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# 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, homotrimerizes 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; Schuetz *et 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 carboxyl-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, intra-molecular interactions between the HSF amino- and carboxyl-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 nGAAn (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; Wiederrecht *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 developmental-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 ($\alpha$ and $\beta$) 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 *yhsf*Δ 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 *yhsf*Δ viability defect. However, expression of hHSF2, even at ~10-fold lower levels, complemented the *yhsf*Δ defect indistinguishably from that shown in Figure 1A (data not shown). Furthermore, we ascertained whether the viability defect associated with *yhsf*Δ cells

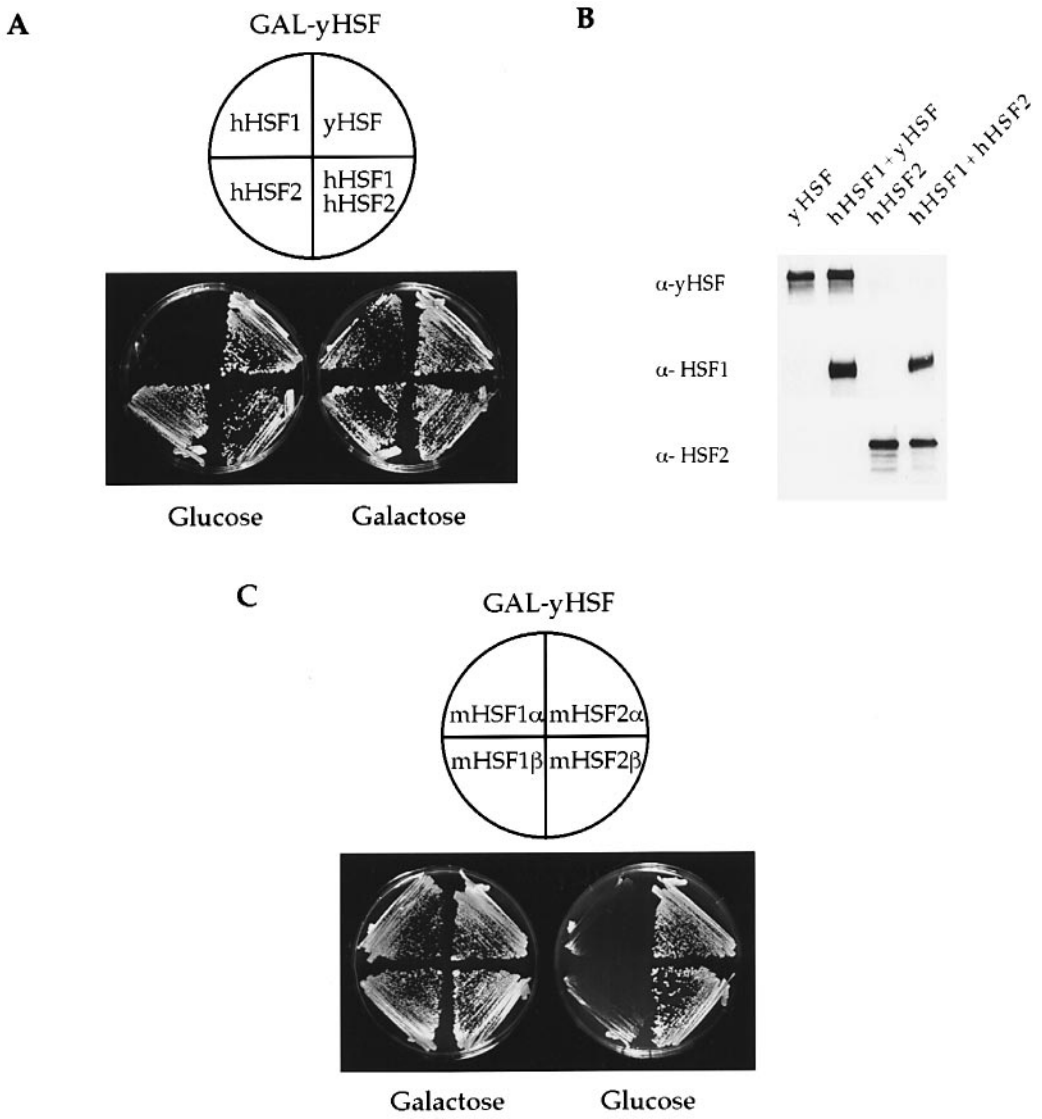


**Fig. 1.** The human and mouse HSF2 isoforms functionally substitute for the *S.cerevisiae* HSF. (**A**) The *S.cerevisiae hsf*Δ strain PS145, harboring a plasmid-borne *GAL1-yHSF*, was transformed with pRS313yHSF, p413GPD-hHSF2, p424GPD-hHSF1 or both p424GPD-hHSF1 and p413GPD-hHSF2. Recipient cells were streaked onto synthetic complete medium containing either 2% glucose or 2% galactose as carbon source, incubated at 30°C for 2 days and photographed. The individual transformants are indicated in the key shown above. (**B**) Extracts from cells transformed with plasmids expressing the indicated HSF molecules were analyzed by immunoblotting using anti-yHSF polyclonal antiserum, anti-hHSF1 polyclonal antibody or anti-mHSF2 polyclonal antibody, which specifically cross-reacts with hHSF2. The plasmids contained in PS145 cells are indicated on the top and the antibody preparations used for probing the immunoblot are indicated on the left. (**C**) PS145 cells transformed with plasmids p424GPDmHSF1α, p424GPDmHSF1β, p413GPDmHSF2α or p413GPDmHSF2β were treated as in (A).

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 *yhsf*Δ cells (data not shown), mHSF2α and β, but not mHSF1α or β, complemented the *yhsf*Δ 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 *yhsf*Δ strain PS145. YHSF–GFP and hHSF2–GFP, but not hHSF1–GFP, complemented the *yhsf*Δ 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

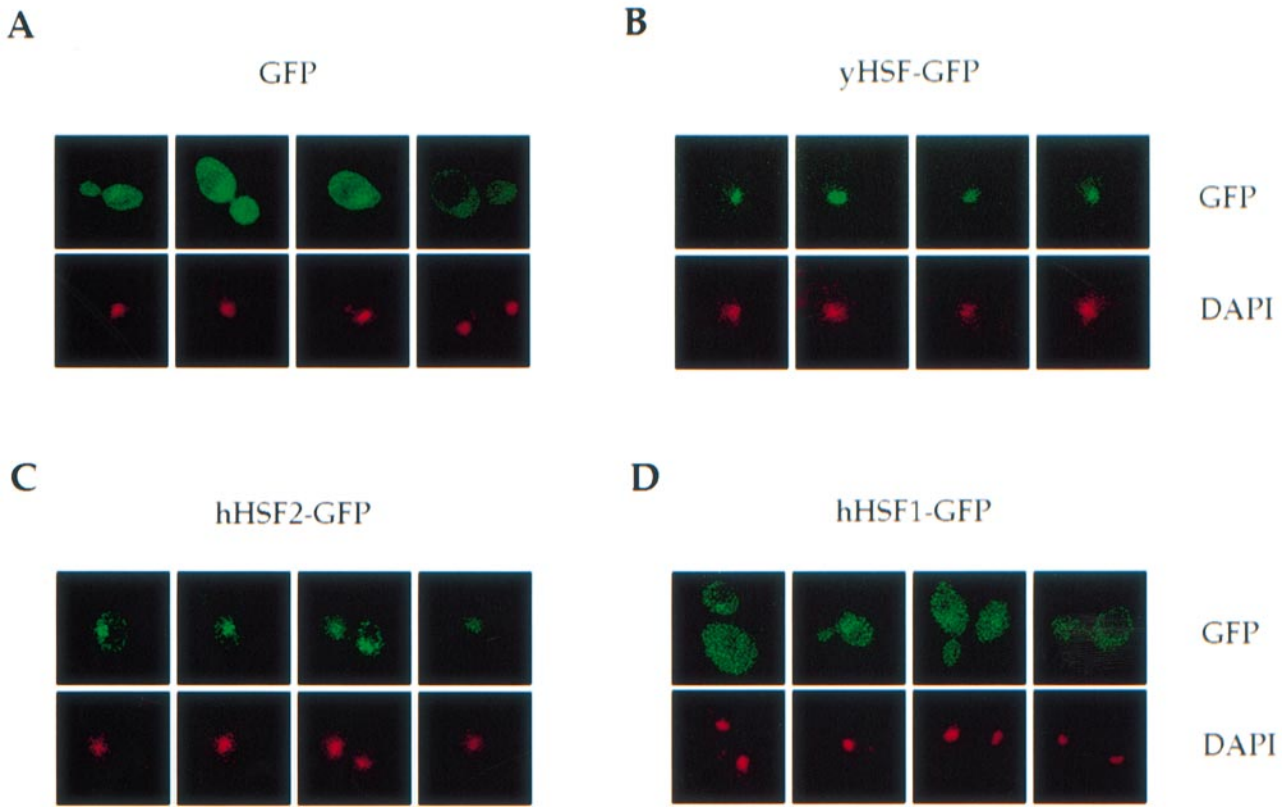


**Fig. 2.** Localization of functional yHSF, hHSF1 and hHSF2 green fluorescent fusion proteins in *S.cerevisiae*. The yHSF, hHSF1 and hHSF2 proteins were fused at their carboxyl-termini to green fluorescent protein (GFP), transformed into PS145 cells and their subcellular locations determined by confocal 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 homotrimers 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

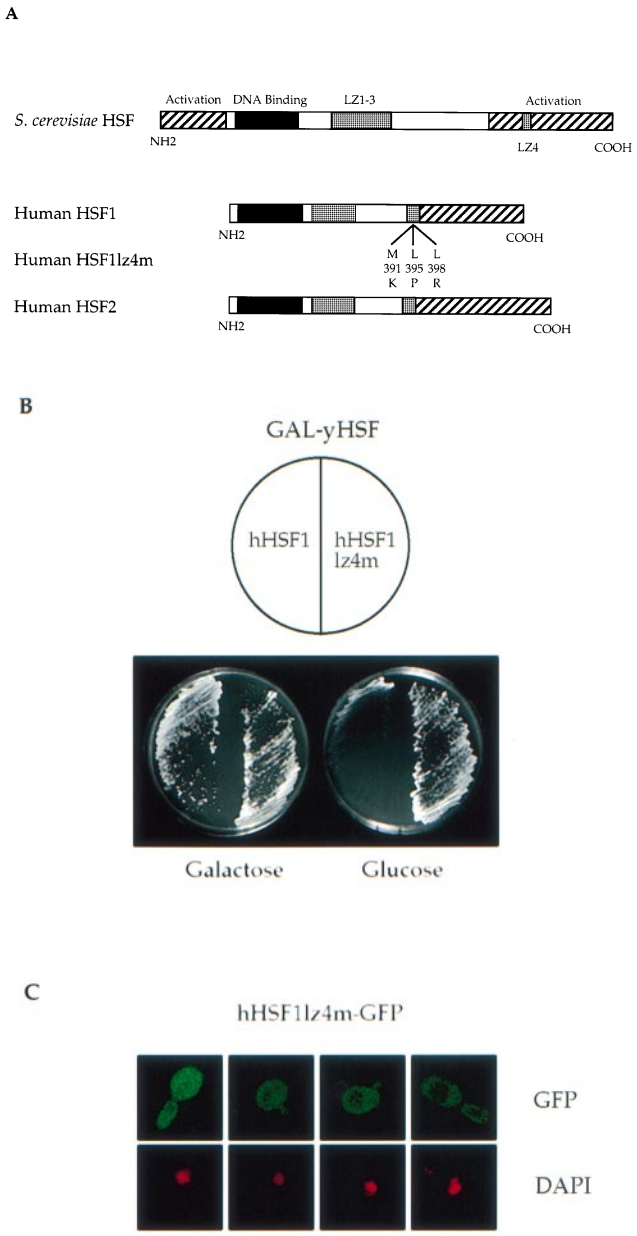


**Fig. 3**. Human HSF1 with a mutationally altered leucine zipper 4 functionally substitutes for yHSF. (**A**) Anatomy of the yHSF, hHSF2, hHSF1 and hHSF1lz4m proteins. The three point mutations in hHSF1lz4m, M391K, L395P and L398R are indicated. (**B**) Complementation analysis. *S.cerevisiae* strain PS145, transformed with p424GPD-hHSF1 or p424GPD-hHSF1lz4m, was streaked onto SC medium containing glucose or galactose as the carbon source, incubated at 30°C for two days and photographed. (**C**) Localization of hHSF1lz4m. Strain PS145, containing p424GPD-hHSF1lz4mGFP as the sole source of HSF, was examined by confocal microscopic analysis for GFP and DAPI as described in Figure 2. Four representative fields are shown.

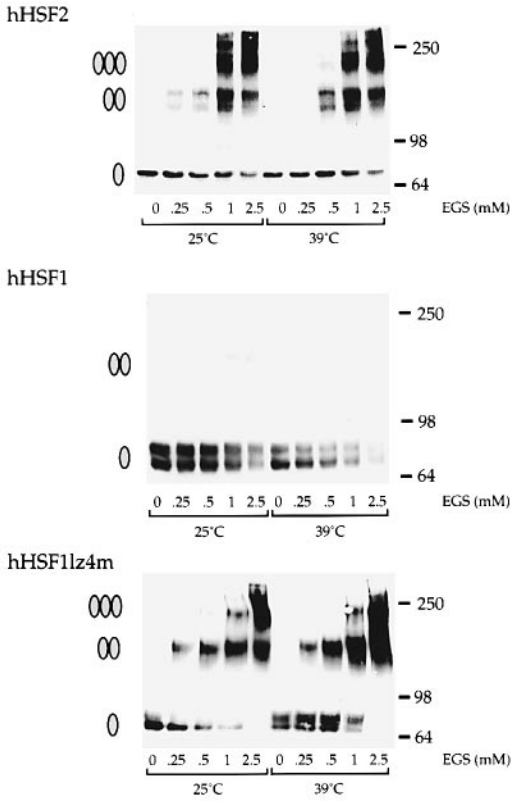


**Fig. 4.** Functionally competent hHSF isoforms trimerize in yeast cells. EGS cross-linking was carried out using native whole cell extracts from PS145 cells expressing either hHSF2 (top), hHSF1 in the presence of yHSF (middle) or hHSF1lz4m (bottom) at 25°C or subjected to 39°C heat shock for 15 min. EGS-treated and DMSO control extracts were electrophoretically fractionated on a 6% SDS–PAGE gel and immunoblotted with anti-mHSF2 or anti-hHSF1 antibody respectively. The positions of molecular weight markers are indicated on the right, EGS concentrations used in the cross-linking indicated on the bottom and ellipses indicate the expected migration of HSF monomers, dimers and trimers.

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 *yhsf*Δ 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 *yhsf*Δ 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): *Sph*I 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 *Afl*II chimeras divide the HSF molecules between the DNA binding and first oligomerization domain. The *Sph*I 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 *Afl*II chimeras, however, retain the intact LZ1–4 domains unique to HSF1 or HSF2. Both *Sph*I chimeras and the hHSF2/1*Afl*II fusion complemented *yhsf*Δ cells

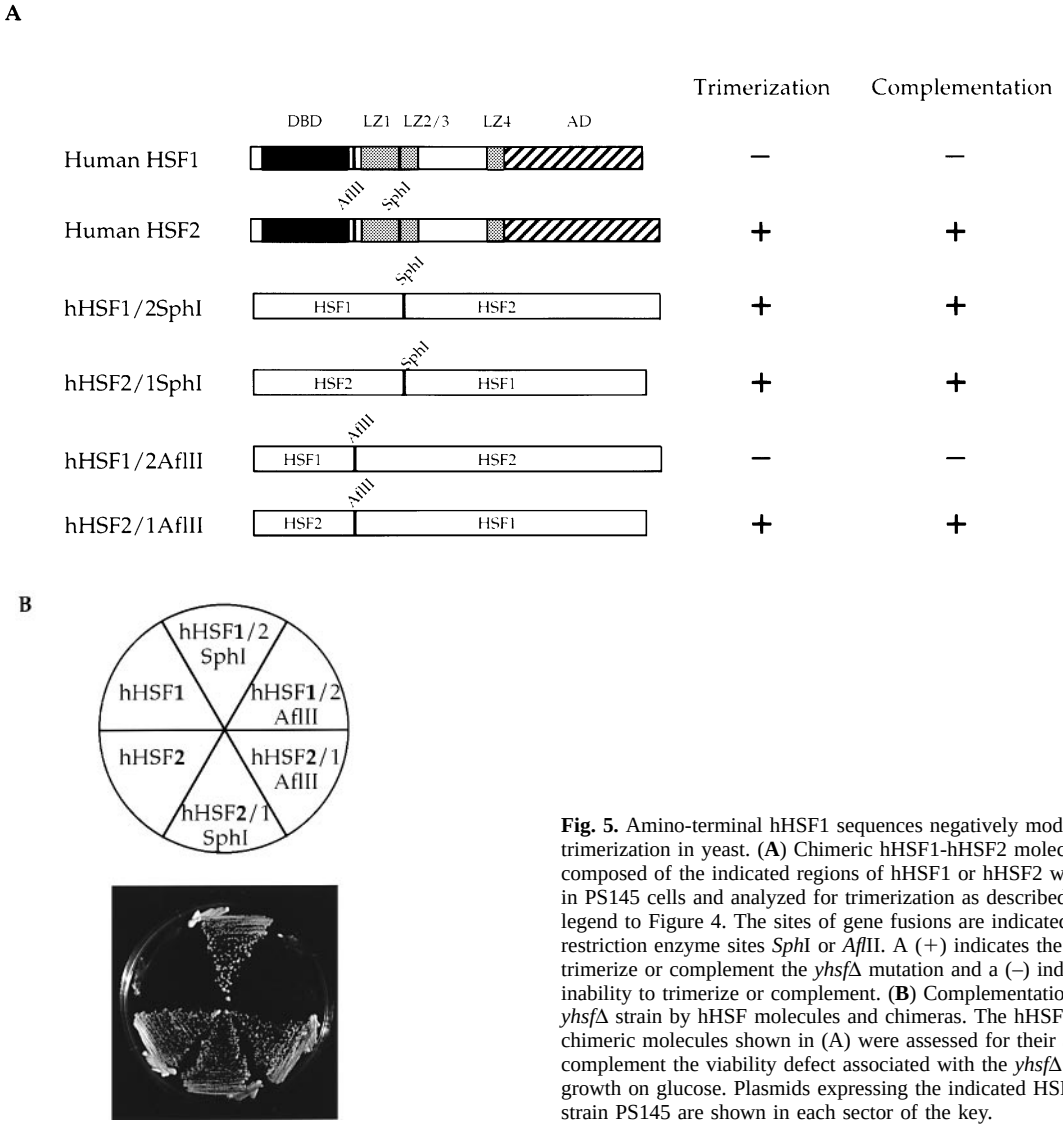


**Fig. 5.** Amino-terminal hHSF1 sequences negatively modulate trimerization in yeast. (**A**) Chimeric hHSF1-hHSF2 molecules composed of the indicated regions of hHSF1 or hHSF2 were expressed in PS145 cells and analyzed for trimerization as described in the legend to Figure 4. The sites of gene fusions are indicated as the restriction enzyme sites *Sph*I or *Afl*II. A (+) indicates the ability to trimerize or complement the *yhsf*Δ mutation and a (–) indicates an inability to trimerize or complement. (**B**) Complementation of the *yhsf*Δ 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 *yhsf*Δ 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*Afl*II 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 *Sph*I fusions showed constitutive trimerization under both control and heat shock conditions (Figure 5A). The hHSF2/1*Afl*II chimera also formed trimers, even though this molecule possesses the intact coiled coil domains 1 through 4 from hHSF1. In contrast, hHSF1/2*Afl*II, 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 *Afl*II site. This is demonstrated by the ability of hHSF2/1*Afl*II to trimerize and complement the *yhsf*Δ mutation and the inability of the hHSF1/2*Afl*II 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*, GTGGAAAGTTATAGAATATTACAGAAGC) and an atypical HSE, containing two consecutive pentamers followed by a third unit after a gap in the periodicity (*CUP1*, CTTCTAGAAGCAAAAAGAGC). Furthermore,

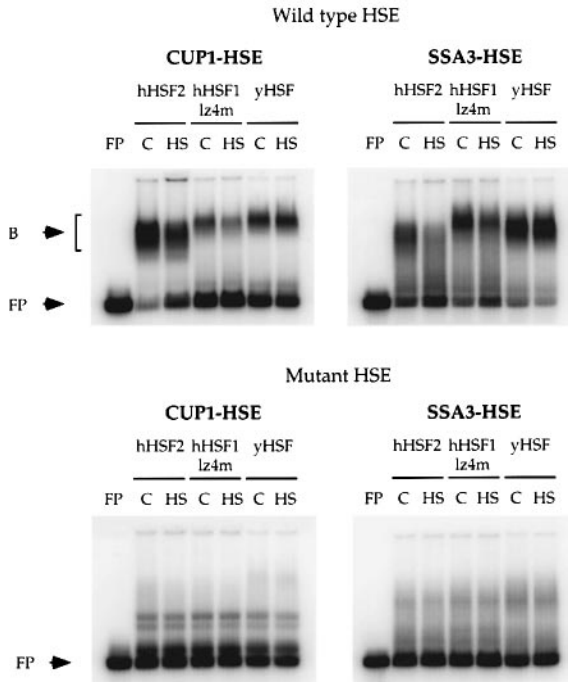


**Fig. 6.** The human HSF2 and HSF1lz4m proteins expressed in yeast bind to HSEs. Electrophoretic mobility shift assays using HSE-containing DNA fragments from the *CUP1* and *SSA3* promoters. *S.cerevisiae* PS145 cells expressing either yHSF, hHSF2 or hHSF1lz4m as their sole source of HSF were grown at 25°C (C) or subjected to a 39°C heat shock (HS) for 15 min, whole cell extracts prepared and 20 μg of each extract incubated with $^{32}$P-labeled DNA fragments encompassing the CUP1-HSE or SSA3-HSE, followed by electrophoretic fractionation on a 1.5% agarose gel. The arrows indicate the location of free DNA probes (FP) and the protein-bound probes (B). Electrophoretic mobility shift experiments carried out with probes containing a wild type HSE are shown on the top. The bottom panel shows the results of DNA binding studies using the same extracts as in the top panel, but with probes containing non-functional HSEs.

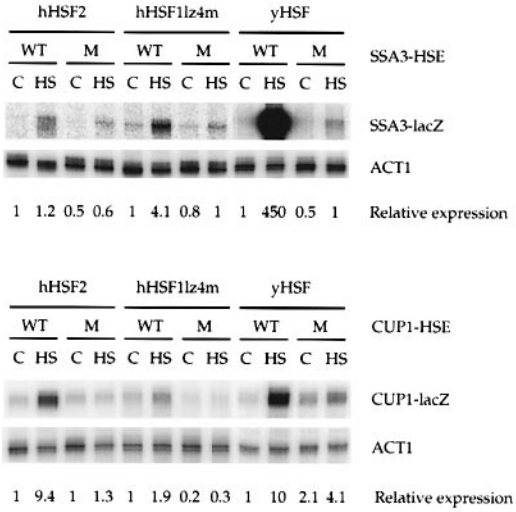


**Fig. 7.** Human HSF2 and HSF1lz4m activate target gene transcription in *S.cerevisiae hsf*Δ cells. *SSA3-lacZ* and *CUP1-lacZ* mRNA levels in response to heat shock. Strain PS145 cells expressing either yHSF, hHSF2 or hHSF1lz4m were independently transformed with the following plasmids: YepCUP1-HSEWT-lacZ, YepCUP1-HSE-M-lacZ, YepSSA3-HSEWT-lacZ and YepSSA3-HSE-M-lacZ. Transformants were grown at 25°C, subjected to control (C, 25°C) or heat shock (HS) treatment at 39.5°C for 15 min, total RNA isolated and *lacZ* mRNA levels were analyzed by RNase protection assays. WT and M refer to the SSA3 or CUP1 promoter derivatives with a wild type or functionally inactive HSE. The *S.cerevisiae* actin mRNA was used as an internal control. ACT1, SSA3-lacZ and CUP1-lacZ refer to the protected mRNA fragments using radio-labeled RNA probes specific for actin, SSA3-lacZ or CUP1-lacZ mRNA respectively. The relative expression levels for CUP1-lacZ and SSA3-lacZ mRNA, as determined by PhosphorImager analysis, are normalized to that of actin mRNA, with each wild type control assigned a value of 1.

a derivative of each promoter in which each of the functional *SSA3* or *CUP1* HSE elements were mutationally inactivated, was used as a control for HSE-dependent DNA binding. Extracts from cells expressing the yHSF, hHSF2 and 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 nGAAn 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* promoter (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

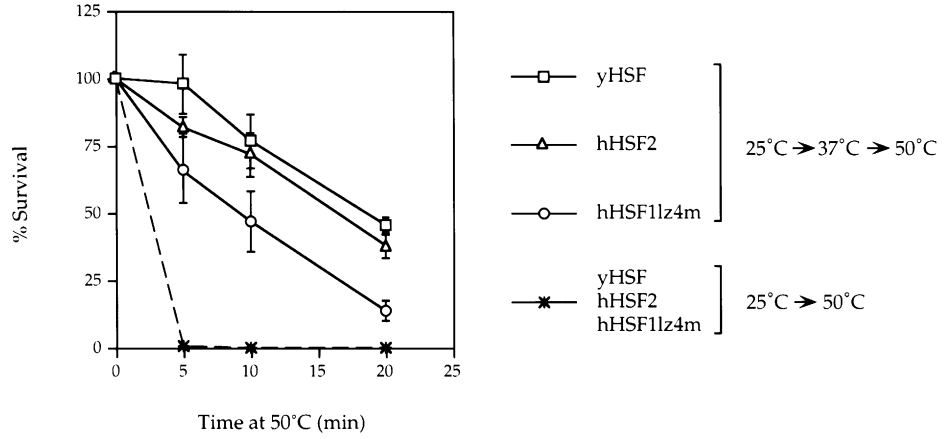


**Fig. 8.** Acquired thermotolerance of cells expressing yHSF, hHSF2 or hHSF1lz4m. PS145 cells expressing yHSF, hHSF2, or hHSF1lz4m were grown to early log phase at 25°C, shifted to 37°C for 30 min and subjected to 50°C heat treatment. Control cells were shifted directly from 25°C to 50°C. Cell aliquots were withdrawn at the indicated times, diluted, plated on YPD agar and plates incubated at 30°C. Percentage cell survival was plotted against the 0 time point samples. Shown are the average of five independent experiments (control 25°C→50°C represents the average of three independent experiments).

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 hsps 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 and *Drosophila* 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 episomal 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 episomal 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 *HSP* 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 *yhsf*Δ 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 *yhsf*Δ 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 *Afl*II restriction site negatively modulate the monomer to trimer transition of hHSF1 in yeast cells. The hHSF2/1*Afl*II 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 carboxyl-terminal coiled coil domains of hHSF1. The reciprocal hHSF1/2*Afl*II 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 carboxyl-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* (*hsp70*) 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 *yhsf*Δ 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

isoforms 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 introducing M391K, L395P and L398R mutations in the hHSF1 cDNA at the appropriate codons using PCR mutagenesis (Ausubel *et al.*, 1987).

Human HSF *Sph*I chimeras were generated by interchanging sequences 3′ of the conserved *Sph*I restriction site at codons 180 and 169 of hHSF1 and hHSF2 respectively, to generate plasmids p424GPDHSF1/2*Sph*I (pHSF1/2*Sph*I) and p423GPDHSF2/1*Sph*I (pHSF2/1*Sph*I). Like the mouse HSF1/2 *Sph*I chimeras (Kroeger and Morimoto, 1994), the human *Sph*I 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*Sph*I 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*Sph*I is precisely the reciprocal of pHSF1/2*Sph*I. To generate the *Afl*II chimeras, a silent mutation was introduced at codon 125 (Leu) of hHSF1 to create an *Afl*II site (CTTAAG, underlining indicates silent G→T mutation). A corresponding *Afl*II 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 p423GPDHSF2-*Afl*II. In both HSFs, this region corresponds to the flexible linker which joins the DNA binding domain to the oligomerization domain. The two *Afl*II chimeras were then created by exchanging the sequences 3′ of the *Afl*II sites in plasmids p424GALHSF1 and p423GPDHSF2-*Afl*II. The chimeras resulting from this exchange were fused in frame. p424GALHSF1/2*Afl*II (pHSF1/2*Afl*II) has amino acids 1–125 of hHSF1 corresponding to the DNA binding domain and amino acids 115–536 of hHSF2 containing the LZ1–3, LZ4, and the regulatory and transactivation domains. p423GPDHSF2/1*Afl*II (pHSF2/1*Afl*II) 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 to the protein corresponding to the carboxyl-terminal portion of each chimera was used. All of the chimeras were expressed from the GPD promoter, except for pHSF1/2*Afl*II which was driven by the *GAL1* promoter. The *GAL* promoter had no effect on either complementation or trimerization assays, but provided higher levels of protein expression than the *GPD* 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 carboxyl terminus of the yeast, hHSF1, hHSF1lz4m and hHSF2 open reading frames to create the corresponding GFP fusion proteins. The function of the non-fused and GFP fusion proteins and their integrity in yeast cells was verified by viability and target gene expression assays and by immunoblotting. Target gene expression was assayed by RNase protection experiments as previously described (Koch and Thiele, 1996) and quantitated by PhosphorImaging. The *CUP1* promoter region containing the HSE (CTTCTAGAAGCA-AAAAGAGC) was fused to the coding region of *Escherichia coli lacZ* gene to create YepCUP1-HSEWT-lacZ. YepCUP1-HSEM-lacZ contains two base mutations in the $HSE_{CUP1}$ as described (Tamai *et al.*, 1994), which renders it unable to be bound by yHSF and non heat shock responsive. The HSE from the *SSA3* promoter (–182 to –135) was fused to the *CYC1* basal promoter to generate YepSSA3-HSEWT-lacZ, and the HSE (GTGGAAAGTTATAGAATATTACAGAAGC) was mutated (GTGTAAAGATATATATTATAACAGCGGC) in YepSSA3-HSEM-lacZ (Boorstein and Craig, 1990).

Yeast strains used in this study are derived from PS145, a gift of Dr Hillary Nelson (*ade2-1 trp1 can1-100 leu2-3,-112 his3-11,-15 ura3 hsf::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 strains designated 'yHSF', 'hHSF2' or 'hHSF1lz4m', plasmids pRS313-yHSF, p413GPD-hHSF2 or p424GPD-hHSF1lz4m, were transformed into yeast strain PS145. Expression of yHSF protein in these strains from YCpGAL1-yHSF was eliminated either by repressing the *GAL1* promoter by growth on glucose, or by forced loss of the *URA3* containing YCpGAL1-yHSF plasmid using 5-fluoro-orotic acid selection (Boeke *et al.*, 1987). The lack of yHSF expression in these strains was confirmed by immunoblot analysis. For cross-linking analysis of the oligomerization status of the HSF species and target gene expression studies, cell cultures were grown in SC medium minus the indicated nutrients at 25°C for 24 h to saturation, re-inoculated to $OD_{650nm}$ of 0.03–0.1 in 5 ml of the same medium and grown at 25°C to early to mid-logarithmic phase ($OD_{650nm}$ of 1.0–1.5). 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 $OD_{650nm}$ of 0.02–0.03, grown to 0.15–0.2 (~$2\times10^6$/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 mM Tris–HCl at pH 7.4, 1 mM EDTA) with an equivalent volume of glass beads and the following protease inhibitors: 1 mM pefabloc (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*-(succinimidylsuccinate) (EGS) cross-linking was carried out as described (Sarge *et al.*, 1993). Immunoblotting was carried out with reagents and protocols from Amersham, using yHSF antiserum (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 $HEGN_{50}$ 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 $^{32}$P end-labeled oligonucleotides derived from either the *CUP1* or *SSA3* promoter encompassing the $HSE_{CUP1}$ or $HSE_{SSA3}$, in the presence of binding buffer (12% glycerol, 12 mM HEPES, pH 7.9, 60 mM KCl, 2 mM $MgCl_2$, 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.

### *Confocal 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 $OD_{650nm}$ of ~0.7. DAPI (4′,6-diamidino-2-phenylindole) was added to 1 ml 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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