Conservation of a stress response: human heat shock transcription factors functionally substitute for yeast HSF

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Heat shock factors (HSF) are important eukaryotic stress responsive transcription factors which are highly structurally conserved from yeast to mammals. HSFs bind as homotrimers to conserved promoter DNA recognition sites called HSEs. The baker's yeast Saccharomyces cerevisiae possesses a single essential HSF gene, while distinct HSF isoforms have been identified in humans. To ascertain the degree of functional similarity between the yeast and human HSF proteins, human HSF1 and HSF2 were expressed in yeast cells lacking the endogenous HSF gene. We demonstrate that human HSF2, but not HSF1, homotrimersize and functionally complements the viability defect associated with a deletion of the yeast HSF gene. However, derivatives of hHSF1 that give rise to a trimerized protein, through disruption of a carboxyl- or amino-terminal coiled-coil domain thought to engage in intramolecular interactions that maintain the protein in a monomeric state, functionally substitute for yeast HSF. Surprisingly, hHSF2 expressed in yeast activates target gene transcription in response to thermal stress. Moreover, hHSF1 and hHSF2 exhibit selectivity for transcriptional activation of two distinct yeast heat shock responsive genes, which correlate with previously established mammalian HSF DNA binding preferences in vitro. These results provide new insight into the function of human HSF isoforms, and demonstrate the remarkable functional conservation between yeast and human HSFs, critical transcription factors required for responses to physiological, pharmacological and environmental stresses.

Keywords: heat shock/human/HSF/transcription/yeast

Introduction

The ability of organisms to cope with environmental insults and physiological stresses depends upon a rapid and coordinated defense. A highly-conserved response to elevated temperatures in all prokaryotes and eukaryotes examined is the induction, at the level of transcription, of a group of genes encoding proteins known as heat shock proteins (HSPs) (Gross et al., 1990; Morimoto et al., 1994; Feige et al., 1996). A number of HSPs are essential even during non-stress conditions, consistent with their established roles in normal cellular growth and maintenance that include protein folding, translocation and proteolysis (Morimoto et al., 1994). In eukaryotes, a regulatory protein denoted Heat Shock Factor (HSF) controls the inducible transcription of many heat shock-responsive genes (Wu, 1995). Molecular cloning of the genes encoding HSFs from a variety of organisms has thus far revealed the presence of a single gene in the yeasts Saccharomyces cerevisiae, Schizosaccharomyces pombe and Kluyveromyces lactis, and in Drosophila; two genes in mouse cells; and three genes in human, chicken and tomato (Sorger and Pelham, 1988; Wiederrecht et al., 1988; Clos et al., 1990; Scharf et al., 1990; Jakobsen and Pelham, 1991; Rabindran et al., 1991; Sarge et al., 1991; Schuettet al., 1991; Gallo et al., 1993; Nakai and Morimoto, 1993; Nakai et al., 1997).

The analysis of HSF molecules from a number of organisms has revealed the presence of highly-conserved motifs: a DNA-binding domain contained within the amino-terminus that is also conserved in its three dimensional structure, an adjacent trimerization domain composed of three hydrophobic heptad repeats, and a fourth hydrophobic heptad repeat (Rabindran et al., 1993; Harrison et al., 1994; Vuister et al., 1994). Adjacent to the carboxy-terminal coiled coil lies a stress-responsive transcriptional activation domain (Green et al., 1995; Shi et al., 1995; Wisniewski et al., 1996). It is currently thought that in the absence of stress, intramolecular interactions between the HSF amino- and carboxy-terminal coiled coil domains sequester the protein in an inactive form (Rabindran et al., 1993; Zuo et al., 1994). Distinct from other HSFs thus far described, HSF from the yeasts S.cerevisiae and K.lactis also contain an amino-terminal transactivation domain (Nieto-Sotelo et al., 1990; Sorger, 1990).

HSFs bind to and activate transcription from a highly conserved promoter DNA sequence known as the heat shock element (HSE). The HSE is composed of two or more contiguous inverted repeats of the 5-base pair sequence nGAAAn (Xiao et al., 1991; Bonner et al., 1994). High affinity binding of HSF to the HSE requires the homo-trimerization of monomeric subunits, with each monomer contacting an individual pentameric sequence in the major groove (Perisic et al., 1989; Sorger and Nelson, 1989; Fernandes et al., 1994). The HSF of S.cerevisiae is thought to be largely constitutively trimeric and binds to many HSEs constitutively, however, some HSEs are inducibly bound by HSF in response to heat stress and pharmacological agents (Jakobsen and Pelham, 1988; Sorger and Nelson, 1989; Giardina and Lis, 1995). Drosophila HSF and mammalian HSF1 are activated in response to heat stress at the levels of trimerization, phosphorylation and DNA binding (Sarge et al., 1993; Zuo et al., 1995; Cotto et al., 1996; Orosz et al., 1996).
Once HSF binds to an HSE, transcriptional activation appears to be distinctly controlled and may involve additional regulatory events such as phosphorylation (Voellmy, 1994; Morimoto et al., 1996).

The yeast HSF gene is essential even in the absence of heat stress (Sorger and Pelham, 1988; Wiederrrecht et al., 1988). Furthermore, recent studies have shown that the Drosophila HSF is essential for oogenesis and early larval development (Jedlicka et al., 1997). Therefore, HSFs appear to activate the expression of genes required for cellular function under both physiological and stressful conditions. The presence of multiple higher eukaryotic HSF isoforms, in contrast to a single yeast or fly HSF, raises the possibility that distinct isoforms may mediate different biological functions. This hypothesis is supported by recent physiological and biochemical data indicating differences in HSF isoform tissue and development-specific expression patterns, ability to respond to distinct stimuli, and preferences for in vitro binding to different tandem arrangements of HSEs (Theodorakis et al., 1989; Schuetz et al., 1991; Sistonen et al., 1992; Kroeger and Morimoto, 1994; Sarge et al., 1994; Nakai et al., 1997). The mouse and human HSF1 isoforms have been demonstrated to activate transcription of a number of chaperone genes upon exposure to heat and other environmental or pharmacological stresses. Although not known to be activated by heat stress, HSF2 binds DNA in human erythroleukemia cells upon treatment of the cells with hemin, which simultaneously leads to differentiation along an erythroid lineage (Theodorakis et al., 1989; Sistonen et al., 1992). Human cells also contain a third isoform, HSF4, which has a similar anatomy to other HSF species, however little is known about its function in response to stress (Nakai et al., 1997). The presence of at least two different spliced isoforms for both the mouse HSF1 and HSF2 proteins (α and β) further underscores the potential different functions of HSF and its complex role in cellular growth and responses to stress (Fiorenza et al., 1995; Goodson et al., 1995).

Although HSF1 and HSF2 are differentially activated to bind DNA in mammalian cells, it is unclear whether these HSF isoforms have completely distinct roles or might functionally overlap. In most human tissues all three known HSF isoforms are expressed, complicating the assignment of their individual functional roles and regulatory responses (Nakai et al., 1997). To explore the degree of functional conservation between yeast and mammalian HSF molecules, human HSF1 and HSF2 were expressed in yeast cells lacking the single essential endogenous HSF gene. Interestingly, human HSF2, but not hHSF1, is capable of complementing the viability defect and conferring thermotolerance. However, derivatives of hHSF1 that give rise to a trimerized protein, through disruption of a carboxyl- or amino-terminal coiled-coil domain thought to engage in intra-molecular interactions that maintain the protein in a monomeric state, functionally substitute for yeast HSF. Analysis of the oligomerization status of these HSFs demonstrate a strict correlation between complementation and the ability of the proteins to trimerize. Surprisingly, hHSF2 expressed in yeast activates target gene transcription in response to thermal stress. Moreover, hHSF1 and hHSF2 exhibit selectivity for activation of two distinct yeast heat shock-responsive genes, which correlate with previously described mammalian HSF DNA binding preferences in vitro. These results demonstrated the remarkable functional conservation between yeast and human HSFs in their ability to sense stress signals and respond by activating target gene transcription.

Results

**Human HSF2 functionally substitutes for the S. cerevisiae HSF**

HSFs are highly conserved in the basic arrangement of functional domains, structure of the DNA binding domain and sequence of their cognate promoter element, the HSE (Wu, 1995). Therefore, we tested whether either of the two human isoforms previously shown to activate gene transcription in mammalian cells, hHSF1 and hHSF2, could functionally complement the viability defect of *S. cerevisiae* cells lacking the single endogenous HSF gene. The recipient for these investigations is *S. cerevisiae* strain PS145 (Sorger and Pelham, 1988), which bears a disruption of the chromosomal HSF gene and a multi-copy episomal plasmid in which the yHSF gene is under the control of the GAL1 promoter (GAL1-yHSF). Because GAL1 is induced by galactose and repressed by glucose, this strain grows well on galactose but does not grow on glucose, where yHSF expression is repressed. The hHSF1 and hHSF2 cDNAs were placed under the control of the constitutive yeast glyceraldehyde-3-phosphate dehydrogenase (GPD) gene promoter (Mumberg et al., 1995), transformed into PS145 cells, and the ability of these cells to grow on glucose was tested. Plasmid pRS313yHSF, a single copy plasmid with yHSF under control of the yeast HSF promoter, was used as a positive control. As shown in Figure 1A, strains expressing either yHSF, hHSF1, hHSF2 or both hHSF1 and hHSF2 grow at 30°C on plates containing galactose. However, when these strains are streaked to medium containing glucose as the sole carbon source, expression of either yHSF or hHSF2 allows cells to grow, while hHSF1 does not suppress the viability defect associated with yhsfΔ cells at temperatures up to 42°C (Figure 1A and data not shown). Co-transformation of both hHSF1 and hHSF2 has the same effect as hHSF2 alone, conferring no obvious growth advantage or disadvantage at any temperature tested. Furthermore, when the GAL1-yHSF plasmid was cured from these four strains by growth on 5-fluororotic acid (Boeke et al., 1987), complementation results identical to the glucose shut-off experiment were obtained (data not shown). To verify that hHSF1 and hHSF2 proteins are expressed in PS145 cells, the four strains were analyzed for expression of hHSF1 and hHSF2 proteins by immunoblotting (Figure 1B). This analysis clearly demonstrated that readily detectable levels of both hHSF1 and hHSF2 were expressed in the PS145 yeast strain, indicating that the lack of functional complementation by hHSF1 was not due to lack of expression. The expression of hHSF1 at higher levels using the strong GAL1 promoter on a multicopy plasmid still failed to suppress the yhsfΔ viability defect. However, expression of hHSF2, even at ~10-fold lower levels, complemented the yhsfΔ defect indistinguishably from that shown in Figure 1A (data not shown). Furthermore, we ascertained whether the viability defect associated with yhsfΔ cells
could be complemented by expression of the functionally homologous mouse HSF isoforms, mHSF1 and mHSF2, which exist as two splicing isoforms (α and β). Although all four mouse HSF isoforms were expressed in yhsfΔ cells (data not shown), mHSF2α and β, but not mHSF1α or β, complemented the yhsfΔ viability defect (Figure 1C). These results demonstrate that the functionally homologous human and mouse HSF2 proteins, but not HSF1, can substitute for the essential functions of yeast HSF.

**Localization of human HSFs in yeast**

To understand the mechanisms of human HSF isoform function in *S. cerevisiae* more completely, the intracellular location of hHSF1 and hHSF2 was analyzed in living yeast cells using green fluorescent protein (GFP) (Heim and Tsien, 1996). Fusions of GFP to the carboxyl-terminus of the complete coding regions yHSF, hHSF1 and hHSF2 were constructed and the function of these HSF–GFP fusion proteins ascertained by testing for their ability to complement the viability defect of the *yhsfΔ* strain PS145. YHSF–GFP and hHSF2–GFP, but not hHSF1–GFP, complemented the *yhsfΔ* viability defect in a manner indistinguishable from the unadulterated HSF proteins (data not shown). Furthermore, immunoblotting with anti-HSF-specific antiserum demonstrated the presence of the fusion protein, but no detectable unmodified HSF molecules in these cells. PS145 cells transformed with plasmids that express yHSF–GFP, hHSF2–GFP, or hHSF1–GFP (in the presence of p313yHSF) were grown on medium containing 5-FOA to cure the pGAL1-yHSF plasmid, and confocal microscopy was carried out to localize the HSF–GFP fusion proteins. As shown in Figure 2, GFP expressed in
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Fig. 2. Localization of functional yHSF, hHSF1 and hHSF2 green fluorescent fusion proteins in *S.cerevisiae*. The yHSF, hHSF1 and hHSF2 proteins were fused at their carboxyl-termini to green fluorescent protein (GFP), transformed into PS145 cells and their subcellular locations determined by confocal microscopy. GFP alone was expressed from the GPD promoter as a control. Cells with the GFP fusion proteins were treated with DAPI for nuclear DNA staining. Pseudo-red color was used for DAPI signals. (A) p414GPD–GFP (in the presence of p313yHSF). (B) p413GPD-yHSF–GFP. (C) p413GPD-hHSF2–GFP. (D) p424GPD-hHSF1–GFP (in the presence of p313yHSF). *GAL1*-yHSF were removed from these cells with 5-FOA selection. Each field shown represents projections of a number of Z series at 0.2–0.4 μm increments, with four fields represented per transformant.

these cells as an unfused protein is localized throughout the cell (panel A), while both yHSF–GFP and hHSF2–GFP are predominantly concentrated in the cell nucleus, as revealed by co-localization with nuclear DNA stained with DAPI (panels B and C). Similar to GFP, however, the hHSF1–GFP protein appears to be located throughout cells (panel D). Analysis of individual Z planes (0.2–0.4 μm increment) of the multiple-plane confocal images reveals a pattern of signals indistinguishable from the stacked images. Furthermore, no changes in the localization of any of these proteins were apparent after cells were subjected to heat shock at 40°C (data not shown). Therefore, the functional yHSF and hHSF2 proteins are primarily concentrated in the yeast nucleus while hHSF1, which is unable to function in yeast cells, is localized throughout the cells (cytosol and nucleus).

Functional complementation correlates with trimerization

High affinity binding of HSF molecules to HSE promoter elements requires homo-trimerization (Perisic *et al.*, 1989; Sorger and Nelson, 1989). Currently, it is thought that latent HSF1 molecules are rendered inactive for DNA binding by intra-molecular interactions between leucine zipper (coiled coil) domains located in the central (lz1, -2, -3) and at the carboxyl-terminal end (lz4) of the protein respectively (Figure 3A). One hypothesis is that heat stress or other signals activate HSF1 by unleashing this interaction and therefore allow HSF to form homotrimer via intermolecular interactions mediated by the coiled coil regions (Wu, 1995; Morimoto *et al.*, 1996; Voellmy, 1996). Therefore, one possible explanation for the lack of hHSF1 function in yeast was that it may fail to form homotrimers. To test this hypothesis, we introduced amino acid substitutions in three hydrophobic residues in the lz4 domain in hHSF1 that would be predicted to disrupt interactions between lz4 and lz1, -2, -3 (Figure 3A). A similar set of mutations in lz4 has previously been shown to render hHSF1 constitutively trimerized and competent for DNA-binding when transfected into human cells (Rabindran *et al.*, 1993; Zuo *et al.*, 1994). This mutationally altered version of hHSF1, designated hHSF1lz4m, complements the viability defect of the *yhsf*Δ deletion (Figure 3B). Intracellular localization experiments were carried out using an hHSF1lz4m–GFP fusion protein that also functionally complements *yhsf*Δ cells (data not shown). We found that hHSF1lz4m–GFP is present throughout the cells, suggesting that the protein is localized to both the cytosol and nucleus (Figure 3C).

To ascertain if functional complementation of *S.cerevisiae yhsf*Δ cells correlated with human HSF homotrimerization, cross-linking experiments were carried out on whole cell extracts prepared from PS145 cells transformed with plasmids expressing hHSF2, hHSF1lz4m, or hHSF1 (in the presence of yHSF). Crosslinking of cell extracts, followed by electrophoretic fractionation and
immunoblotting analysis clearly demonstrate that hHSF2 is constitutively trimerized in cells grown at 25°C, with no stimulation upon heat shock at 39°C (Figure 4, top). However, under both control and heat shock conditions, hHSF1 exists predominantly as a monomer, with a very small percentage of the total hHSF1 migrating at the position expected for a homodimer (Figure 4, center). As with hHSF2, the bulk of the hHSF1lz4m derivative, which complements the yhsfΔ viability defect, forms homotrimers both at 25 and 39°C (Figure 4, bottom). Under these conditions yHSF is constitutively trimerized (data not shown).

The ability of hHSF1lz4m to trimerize and complement the yhsfΔ viability defect suggested that hHSF1 could function in yeast once unleashed from its inactive monomeric state. Since hHSF2 efficiently trimerizes in yeast, whereas hHSF1 remained monomeric even under heat shock conditions, we ascertained which regions of hHSF1 are necessary for the maintenance of inactive monomers in yeast cells by constructing reciprocal chimeras between hHSF1 and hHSF2. Two sets of fusion proteins were made (Figure 5A): SphI chimeras join the DNA binding domain and LZ1 of one HSF to LZ2–3, LZ4 and the transcriptional activation domain of the other HSF; and AflII chimeras divide the HSF molecules between the DNA binding and first oligomerization domain. The SphI chimeras, therefore, contain heterologous sequences within leucine zippers 1–3, which would be predicted to disrupt intra-molecular interactions between amino-terminal and carboxyl-terminal coiled coil regions in both hHSF1/2 and hHSF2/1 (Kroeger and Morimoto, 1994; Zuo et al., 1994). The AflII chimeras, however, retain the intact LZ1–4 domains unique to HSF1 or HSF2. Both SphI chimeras and the hHSF2/AflII fusion complemented yhsfΔ cells...
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Fig. 5. Amino-terminal hHSF1 sequences negatively modulate trimerization in yeast. (A) Chimeric hHSF1-hHSF2 molecules composed of the indicated regions of hHSF1 or hHSF2 were expressed in PS145 cells and analyzed for trimerization as described in the legend to Figure 4. The sites of gene fusions are indicated as the restriction enzyme sites SphI or AflII. A (+) indicates the ability to trimerize or complement the yhsfΔ mutation and a (−) indicates an inability to trimerize or complement. (B) Complementation of the yhsfΔ strain by hHSF molecules and chimeras. The hHSF proteins and chimeric molecules shown in (A) were assessed for their ability to complement the viability defect associated with the yhsfΔ strain by growth on glucose. Plasmids expressing the indicated HSF proteins in strain PS145 are shown in each sector of the key.

for growth, however the hHSF1/2/AflII fusion, like hHSF1, did not (Figure 5B). To determine if viability correlated with trimerization, we conducted EGS cross-linking of each chimeric HSF protein expressed in yeast (summarized in Figure 5A). Both of the SphI fusions showed constitutive trimerization under both control and heat shock conditions (Figure 5A). The hHSF2/1/AflII chimera also formed trimers, even though this molecule possesses the intact coiled coil domains 1 through 4 from hHSF1. In contrast, hHSF1/2/AflII, which has the oligomerization domains of hHSF2, showed only trace levels of the expressed proteins as trimers. These observations strongly suggest that the monomer to trimer transition of hHSF1 is controlled at two levels. At one level, this transition is governed by intramolecular interactions between LZ1–3 and LZ4, as demonstrated by the analysis of the hHSF1lz4m derivative. Secondly, the monomer to trimer transition is negatively modulated by hHSF1 sequences encoded amino-terminally to the AflII site. This is demonstrated by the ability of hHSF1lz4m to trimerize and complement the yhsfΔ mutation and the inability of the hHSF1/2/AflII chimera to function. Furthermore, these data establish that the ability of human HSF isoforms to substitute for yeast HSF in the cell viability assay strictly correlates with the ability of these molecules to form homotrimers.

**Human HSF isoforms exhibit specificity for target gene activation in yeast**

HSFs bind to promoter HSEs and activate both basal and stress-inducible target gene transcription. To ascertain if the human HSF isoforms activate gene expression in yeast, we first determined whether the functional human HSF isoforms, expressed in yeast, are capable of binding to HSEs within yeast promoters by electrophoretic mobility shift experiments. For these experiments, HSE-containing promoter DNA fragments were used from the SSA3 gene, encoding a member of the yeast hsp70 family, and the CUP1 gene, encoding the yeast copper-binding metallothionein protein (Boorstein and Craig, 1990; Tamai et al., 1994; Liu and Thiele, 1996). These genes provide examples of a consensus heat shock responsive promoter HSE, containing five consecutive pentameric elements (SSA3, GTGGAAAGTTATAAGAAATACGAGAAGC) and an atypical HSE, containing two consecutive pentamers followed by a third unit after a gap in the periodicity (CUP1, CTTCAGAACGAAAAAGAAGC). Furthermore,
a derivative of each promoter in which each of the functional SSA3 or CUP1 HSE elements were mutagenically inactivated, was used as a control for HSE-dependent DNA binding. Extracts from cells expressing the yHSF, hHSF2 or hHSF1lz4m proteins bound to both the CUP1 and SSA3 promoters in vitro (Figure 6A). Furthermore, consistent with the constitutive trimerization of yHSF, hHSF2 and hHSF1lz4m expressed in yeast, this binding is not induced (and may be slightly inhibited) by heat shocking the cells prior to extract preparation but is absolutely dependent on the presence of a functional HSE (Figure 6B). Although a quantitative analysis of binding was not carried out, we observed a preference of hHSF2 for binding to the CUP1 HSE and hHSF1lz4m for binding to the SSA3 HSE when equivalent amounts of extract were used in each binding reaction. This is consistent with previous in vitro binding site selection studies carried out with mouse HSF1 and HSF2 (Kroeger and Morimoto, 1994), which demonstrated that HSF1 prefers an array of 4 to 5 units of nGAAAn pentameric consensus sequences, while HSF2 prefers 2 to 3 units.

Since both hHSF2 and hHSF1lz4m bind specifically to the CUP1 and SSA3 HSEs, RNase protection experiments were carried out using plasmid-borne CUP1- and SSA3-lacZ fusions to determine if these genes are activated by functional human HSF derivatives in yeast cells (Figure 7). In response to heat shock hHSF2 activated CUP1-lacZ transcription nearly 10-fold, and hHSF1lz4m activated CUP1-lacZ expression ~2-fold (Figure 7, bottom panels). In contrast, the SSA3-lacZ fusion was barely induced after heat shock in cells expressing hHSF2 as compared to ~4-fold induction by hHSF1lz4m (Figure 7, top panels). Both yeast promoters were strongly activated by heat shock in cells expressing the yHSF protein and in all cases, activation was dependent on functional CUP1 or SSA3 HSE elements (Figure 7). Therefore, the higher activity of hHSF2 for transcriptional activation of CUP1-lacZ and the stronger transcription of the SSA3-lacZ fusion by hHSF1lz4m is consistent with the different binding preferences of the two HSFs on CUP1 or SSA3 HSE elements (Figure 6). Expression from the lacZ reporter genes faithfully represented expression of the endogenous chromosomal genes (data not shown). Therefore, although both hHSF2 and hHSF1lz4m are constitutive with respect to trimerization and DNA binding in yeast cells like yHSF, target gene expression is significantly induced by heat shock. Importantly, these data demonstrate that hHSF2 is capable of gene activation in response to heat stress in yeast cells, via a promoter with an atypical HSE similar to that selected in vitro as a preferred binding site (Kroeger and Morimoto, 1994).

**Human HSFs foster acquired thermotolerance in yeast cells**

Since both hHSF2 and hHSF1lz4m activate target gene transcription in yeast in response to heat stress, we tested
whether yeast cells expressing hHSF2 or hHSF1lz4m can foster acquired thermotolerance. Yeast cells pre-treated with a moderately high temperature (37°C) can be subsequently protected from exposure to lethal temperature (50°C), which is called acquired thermotolerance. Induced synthesis of hsp104 during the pre-treatment has been shown to be important for acquired thermotolerance (Sanchez and Lindquist, 1990; Sanchez et al., 1993). Cells expressing yeast HSF, hHSF2 or hHSF1lz4m each supported the development of acquired thermotolerance at 50°C after a 30 min pre-incubation at 37°C, with hHSF1lz4m somewhat less active in this assay than yHSF or hHSF2 (Figure 8). Regardless of which HSF was expressed in PS145, a direct shift from 25 to 50°C resulted in rapid loss of cell survival.

**Discussion**

In eukaryotes, HSFs orchestrate coordinated cellular defenses in response to thermal stress and other stressful conditions. HSFs harbor similar functional domains and activate target gene transcription through a highly-conserved cis-acting promoter DNA binding site (Wu, 1995). However, the existence of multiple genes encoding distinct HSFs in higher plants and animals in contrast to the single, essential gene in yeasts raises two important questions: (i) How are the mammalian HSF isoforms functionally and evolutionarily related to the single yeast HSF? and (ii) What are the individual regulatory modes and contributions to gene transcription via HSEs by each HSF isoform? Since HSF1 and HSF2 are co-expressed in nearly all mammalian tissues, it is difficult to establish unequivocally their individual functions and contributions to the stress response. To address these questions, we have devised a yeast system to examine the functional conservation between *S.cerevisiae* and human HSFs and to analyze the activity of each human isoform independently. Our analysis revealed a significant difference in the ability of HSF1 and HSF2 to complement the viability defect associated with the deletion of the *yHSF* gene.

We show that HSF2, but not HSF1, could rescue yeast cells lacking *yHSF*, and this function correlated with the ability of these proteins to form homotrimers. Although the basis for the essential nature of the *S.cerevisiae* HSF, even under non-stress conditions, is currently unclear, these results suggest that HSF2 serves to activate transcription or perhaps play an essential promoter architectural role for genes whose products are essential for viability (Sorger and Pelham, 1988; Wiederrecht et al., 1988; Gross et al., 1993). Previously it has been demonstrated that episoal expression of the single *Drosophila* HSF gene could suppress the viability defect associated with deletion of the essential HSF gene in the fission yeast *Schizosaccharomyces pombe* (Gallo et al., 1993). However, *S.pombe* strains harboring dHSF had altered cell morphology and slow growth rates, due to a consequence of the episcopal expression system. In *S.cerevisiae*, however, no differences in growth between strains harboring hHSF2 or yHSF were detected. Surprisingly, HSF2 was found to respond to thermal stress, a stimulus not previously thought to activate HSF2 in mammalian cells (Sistonen et al., 1992). Furthermore, activation of target gene expression in response to heat shock occurred at 39°C, the heat shock activation temperature of *S.cerevisiae*. These data are consistent with previous reports in which HSFs expressed in heterologous systems adopt the thermal activation profile of the host (Clos et al., 1990, 1993; Gallo et al., 1993; Treuter et al., 1993; Hubel et al., 1995). A more significant observation is that hHSF2 is trimerized and capable of binding to HSEs at control temperatures in yeast while hHSF2-mediated expression of SSA3 and *CUP1* is clearly inducible in response to heat shock. These data imply that the thermal stress signal to activate transcription of these genes via hHSF2 is separable from trimerization.

Expression of hHSF2 also allowed yeast cells to acquire thermotolerance, protecting them from a lethal heat shock that was indistinguishable from the endogenous *yHSF*. The ability to survive heat shock may not be solely attributable to HSF-induced *HSF* gene expression. Rather, HSF may confer viability and allow cells to mount a thermotolerance response via an alternative pathway, such as the Msn2, Msn4-mediated activation of hsp104 (*Lindquist and Kim, 1996; Martinez-Pastor et al., 1996;
Schmitt and McEntee, 1996). Nevertheless, the current observations clearly establish the ability of hHSF2 to respond to heat shock to activate transcription of a target gene in an HSE-dependent manner. Thus, in addition to its putative roles in development, HSF2 may regulate a subset of unknown target genes under thermal stress conditions in mammalian cells.

Our results clearly establish a direct correlation between the ability of human HSF isoforms to trimerize and their ability to complement the viability defect of yhsfΔ mutants. A number of studies of mammalian, fly, and yeast HSF have identified key structural determinants for trimerization (Sorger and Nelson, 1989; Peteranderl and Nelson, 1992; Zuo et al., 1995; Orosz et al., 1996). As in mammalian cells, HSF1 is sequestered as an inactive monomer in yeast via intra-molecular interactions between LZ1–3 and LZ4. Indeed, mutations of the LZ4 coiled coil domain, previously shown to result in constitutive hHSF1 trimerization in human 293 cells (Rabindran et al., 1993), resulted in a constitutively trimeric hHSF1lz4m molecule which complemented the yhsfΔ viability defect. Unexpectedly, hHSF1lz4m did not show increased distribution to the nucleus at any temperature despite significant levels of trimer formation. Our confocal microscopy images revealed that both hHSF1 and hHSF1lz4m were distributed throughout all cellular compartments, including the nucleus. It is possible that, while overall localization did not change, the form of the nuclear hHSF1lz4m was trimeric and thus able to bind HSEs in essential target genes with high affinity and confer viability upon these cells. It is also possible that a higher percentage of hHSF1lz4m molecules are nuclear localized due to better trimerization than hHSF1, but the differences are not readily detectable with confocal microscopy.

In addition to the regulation of the HSF1 monomer to trimer transition via intra-molecular interactions across the hydrophobic coiled coils, other regions of hHSF1 clearly play an important regulatory role. Our analysis of hHSF1-hHSF2 chimeric molecules indicates that codons amino-terminal to the AflII restriction site negatively modulate the monomer to trimer transition of hHSF1 in yeast cells. The HSF2/AflII fusion, which contains the DNA-binding domain and a portion of the flexible linker from hHSF2, is functional in yeast despite harboring the amino- and carboxy-terminal coiled coil domains of hHSF1. The reciprocal hHSF1/2AflII fusion, however, does not form trimers or functionally replace the yeast HSF. These results indicate that sequences external to the coiled coil domains, encompassing the DNA binding domain and less-well conserved linker region, modulate the ability of hHSF1 to switch from the inactive monomer to the trimerized form in yeast cells. Consistent with this observation, amino acid residues found near the carboxyl-terminal transactivation domain, central region and carboxy-terminal end of the DNA binding domain all serve to regulate trimerization of the Drosophila HSF (Orosz et al., 1996). Since the amino-terminal end of the DNA binding domain has been demonstrated to play no significant role in the regulation of hHSF1 or dHSF trimerization in vivo (Rabindran et al., 1993; Orosz et al., 1996), it is possible that differences in the flexible linker between hHSF1 and hHSF2 may account for the differences in trimerization in yeast. The unstructured linker joining the DNA binding domain to the first coiled coil is thought to close off the hydrophobic core of the DNA binding domain and promote high affinity binding to the HSE by proper alignment of the DNA binding domains in the trimer (Flick et al., 1994; Harrison et al., 1994; Vuister et al., 1994). Deletion analysis of the Drosophila HSF demonstrated that this linker region is important for retention of the monomer under non-stress conditions (Orosz et al., 1996). It is currently unknown whether such elements represent sites involved in additional intra-molecular regulatory interactions or sites for the action of trans-acting factors that positively or negatively regulate HSF1 multimerization.

The yeast system that we have developed for human HSF expression has permitted an analysis of the differences in function between HSF1 and HSF2. The data presented here clearly demonstrate that hHSF2 and trimerizable derivatives of hHSF1 are capable of responding to thermal stress to activate gene expression in yeast. Furthermore, these results suggest that hHSF1 both binds to and activates transcription more strongly from the SSA3 (ksp70) promoter while hHSF2 transcriptional activity is higher for the yeast metallothionein (CUP1) promoter. Previous in vitro DNA binding and footprinting studies using purified mouse HSF1 and HSF2 proteins have demonstrated clear differences in HSF–HSE binding interactions (Kroeger et al., 1993; Kroeger and Morimoto, 1994). Binding site selection experiments have demonstrated that mHSF1 binds cooperatively to extended HSE elements much like those found in the SSA3 gene promoter, however, mHSF2 has a binding preference for HSEs harboring two or three pentameric consensus sequences, much like those found in the CUP1 promoter (Kroeger and Morimoto, 1994). Therefore, the differential transcriptional activity of hHSF2 and hHSF1 on the CUP1 and SSA3 promoters parallels HSF1 and HSF2 DNA binding preferences, and provides examples of target gene selectivity for the two HSF isoforms. However, this difference could be due to promoter specificity dictated by elements or factors other than the HSE alone. The fact that yeast HSF can activate both types of HSEs well suggests that the DNA binding domain of yHSF might be more conformationally flexible than either hHSF1 or hHSF2 (Flick et al., 1994). A previous observation that a single amino acid substitution in the yHSF DNA binding domain (V203A) can alter the specificity of yHSF on different promoters, resulting in an increased affinity for the CUP1 promoter and a decreased affinity for the SSA3 promoter, is consistent with this idea (Silar et al., 1991). Despite the promoter selectivity, both hHSF1lz4m and hHSF2 must act on essential target genes with similar potency, since they both complemented the viability defect of yhsfΔ when expressed in yeast. This work establishes the utility of yeast for exploring the precise mechanisms for stress signaling to the human HSF isoforms and demonstrates the striking conservation of this stress response from yeast to humans.

Materials and methods

Yeast plasmids, strains and growth conditions

Human or mouse cDNAs encoding HSF1 and HSF2 were subcloned into the yeast expression vectors p413GPD and p424GPD respectively (Mumberg et al., 1995). Mouse and human cDNAs encoding HSF...
isofoms were the generous gifts of Drs Carl Wu, Robert Kingston, Richard Morimoto and Kevin Sarge. *S.cerevisiae* HSF, under the control of the yeast *HSF1* promoter, was subcloned into pRS313 (Sikorski and Hieter, 1989). p424GPD-hHSF1lz4m was generated by inserting M391K, L395P and L398R mutations in the *hHSF1* DNA at the appropriate codons using PCR mutagenesis (Ausubel et al., 1987).

Human HSF *Spml* chimeras were generated by interchanging sequences 3’ of the *Spml* restriction site at codons 180 and 169 of *hHSF1* and *hHSF2* respectively, to generate plasmids p424GPDHSF1/2-Spml (pHSF2/1-Spml) and p423GPDHSF2/1-Spml. Like the mouse *HSF1* and *HSF2* chimeras (Kroeger and Morimoto, 1994), the human HSF chimeric proteins divide the molecules between leucine zipper 1 (LZ1) and leucine zippers 2–3 (LZ2–3) of the amino-terminal oligomerization domain. Thus pHSF1/2-Spml has the DNA binding domain and LZ1 from *hHSF1* and LZ2–3, the regulatory domain, LZ4, and the carboxyl-terminal activation domain from *hHSF2*, while pHSF2/1-Spml is precisely the reciprocal of pHSF1/2-Spml. To generate the *Af/Af* chimeras, a silent mutation was introduced at codon 125 (Leu) of *hHSF1* to create an *Af/Af* site (CTTAAAG, underlining indicates silent G→T mutation). A corresponding *Af/Af* site was introduced into *hHSF2* by the insertion of a single codon (GAA) encoding leucine (Leu114*) between amino acids 114 (Ser) and 115 (Lys), and making a silent A→G mutation in *Lys115* to create plasmid p423GPDPSH1Af/1Af. In both HSFs, this region corresponds to the flexible linker which joins the DNA binding domain to the oligomerization domain. The two *Af/1Af* chimeras were then created by exchanging the sequences 3’ of the *Af/1Af* sites in plasmids p424GALHSF1 and p424GDPHSF2-Af/1Af. The chimeras resulting from this exchange were fused in frame. p424GALHSF1/2-Af/1Af (pHSF1/2-Af/1Af) has amino acids 1–114* of *hHSF1* according to the DNA binding domain and amino acids 115–536 of *hHSF1* containing the LZ1–3, LZ2, and the regulatory and transactivation domains. p423GPDHSF2/1Af/1Af (pHSF2/1Af/1Af) contains amino acids 1–Leu114* of *hHSF2* and amino acids 126–517 of *hHSF1*; this construct has the single additional amino acid insertion (Leu114*). All constructs were sequenced to confirm that only desired mutations were introduced, and expression of each fusion protein confirmed by immunoblot. For immunoblot analysis, HSF antisera specific to the protein corresponding to the carboxyl-terminal portion of each chimera was used. All of the chimeras were expressed from the GDP promoter, except for pHSF1/2-Af/1Af which was driven by the GAL1 promoter. The GAL promoters had no effect on either complementation or trimerization assays, but provided higher levels of protein expression than the GDP promoter. The cDNA encoding green fluorescence protein (GFP) with S65T mutation (gift from Dr R.Tsien) (Heim and Tsien, 1996) was fused in-frame to the carboxy terminus of the yeast, *hHSF1*, *hHSF1* and *hHSF2* open reading frames to create the corresponding GFP fusion proteins. The function of the non-fused and GFP fusion proteins and the integrity in yeast cells was verified by viabilities of target gene expression assays and by immunoblotting. Target gene expression was assayed by RNSa protection experiments as previously described (Koch and Thiele, 1996) and quantitated by PhosphorImaging.

The *CUP1* promoter region containing the HSE (CTTCTAGGAAGCAAAAAGATC) was fused to the coding region of *Echerichia coli lacZ* gene to create YepSSA3-HSEWT-lacZ. YepSSA3-HSE-lacZ contains two base mutations in the HSECR that respectively render it unable to be bound by yHSF and non heat shock responsive. The HSE was from the *Ss43* promoter (182 to 135) was fused to the *Cyc1* basal promoter to generate YepSSA3-HSEWT-lacZ, and the HSE (GTGGAAATGTTAAGATTATCAGAACGCC) in YepSSA3-HSE-lacZ (Boorstein and Craig, 1990).

Yeast strains used in this study are derived from PS145, a gift of Dr Hillary Nelson (ade2-1 trpl can1-100 leu2-3,112 his3-11,15 ura3 his::LEU2 YcpGAL1-yHSF) and were all grown in synthetic complete (SC) medium or agar plates minus the indicated nutrients as selectable markers (Sorger and Pelham, 1988). In the strain designated ‘yHSF’, ‘hHSF1’ or ‘hHSF2’, the HSE of *hHSF1* or *hHSF2* was replaced by the *URA3* gene introduced by immunoblot ‘yHSF’, ‘hHSF1’ or ‘hHSF2’. Cells were then pelleted and resuspended in the same volume of fresh medium, and incubated at 39.5°C for 15 min before harvesting.

**Acquired thermotolerance assay**

Cells were grown overnight at 25°C to midlog phase, re-inoculated to OD650nm of 0.02–0.03, grown to 0.15–0.2 (~2x10^6/ml) at 25°C, and subjected to 37°C treatment in a waterbath shaker for 30 min before shifting to 50°C (Sanchez and Lindquist, 1990). For control experiments, cells were shifted directly from 25°C to 50°C. One hundred μl of each sample were withdrawn at the indicated time points, kept on ice and diluted 500-fold with ice-cold YPD medium. One hundred μl of the diluted cells were plated on YPD agar plates and incubated at 30°C for 2 days. Colonies were counted and normalized to the number of colonies at the zero time point.

**Immunoblot and cross-linking analysis**

Harvested cells were washed once in ice-cold sterile water, resuspended in SDS harvest buffer (0.5% SDS, 10 m Tris–HCl at pH 7.4, 1 mM EDTA) with an equivalent volume of glass beads and the following protease inhibitors: 1 mM pepstatin (Boehringer Mannheim, Indianapolis, IN), 8 μg/ml aprotinin, 4 μg/ml pepstatin, 2 μg/ml leupeptin. The mixture was vortexed for 1 min at top speed at 4°C for 3 times with 15 s intervals on ice. After centrifugation at 4°C, the protein concentration of the supernatant was determined by the Bradford assay and equal amounts of total cellular protein were subjected to SDS–PAGE. Cell extracts for cross-linking were prepared similarly in non-denaturing HEGN buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 10% glycerol, 0.4 M NaCl) and ethylene glycol bis-(sucinimidylsuccinate) (EGS) cross-linking was carried out as described (Sarge et al., 1993). Immunoblotting was carried out with reagents and protocols from Amersham, using yHSF antisera (gift from Peter Sorger), hHSF1 polyclonal antibody (gift from Carl Wu), and mHSF2 polyclonal antibody which specifically cross-reacts with hHSF2 (gift from Richard Morimoto).

**Electrophoretic mobility shift assay**

Harvested cells were washed and resuspended in HEGN0 buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 10% glycerol, 50mM NaCl), and cell extracts were similarly prepared as above (immunoblot). Twenty μg of total cellular protein was incubated for 20 min at room temperature with 2μP end-labeled oligonucleotides derived from either the *CUP1* or *SSA3* promoter encompassing the HSECUP or HSECASA, in the presence of binding buffer (12% glycerol, 12 mM HEPES, pH 7.9, 60 mM KCl, 2 mM MgCl₂, 4 mM Tris–HCl, pH 7.9, 0.12 mM EDTA, 0.6 mM DTT). One μl of polydI-Dc (1 μg/μl) was added to each reaction. The samples were electrophoretically fractionated on a 1.5% agarose gel at 120 V at 4°C, the gel dried and exposed to X-ray film (BioMax, Kodak) and subjected to PhosphorImager analysis.

**Conflocal microscopy**

The GFP fusion proteins were visualized by use of a Meridian confocal microscope (Ultima). Cells were inoculated from saturated cultures and allowed to grow in SC medium to OD650nm of ~0.7. DAPI (4′,6-diamino-2-phenylindole) was added to 1 μl of each cell culture to final concentration of 3 μg/ml for DNA staining. Cells were incubated on a rotating wheel for 3 h and washed with SC medium. Cells were then mixed with an equal volume of 1% low melting agarose and applied to slides. The samples were subjected to confocal microscopy analysis, using 100× objective lens, Argon-ion laser and the following filter sets: 530/30 BP (GFP), 460/40 BP (DAPI) and 485 LP dichroic. A Blue laser line of 180 mW laser power was used for detection of GFP signals, while a UV laser line of 60 mW laser power was used for DAPI signals. A pinhole size of 80 μm and a 3× digital zoom was used. A Z-series of the cells at 0.2–0.4 μm increments was projected to give the final image, according to the manufacturer’s software (Ultima System Software V4.19).

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