THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 24, Issue of June 11, pp. 17219–17225, 1999

Printed in U.S.A.

Modulation of Human Heat Shock Factor Trimerization by the Linker Domain*

(Received for publication, February 11, 1999, and in revised form, April 6, 1999)

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Heat shock transcription factors (HSFs) are stress-responsive proteins that activate the expression of heat shock genes and are highly conserved from bakers' yeast to humans. Under basal conditions, the human HSF1 protein is maintained as an inactive monomer through intramolecular interactions between two coiled-coil domains and interactions with heat shock proteins; upon environmental, pharmacological, or physiological stress, HSF1 is converted to a homotrimer that binds to its cognate DNA binding site with high affinity. To dissect regions of HSF1 that make important contributions to the stability of the monomer under unstressed conditions, we have used functional complementation in bakers' yeast as a facile assay system. Whereas wild-type human HSF1 is restrained as an inactive monomer in yeast that is unable to substitute for the essential yeast HSF protein, mutations in the linker region between the DNA binding domain and the first coiled-coil allow HSF1 to homotrimerize and rescue the viability defect of a *hsf*Δ strain. Fine mapping by functional analysis of HSF1-HSF2 chimeras and point mutagenesis revealed that a small region in the amino-terminal portion of the HSF1 linker is required for maintenance of HSF1 in the monomeric state in both yeast and in transfected human 293 cells. Although linker regions in transcription factors are known to modulate DNA binding specificity, our studies suggest that the human HSF1 linker plays no role in determining HSF1 binding preferences *in vivo* but is a critical determinant in regulating the HSF1 monomer-trimer equilibrium.

The response of all eukaryotic organisms to thermal, environmental, and physiological stress involves the rapid production of a group of proteins called heat shock proteins (Hsps)[1] (1). At the level of transcription, this response is orchestrated by the heat shock transcription factor (HSF). HSF binds as a homotrimer to a conserved regulatory site, the heat shock element (HSE), composed of inverted repeats of the 5-base pair sequence 5′-nGAAn-3′ located in the promoters of heat-inducible Hsp genes (2, 3). The architecture of HSF is modular, conserved among all characterized HSFs, and composed of a winged helix-turn-helix DNA binding domain, an adjacent coiled-coil trimerization domain, a central regulatory domain, and a transcriptional activation domain at the carboxyl terminus (4). Additionally, many metazoan HSF molecules harbor a second hydrophobic repeat abutting the activation domain that is believed to play an important role in suppressing the activity of HSF under normal growth conditions (5, 6). In yeast and *Drosophila*, HSF is encoded by a single gene, but in mammals and higher plants, multiple HSF genes have been cloned (4, 7). Mammalian HSF1 is activated in response to many stresses, but the regulation and contribution of other HSF isoforms to stress responses and normal physiology remain largely unknown.

The activation of HSF1 occurs via a multistep process. Under non-stress conditions, mammalian HSF1 and *Drosophila* HSF (dHSF) appear as monomeric forms that exhibit little DNA binding activity (8–11). These latent HSF molecules are negatively regulated through intramolecular interactions between the amino-terminal and carboxyl-terminal hydrophobic domains. In response to stress, HSF converts from a monomer to a trimer (Fig. 1*A*). This level of regulation can be abrogated in the absence of stress by creating deletions or mutations within either hydrophobic domain that are predicted to disrupt the integrity of the hydrophobic heptad repeat, resulting in the formation of constitutively trimeric HSF molecules with a concomitant loss of monomeric species (5, 6, 12–15). Several deletion mutations of the amino, carboxyl, and internal regions of dHSF also result in constitutive trimerization, suggesting that the coiled-coil interactions that restrain the inactive protein may be stabilized by additional regions within the HSF monomer (5, 13, 16). Recently, interactions between HSF1 and the molecular chaperone Hsp90 have been detected, suggesting a role for additional factors in its regulation (17, 18). Upon trimerization, HSF undergoes a significant conformational change with the amino-terminal hydrophobic domains from individual monomeric units, forming a stable α-helical structure consistent with a triple-stranded coiled-coil (10, 19). Trimerization leads to high affinity DNA binding to the HSE (10, 20); however, transcriptional activation appears to be distinctly regulated from DNA binding because these events can be uncoupled by some nonsteroidal anti-inflammatory drugs (21). Transcriptional activity requires the unmasking of the carboxyl-terminal activation domain and correlates with hyperphosphorylation of HSF1 in many, but not all, instances (11, 22, 23). Attenuation of the heat shock response occurs through feedback regulation of active HSF trimers by interactions with Hsp70 and heat shock factor-binding protein 1 (24–26).

Significant structural, functional, and regulatory aspects of metazoan and yeast HSF proteins are conserved. An intriguing difference is that the single HSF gene in both budding and

* This work was supported in part by a Taisho Excellence in Research Program Award from Taisho Pharmaceuticals, Co. Ltd and a grant from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a National Research Service Award (GM18858) Postdoctoral Fellowship.

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[1] The abbreviations used are: Hsp, heat shock protein; HSF, heat shock transcription factor; hHSF, human HSF; dHSF, *Drosophila* HSF; yHSF, *Saccharomyces cerevisiae* HSF; HSE, heat shock element; LZ, leucine zipper; GFP, green fluorescent protein; HEGN, 20 mM Hepes, pH 7.9, 0.5 mM EDTA, 10% glycerol, and 100 mM NaCl.

fission yeast is essential for viability even under non-stress conditions, whereas dHSF is dispensable for cell growth (27–30). Flies lacking HSF, like mouse embryonic stem cell knockouts of HSF1, are sensitive to heat shock, indicating a primary role for HSF1 in protection against thermal stress (30, 31). Consistent with their requirement under all growth conditions, HSFs from the yeasts *Saccharomyces cerevisiae* and *Klyveromyces lactis* are found as trimers and exhibit constitutive DNA binding activity at all temperatures and conditions tested (20, 32, 33). The occupancy of specific HSEs in the *S. cerevisiae HSP82* promoter increases upon heat shock, suggesting that some yeast HSEs are inducibly bound, as observed in higher eukaryotes (34). We previously established an assay to examine the functional relatedness between the human HSF (hHSF) isoforms and *S. cerevisiae* HSF (yHSF) by testing for the ability of hHSF1 and hHSF2 to support the growth of *hsf*Δ yeast cells (35). We found that hHSF2, but not hHSF1, was capable of complementing the viability defect of *hsf*Δ yeast. Human HSF1 expressed in *S. cerevisiae* was found as an inactive monomer under both control and heat shock conditions. A derivative of hHSF1 with point mutations in the carboxyl-terminal hydrophobic heptad repeat (hHSF1-LZ4) or chimeric molecules between hHSF1 and hHSF2 joined within the amino-terminal trimerization domain could both trimerize and support *hsf*Δ cell growth. These observations indicate that the defect in hHSF1 function in yeast is related to the regulation of the monomer-to-trimer transition of hHSF1, rather than an inability of hHSF1 to activate appropriate target genes.

In this investigation, we have used the yeast assay system to identify novel hHSF1 amino acid residues that regulate the monomer-to-trimer transition. We demonstrate that sequences between the DNA binding domain and first trimerization domain, known as the flexible linker region, strongly modulate the equilibrium between the hHSF1 monomer and trimer in yeast and mammalian cells. In yeast, linker mutants of hHSF1 trimerize constitutively and support the growth of *hsf*Δ cells. Importantly, when expressed in human cells, these same hHSF1 derivatives are constitutively trimerized and bound to HSEs. The linker sequences do not alter the preferences for target gene activation by hHSF1 and hHSF2, suggesting that the hHSF1 linker domain plays a selective role in modulating oligomerization. These results illustrate the complexity in the regulation of the hHSF1 monomer-to-trimer transition and demonstrate the utility of the yeast model for analysis of the regulation and function of individual mammalian HSF proteins.

EXPERIMENTAL PROCEDURES

Yeast Strains, Growth Conditions, and Plasmids—Yeast expression plasmids p424GPDHSF1 and p413GPDHSF2 harboring the complete hHSF1 and hHSF2 cDNAs, respectively, under the control of constitutive yeast promoters were described previously (35). All linker mutations were constructed by oligonucleotide-directed mutagenesis and sequenced to confirm that only desired mutations were introduced. A complete listing of the mutants generated and used in these studies is shown in Fig. 2*A*. The recipient strain for all experiments was the W303 derivative PS145 (*MAT* ***a*** *ade2*-1 *trp1 can 1*-100 *leu2*,3-112 *his3*-11,15 *ura3 hsf*::*LEU2* YCp*GAL1-yHSF*; a gift from Dr. Hillary Nelson (27)). Plasmids harboring hHSFs were transformed according to the polyethylene glycol-lithium acetate procedure and tested for the ability to complement *hsf*Δ cells as described previously (35). Cells were routinely grown at 30 °C in selective synthetic complete medium and heat shocked for 15 min by submersing tubes into a shaking water bath equilibrated to 40 °C.

Yeast Cell Biological and Biochemical Techniques—Yeast cell extracts were prepared by glass bead disruption in HEGN containing 1% Triton X-100, 0.5 mM dithiothreitol, and protease inhibitors, and immunoblotting was performed as described previously (35) using antibodies specific to HSF1 (a gift from Dr. Carl Wu) or HSF2 (a gift from Dr. Richard Morimoto). Procedures for ethylene glycol bis(succinimidyl succinate) cross-linking, immunoblotting, RNase protection assays, and fluorescence microscopy have been described previously (35).

Mammalian Cell Culture, Plasmids, and Transfections—The mammalian expression vector pBC19 containing wild-type hHSF1 cDNA under the control of the human cytomegalovirus promoter was a gift from Dr. Stuart Calderwood. All linker derivatives were directly subcloned into this vector. Human embryonic kidney 293 cells obtained from American Type Culture Collection were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and supplemented with penicillin and streptomycin. Culture plates containing approximately 4×10^5 cells were transfected by calcium phosphate precipitation of DNA consisting of 0.5 μg of expression plasmid and 19.5 μg of carrier (pKS$^-$). After a 4-h incubation at 37 °C, the precipitate was aspirated, and the cells were washed with phosphate-buffered saline and fed with complete medium. To limit the level of overexpression, transfections were performed with a low amount of expression DNA, and the time of expression was limited to no more than 14 h. Cells were heat shocked for 20 min by submersing sealed plates into a water bath equilibrated to 42 °C. Cells were harvested by washing twice with ice-cold phosphate-buffered saline and scraped in phosphate-buffered saline, and cell pellets were frozen in liquid nitrogen.

Mammalian Cell Extracts and Biochemical Techniques—Whole cell extracts were prepared by thawing frozen cell pellets in 20 mM Hepes, pH 7.9, 0.5 mM EDTA, 10% glycerol (HEG) containing 0.42 M NaCl, 1.5 mM $MgCl_2$, and protease inhibitors (35), dispersing the cells by repeated pipetting and incubating tubes on ice for 15 min. The extracts were clarified in a microcentrifuge at 14,000 rpm for 15 min at 4 °C, and an aliquot of the supernatant (2 μg of protein) was used in DNA binding reactions in buffer containing 20 mM Hepes, pH 7.9, 0.5 mM EDTA, 75 mM NaCl, 10% glycerol, 0.5 μg of poly(dI-dC), and approximately 0.2 ng of ^{32}P-labeled DNA probe containing a consensus HSE with four inverted repeats of the recognition element 5′-nGAAn-3′ (11). Protein-DNA complexes were resolved on a 3.5% native polyacrylamide gel in 0.5× Tris borate-EDTA buffer.

RESULTS

To identify regions of the hHSF1 molecule that could influence the monomer-to-trimer conversion (Fig. 1*A*), we used a yeast assay based on the ability of heterologous HSFs to support the growth of *hsf*Δ cells (35). We observed previously that sequences amino-terminal to the first trimerization domain of hHSF1 appeared to modulate trimerization in yeast because replacing the hHSF1 DNA binding domain and linker with homologous sequences from hHSF2 resulted in a constitutively trimerized chimeric HSF molecule. To examine the role of this region in detail, we exchanged sequences encompassing the linker from hHSF2 into hHSF1 (Fig. 1*B*). In these studies, we define the linker as sequences between the end of β-sheet 4 in the HSF1 DNA binding domain (36, 37) and the first hydrophobic amino acid of LZ1 (amino acids 102–136 for hHSF1; amino acids 94–125 for hHSF2). Replacement of the complete hHSF1 linker by hHSF2 sequences resulted in a molecule (M1+M2) that could complement *hsf*Δ cells for growth (Fig. 1*C*). Subsequently, we interchanged smaller segments of the linker and identified that amino-terminal sequences (M1), but not the carboxyl-terminal region (M2), supported growth of *hsf*Δ cells, although both proteins were expressed to similar levels in yeast cells (Fig. 1*D*). Comparison of the hHSF1 and hHSF2 sequences encompassed by M1 revealed only five amino acid differences. Therefore, to determine the precise residues responsible for complementation by hHSF1, we constructed two additional linker mutations encompassing amino acids 103–106 (M3) and amino acids 109–110 (M4). Only M4 conferred viability, although yeast cells expressing this allele grew more slowly than other hHSF1 alleles that complemented growth, whereas the M3 allele did not support any growth. The M4 allele, harboring the double mutation (E109D, Q110D), was the minimal swap that could confer viability in yeast because a single amino acid (Q110D) substitution did not allow growth, nor did a double alanine substitution for EQ (data not shown). Deletion mutants within the hHSF1 linker, ΔST and ΔTSV, which attempt to mimic the gap in the hHSF2 linker, also did

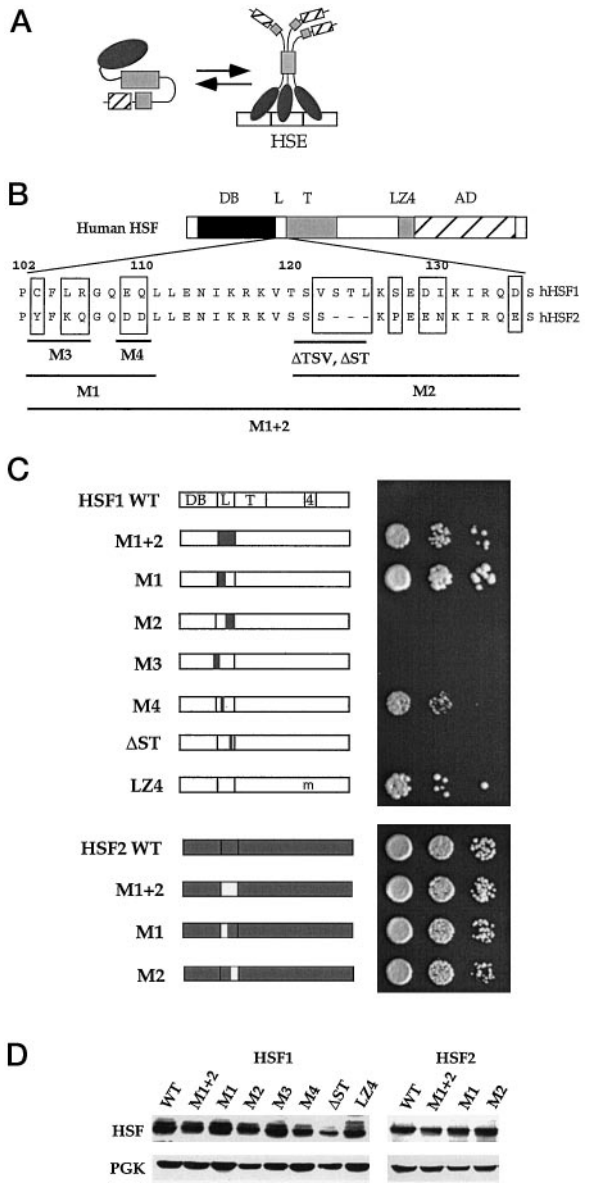

FIG. 1. **Complementation of the yeast *hsf*Δ allele by hHSF1 and hHSF2 linker mutants.** *A*, model for the conversion of latent, monomeric hHSF1 into trimeric HSF that can bind to a HSE with high affinity (6, 10). *B*, comparison of primary amino acid sequences between the hHSF1 and hHSF2 linker regions. *DB*, DNA binding domain; *L*, linker; *T*, trimerization or leucine zippers 1–3; *LZ4*, leucine zipper 4; *AD*, transcriptional activation domain. The *numbers* refer to the hHSF1 amino acid sequence. *M1* through *M4* refer to mutants constructed by directly swapping the indicated sequences from one HSF isoform to the other. Δ*ST* and Δ*TSV* refer to deletion mutations made in the hHSF1 background. *C*, glucose shut-off assays reveal a region of the linker critical for restraining hHSF1 in an inactive state in yeast. Sequences from hHSF1 are represented in *white*, and sequences from hHSF2 are represented in *gray*. *m* refers to a mutation in the LZ4 domain. PS145 cells harboring the indicated hHSF isoforms were grown in galactose medium to permit the expression of a plasmid-borne copy of yHSF under the control of the *GAL1* promoter. Three 10-fold serial dilutions (from *left* to *right*) of these cells were plated to glucose medium that represses *GAL1*-driven yHSF expression. Plates were photographed after a 3-day incubation at 30 °C for hHSF1-expressing cells or a 2-day incubation for hHSF2-expressing cells. *D*, levels of hHSF1 and hHSF2 were detected by immunoblotting with specific antibodies against each protein. Levels of yeast phosphoglycerate kinase (*PGK*) were used to normalize sample loading.

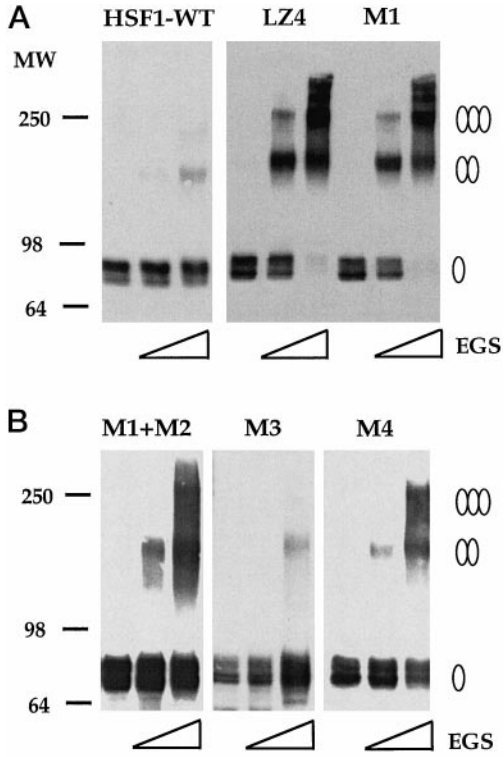

FIG. 2. **Constitutive trimerization of hHSF1 linker derivatives correlates with the ability to confer viability to yeast cells.** Whole cell extracts from yeast expressing the indicated hHSFs were cross-linked with 0 (Me_2SO vehicle alone), 0.5, or 2.5 mM ethylene bis(succinimidyl succinate) (indicated by the ramp from *left* to *right*) and resolved by denaturing SDS-polyacrylamide gel electrophoresis. hHSF1 proteins were detected by immunoblotting with a specific antibody against hHSF1. The monomeric, dimeric, and trimeric states are depicted to the right of the figure as *one, two,* or *three ovals*, respectively. The positions of molecular weight standards are indicated on the *left*. The designations for hHSF1 proteins are described in the legend to Fig. 1*B*.

not complement the viability defect of *hsf*Δ cells (Fig. 1*C*; data not shown); however, these proteins were consistently expressed at lower levels than other hHSF1 linker derivatives, suggesting that they may be unstable in yeast (Fig. 1*D*). These data demonstrate that whereas the M4 mutation is the minimal substitution necessary for allowing hHSF1 to function in yeast, complementation to the extent observed for the LZ4 mutant was obtained by substitution of all five amino acids in the amino-terminal portion of the hHSF1 linker that differ from hHSF2. To determine whether these hHSF1 linker sequences could act autonomously to suppress hHSF2 function, we constructed the reciprocal molecules by substituting hHSF1 linker sequences into hHSF2. All of the hHSF2 derivatives supported the growth of *hsf*Δ cells as well as wild-type hHSF2 (Fig. 1*C*), suggesting that the hHSF1 linker acts through a specific interaction within hHSF1 that is not conserved in hHSF2.

We previously observed a strict correlation between the ability of hHSFs to form trimers in yeast under normal growth temperatures and their ability to confer viability to *hsf*Δ cells (35). To examine whether the linker derivatives described in Fig. 1 also show a similar correlation, we performed ethylene bis(succinimidyl succinate) cross-linking on extracts from yeast cells expressing both hHSFs and yHSF to permit the assay of all hHSF1 derivatives, including those that failed to support viability. In agreement with our previous observations (35), wild-type hHSF1 was found primarily as a monomeric species in yeast, whereas the LZ4 mutant was found almost quantitatively in higher oligomers (Fig. 2). Linker mutants that complemented the growth defect of *hsf*Δ cells (M1+M2, M1, and M4) all formed some trimers, whereas mutants that failed to

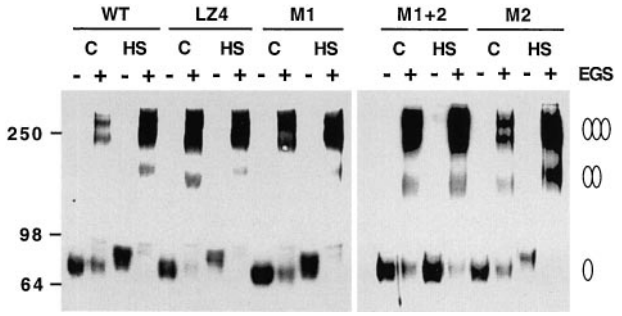

FIG. 3. **Oligomerization states of hHSF1 linker mutants expressed in transiently transfected human embryonic kidney 293 cells.** Cells were transfected with plasmids expressing the indicated isoform of hHSF1. After 14 h, cells were maintained at 37 °C (C) or heat shocked for 20 min in a 42 °C water bath (*HS*). Whole cell extracts were incubated with Me_2SO alone (−) or 0.5 mM ethylene bis(succinimidyl succinate) (+), and the oligomeric status of hHSF1 was detected by immunoblot analysis. The designations for hHSF1 proteins are described in the legend to Fig. 1*B*. The oligomeric states of HSF are depicted as *ovals* to the *right* of each panel, with molecular weight standards indicated to the *left*.

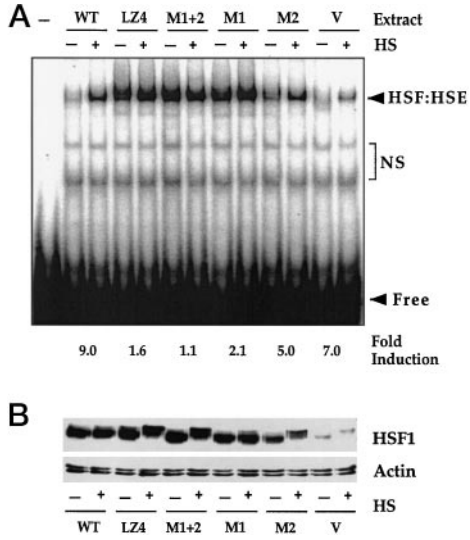

FIG. 4. **Mutations in the linker result in constitutive DNA binding activity of hHSF1.** Cells were transfected and treated as described in the legend to Fig. 3. *A*, mobility shift analysis of HSF binding to a consensus HSE composed of four inverted repeats of the sequence nGAAn; *V* refers to empty vector (pcDNA3.1)-transfected cells. The specific hHSF-HSE complex is indicated; *NS* indicates nonspecific binding by extracts to the probe that cannot be competed by the addition of excess cold oligonucleotide. − and + refer to control and heat shocked cells, respectively. Quantitation of HSF-HSE complexes was performed on a Molecular Dynamics PhosphorImager. Fold induction was calculated by dividing the heat shock HSF:HSE value by the paired control HSF:HSE value. *B*, immunoblot analysis of the same extracts used in *A* for DNA binding to show the levels of hHSF1 proteins expressed in transfected 293 cells. β-Actin was used to normalize the amount of extract in each lane on the same filter after stripping off the HSF1 antibody.

support the growth of *hsf*Δ cells (M3; data not shown) also failed to trimerize. We considered the possibility that complementation by the hHSF1-M1+M2 derivative could have resulted from enhanced nuclear localization because a hHSF2 bipartite nuclear localization signal is contained within the linker sequences (38). We have previously shown that green fluorescent protein-tagged hHSF2 (hHSF2-GFP) was concentrated in the yeast nucleus, like yHSF-GFP, whereas both hHSF1-GFP and LZ4-GFP fusions showed diffuse fluorescence throughout the cell (35). The GFP-tagged linker mutant M1+M2 appeared indistinguishable from LZ4-GFP, indicating that the hHSF2 linker sequences did not visibly affect the intracellular distribution of the hHSF1 linker mutant (data not shown). Thus, only those hHSF1 derivatives capable of constitutive trimerization, through mutations in either the coiled-coil domain or the amino-terminal portion of the linker, can support the growth of yeast cells lacking yHSF.

To test whether the role of the linker in modulating hHSF1 trimerization in yeast could be recapitulated in human cells, we transfected human embryonic kidney 293 cells with two representative linker mutants that complemented the growth of *hsf*Δ cells (M1+M2 and M1) and one that did not (M2). Consistent with previous reports (5), significant overexpression of hHSF1 either by transfection with large quantities of DNA (>1 μg/4 × 10^5 cells) or by allowing transfected cells to express exogenous genes for long periods (>14 h) resulted in constitutively trimerized and DNA binding competent wild-type hHSF1 (data not shown). Therefore, we limited the expression of transfected hHSF1 cDNAs so that we could observe the heat-inducible activation of ectopically expressed hHSF1. Under these conditions, hHSF1 linker mutants (M1+M2 and M1) that supported the growth of *hsf*Δ cells and were constitutively trimerized in yeast also showed significant trimerization under control temperatures in human 293 cells (Fig. 3). In contrast, the M2 linker mutant showed only partial trimerization under control temperatures, like wild-type hHSF1 (*WT*), and was extensively converted to trimers upon exposure to heat shock temperatures.

Because trimerization of HSF1 is obligatory for high affinity binding to HSEs (20, 39), we performed DNA binding assays using extracts from control and heat shocked cells to independently assess the relative level of oligomerization by each hHSF1 derivative. Consistent with the trimerization data, 293 cells transfected with wild-type hHSF1 (*WT*) exhibited significant heat-inducible DNA binding (9-fold) to the HSE probe, although basal levels of DNA binding were higher than in vector-transfected (*V*) cells (Fig. 4*A*). The hHSF1 leucine zipper mutant LZ4 was significantly activated at control temperatures, with little stimulation in DNA binding upon heat shock. The linker mutants M1+M2 and M1 appear to be quantitatively similar to the LZ4 mutant for constitutive DNA binding under non-stress conditions. The M2 mutant, which fails to complement *hsf*Δ cells, showed a 5-fold increase in heat-inducible binding to the HSE probe, suggesting that its DNA binding activity is not deregulated at control temperatures. Immunoblot analysis revealed that similar levels of protein were expressed from each transfected plasmid (Fig. 4*B*). Moreover, heat shocked extracts show the expected reduced electrophoretic mobility of hHSF1 that is associated with heat-induced hyperphosphorylation (9, 11). These results demonstrate that the hHSF1 linker modulates the monomer-to-trimer equilibrium in human cells as well as in yeast.

A linker domain connecting DNA binding and oligomerization domains of the fungal Zn_2Cys_6 family of transcription factors has been demonstrated to enforce DNA binding specificity among the related GAL4, PPR1, and PUT3 proteins to their cognate recognition sequences (40). Similarly, alterations in the length or composition of the yHSF linker significantly decreased the affinity of HSF trimers to bind to a HSE (41). Because mammalian HSF1 and HSF2 isoforms exhibit distinct preferences for binding to different HSEs *in vitro* (42) as well as for differential target gene activation *in vivo* using our yeast assay system (35), we tested the possibility that the hHSF linker might also influence this specificity. Our previous work showed that hHSF1 preferentially activated transcription of a *SSA3* (Hsp70)-*lacZ* reporter that contains five tandem repeats of the HSE pentamer (Fig. 5*A*) and that hHSF2 more potently activated a *CUP1* (metallothionein)-*lacZ* fusion that has a

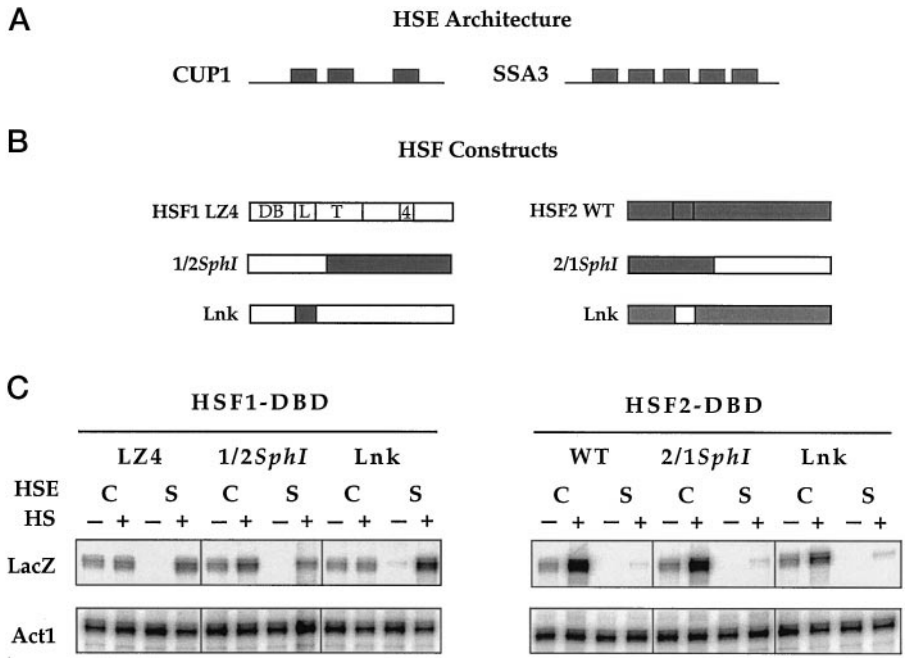

FIG. 5. **Preferential target gene activation by hHSFs in response to heat shock is independent of linker sequences.** *A,* diagram showing the number and arrangement of HSEs within the yeast *CUP1* and *SSA3* promoters. Each *shaded box* represents one HSE pentamer. *B*, schematic of hHSF highlighting the location of the linker and the conserved *Sph*I restriction site located within the first oligomerization domain of both hHSF1 and hHSF2. *DB*, DNA binding; *L*, linker; *T*, trimerization; *4*, leucine zipper four. *C*, levels of *lacZ* mRNA expressed from promoters harboring either the *CUP1* (*C*) or *SSA3* (*S*) HSEs were detected by RNase protection assays using total RNA isolated from yeast cells expressing the indicated hHSF as the sole source of HSF. Cells were maintained at 22 °C (*HS* −) or heat shocked (*HS* +) for 15 min at 40 °C. Levels of actin mRNA were used for normalization.

shorter, noncanonical gapped HSE (35). To examine the potential role of the linker in modulating target gene specificity, we transformed *CUP1-lacZ* or *SSA3-lacZ* reporter plasmids into *hsf*Δ cells expressing the full-length linker mutations (M1+M2), wild-type controls, or chimeras fused at a conserved *Sph*I site (42) that maintains homogeneous DNA binding and linker domains (Fig. 5*B*). The levels of *lacZ* mRNA were detected by RNase protection assays in control and heat shocked cells. As observed previously, the *CUP1* promoter reproducibly yielded higher basal levels of *lacZ* transcripts than the *SSA3* promoter (35); thus, we focused on the degree of heat-inducible expression. All molecules that harbored the hHSF1 DNA binding domain (Fig. 5*C, HSF1-DBD*) displayed stronger heat-inducible activation of the *SSA3-lacZ* (>40-fold) reporter compared with the *CUP1-lacZ* reporter (<2-fold). In contrast, all molecules containing the hHSF2 DNA binding domain (*HSF2-DBD*) potently activated *CUP1-lacZ* in response to heat shock (7–10-fold) but minimally activated the *SSA3-lacZ* reporter. For all HSF constructs, heat-inducible expression of the reporter genes was abolished when the HSEs were mutated (data not shown). The similarity in response by all molecules with the same DNA binding domain, regardless of linker or trimerization sequences, supports a model in which DNA binding preferences, and therefore target gene specificity, are determined by the hHSF isoform DNA binding domain and are not significantly influenced by the linker domain.

DISCUSSION

Regulation of mammalian HSF1 activation is tightly controlled to prevent inappropriate activation of the heat shock response. Under non-stress conditions, HSF1 is repressed as an inactive monomer. Activation of hHSF1 occurs through discrete steps involving trimerization, nuclear accumulation, high affinity binding to HSEs, and transcriptional activation. Here we have used a dual approach, combining analysis of the structure-function of human HSF1 expressed in yeast with transfections in a human cell line to identify a region of the linker that plays an important role in the maintenance of the monomeric state. We have mapped the critical region to the amino-terminal end of the linker that abuts the fourth β-sheet of the DNA binding domain. The M4 construct containing the double mutation (E109D, Q110D) was the minimal swap that supported the growth of *hsf*Δ cells, but a complete swap of the amino-terminal portion of the linker, construct M1 (amino acids 103–110), gave a more robust complementation of yeast cell growth than M4, based on colony growth rates. Therefore, we suggest that these amino acids may form a surface that interacts specifically with another, as yet unidentified, region of hHSF1 to stabilize the monomer. A chimera composed of the hHSF1 DNA binding and linker domains fused to the remainder of hHSF2 (construct hHSF1/2*AflII* (35)) was sequestered as an inactive monomer that failed to complement *hsf*Δ yeast, suggesting a potential site of interaction for the hHSF1 linker within the DNA binding domain. The inability of the hHSF1 linker alone to suppress hHSF2 trimerization further supports the idea that it is working through an intramolecular mechanism. Interestingly, analysis of the solution structure of the dHSF DNA binding domain revealed that two residues located within the linker, Leu-142 and Ile-145 (corresponding to amino acids Leu-112 and Ile-115 of hHSF1), interact with the hydrophobic core of the DNA binding domain (37). These conserved residues lie directly adjacent to the site that we have mapped as being the critical region for modulating trimerization of hHSF1.

The idea that multiple regions of HSF may contribute to monomer regulation has been postulated from deletion analyses of HSF (5, 12, 13). Consistent with the results presented here, truncation of the dHSF DNA binding domain to amino acid 136 did not lead to spontaneous trimerization; however, removing an additional 17 amino acids that impinged upon the dHSF linker and align with the human linker sequence did result in significant trimerization at non-heat shock temperatures (13). The current analysis extends this finding to hHSF1 and clearly demonstrates that the substitution of five critical amino acids at the amino-terminal region of the hHSF1 linker, in the context of the intact molecule, is sufficient to disrupt the monomeric state. Although no direct demonstration of an intramolecular interaction within the hHSF1 monomer has yet been reported, there is abundant biochemical and genetic evidence to support a model (Fig. 6) in which the inactive HSF1 monomer is restrained by contacts between the coiled-coil domains (5, 6, 10). Point mutations within either hydrophobic domain that disrupt the arrangement of heptad repeats lead to

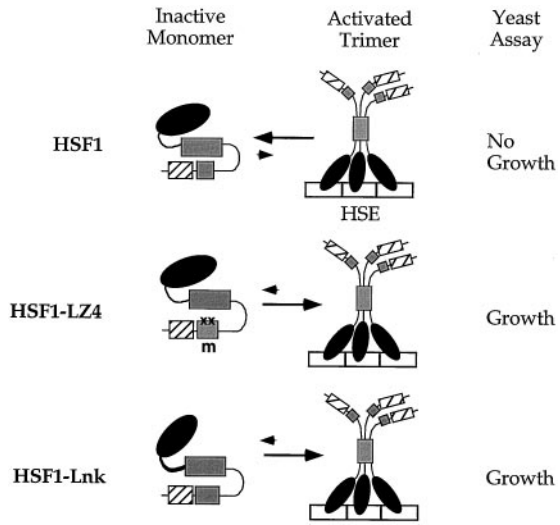

FIG. 6. **Model for activation of hHSF1.** Under non-stress conditions, the bulk of hHSF1 is found as an inactive monomer restrained by intramolecular interactions and, *in vivo*, by interactions with Hsps (data not shown for simplicity). Conversion from the monomer to trimer occurs constitutively when mutations (indicated by *XX*) are introduced within either leucine zipper domain (*e.g. hHSF1-LZ4*) that would be predicted to disrupt intramolecular hydrophobic interactions. Constitutive trimerization also occurs when specific mutations (shown in *bold*) within the hHSF1 linker (*hHSF1-Lnk*) are introduced.

constitutive and nearly quantitative trimerization of hHSF1 when expressed in either yeast (Fig. 2), human cells (Fig. 3 (5)), or *Xenopus* oocytes (6). Furthermore, purified mouse HSF1 or dHSF lacking the last leucine zipper domain result in constitutively trimerized complexes *in vitro* (14, 15). These observations that any number of perturbations to HSF1 structure, including mutations in the linker, lead to a significant shift in the monomer-to-trimer equilibrium suggest that the stability of the inactive monomer depends upon multiple interdependent contacts. Intriguingly, purified dHSF can be reversibly trimerized *in vitro* in response to heat, decreased pH, and some chemical inducers of the heat shock response, indicating that the monomeric HSF protein can directly sense some environmental changes (15, 43). *In vivo*, the stabilization of HSF1 monomers may involve interactions with other cellular factors such as molecular chaperones (17, 18); however, the precise role of these proteins in HSF regulation and the sites of interaction between these proteins remain to be determined.

The presence of structured linkers between DNA binding and oligomerization domains is a common motif among several classes of fungal and mammalian transcription factors. Among the best characterized is the role of the linker in discriminating between similar binding sites within the yeast Zn_2Cys_6 family that includes GAL4, PUT3, and PPR1. Analysis of the chimeras between these proteins, together with three-dimensional structural determinations, revealed that the unique linker, rather than the highly similar DNA binding domains, was the determinant of DNA binding specificity (44–47). In our study, we found that interchanging the hHSF1 and hHSF2 linkers did not significantly alter the preferential activation of *SSA3-lacZ* and *CUP1-lacZ* by all molecules harboring the hHSF1 and hHSF2 DNA binding domains, respectively. We note, however, that the DNA binding specificity is not absolute, and both HSF1 and HSF2 can bind a variety of HSEs (42). Nevertheless, the *SSA3* and *CUP1* promoters represent naturally occurring HSEs that show preferential heat-inducible activation by the hHSF isoforms. Thus the role of the HSF1 linker appears to be distinct from that of the Zn_2Cys_6 family: the HSF1 linker influences trimerization rather than discriminating between different types of HSEs. An implication from the current results is that differential specificity for distinct HSEs and for distinct footprint patterns exhibited by mammalian HSF1 and HSF2 (42, 48) is determined primarily by the highly similar, although not identical, DNA binding domains.[2] This prediction is fully consistent with the previous observation that a single amino acid substitution within the DNA binding domain of yHSF dramatically increased the binding affinity for the *CUP1* promoter and decreased binding to the *SSA3* promoter (49).

Previous studies on the yHSF linker suggested an important role in aligning the DNA binding domains of HSF to the HSE (41). Because of the constraints imposed on a trimeric protein by the necessity to bind three consecutive HSEs on a linear DNA molecule, it is believed that the HSF linker must provide sufficient flexibility for the individual DNA binding domains to contact each cognate binding sequence (20). The minimal yHSF linker required for DNA binding and functional complementation in yeast was mapped to a 21-amino acid sequence that aligns in length and, in part, in composition to the mammalian and *Drosophila* HSF linkers, whereas a 52-amino acid extension unique to the yHSF linker was dispensable for either DNA binding or complementation (41). Thus, the linker appears to play multiple roles: providing flexibility to all HSF trimers, closing off the DNA binding domain through hydrophobic interactions, and modulating trimerization for a subset of HSFs including hHSF1 and dHSF. We propose that within certain HSF contexts, such as in the hHSF1 monomer, the linker may adopt a specific conformation that would allow it to make contacts with another surface of HSF, despite the lack of evidence for a defined conformation in solution. By analogy, it is interesting that the solution structures determined by NMR of the GAL4 and PUT3 DNA binding domains also revealed flexibility in the flanking linker sequences (47, 50, 51), but cocrystal structures of these proteins bound to cognate DNA firmly established that each linker adopted a distinct conformation (45, 52). The precise mechanism by which hHSF1 converts from a monomer to trimer upon sensing stress remains incompletely understood, and our results underscore the complexity of this regulation. How the linker and other regions of HSF1 act to modulate trimerization must await the complete structural determination of the protein.

Acknowledgments—We thank Vincenzo Zimarino for critical reading of the manuscript and helpful suggestions with mammalian transfections. We thank Xiao-Dong Liu, Nicholas Santoro, and Kevin Morano for critical readings, advice, and yeast plasmid constructs; members of the Thiele laboratory for suggestions; and Chen Kuang for excellent technical assistance. The gifts of reagents from Hillary Nelson, Richard Morimoto, Carl Wu, Robert Kingston, and Stuart Calderwood are gratefully acknowledged.

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