the role of HSPs in cancer can provide insights into potential therapeutic targets.

Conclusion

The understanding of HSPs and their regulation has advanced significantly over the past few decades. The continued study of these proteins is essential for the development of new therapeutic strategies for various diseases, including neurodegenerative disorders, cancer, and age-related conditions. The research on HSPs continues to provide important insights into the regulation of stress response and the maintenance of cellular homeostasis.