Expression of human heat shock transcription factors 1 and 2 in HeLa cells and yeast

Chao-Xing Yuan, Eva Czarnecka-Verner and William B. Gurley

Department of Microbiology and Cell Science, Program in Plant Molecular and Cellular Biology, University of Florida, Box 110700, Gainesville, FL 32611–0700, USA

Abstract We have examined reporter gene (β -gal) expression directed by human heat shock transcription factors 1 and 2 (HSF1 and HSF2) in HeLa cells and in yeast (*Saccharomyces cerevisiae*). Transcriptional activation domains of both HSFs were mapped to the C-termini using chimeric proteins containing the GAL4 DNA binding domain (GAL4-DBD). Deletion analysis of HSF1 largely confirmed the mapping and expression pattern of activation domain 2 (AD2) previously reported by Green et al (1995) with the exception of the contribution of the oligomerization domain (hydrophobic region A) to basal repression in yeast, but not in HeLa cells. In addition, a C-terminal activation domain for HSF2 (amino acids 397 to 536) was identified by analysis in yeast. In contrast to HSF1, full length HSF2 and the isolated activation domain of HSF2 showed little activity in HeLa cells. Analysis of point mutations generated by low fidelity PCR within AD2 of HSF1 indicated that hydrophobic and charged amino acids in addition to proline, serine and threonine make critical contributions to transcriptional activity. Co-expression of GAL4-DBD fusions with AD2 of HSF1 and the C-terminal activation domain of HSF2 showed no evidence of synergism in the activation of transcription. Wild-type human HSF1 and HSF2 were both able to substitute for the endogenous yeast HSF under normal growth conditions.

INTRODUCTION

In most organisms a sudden elevation in temperature of approximately 10°C results in a major shift in transcriptional expression. Expression of most normal genes is attenuated and a subset of genes known as heat shock genes are rapidly activated (Ashburner and Bonner 1979; Craig and Gross 1991). The protein that mediates this vigorous induction of transcription is the heat shock transcription factor (HSF). In metazoans, activation of HSF is thought to involve a conformational change of pre-existing HSF accompanied by trimerization of identical subunits converting the protein from a form with low affinity for DNA to one that strongly binds a heat shock element (HSE) (Westwood et al 1991). The HSE is required for heat induced activation of heat shock protein (Hsp) promoters and is usually comprised of at least

three tandem repeats of a 5-base pair (bp) sequence (5'-nGAAn-3') arranged in alternating orientations (Pelham 1982; Amin et al 1988; Xiao et al 1991; Barros et al 1992).

More than 20 HSFs from different species including yeast, *Drosophila*, mouse, human, chicken, tomato, soybean, maize and *Arabidopsis* have been cloned as cDNAs and sequenced (Wu 1995; Nover et al 1996). The various HSFs show relatively little overall amino acid identity, but are considerably conserved in the DNA binding (DBD) and oligomerization domains (OD). The DBD comprises a triple helix cluster typical of helix-turn-helix proteins (Damberger et al 1994; Harrison et al 1994; Vuister et al 1994), and the OD consists of a series of 4, 3-(*abcdefg*)_n pattern hydrophobic heptad repeats (leucine zipper) forming a coiled-coil during trimerization (Sorger and Nelson 1989; Peteranderl and Nelson 1992; Scharf et al 1994).

The transcriptional activation domains of HSFs from yeast, *Drosophila*, mammals and tomato have been mapped in detail. Activation domains of the *Saccharomyces cerevisiae* HSF (ScHSF) are located in both the N-terminal and C-terminal regions of the protein

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Correspondence to: William B. Gurley Tel: +1 352 392 1568; Fax: +1 352 392 5922; E-mail: Wgurley@micro.ifas.ufl.edu

(Nieto-Sotelo et al 1990; Jakobsen and Pelham 1991; Chen et al 1993). Of these, the C-terminal activator is the strongest and best characterized, and appears to be required for the transient heat shock (HS) response as well as sustained growth at high temperatures (Nieto-Sotelo et al 1990; Chen et al 1993). In tomato, three HSFs have been cloned, and in each case a tryptophan motif (trp-repeat) located C-terminally to the OD has been associated with transcriptional activity (Treuter et al 1993). *Kluyveromyces lactis* HSF contains a single activation domain which is similar to the C-terminal domain of ScHSF, both of which overlap a hydrophobic repeat (Chen et al 1993).

Two adjacent activation domains (AD1 and AD2) have been identified in the C-terminal portion of human HSF1 (Green et al 1995; Newton et al 1996). AD1 encompasses 20 amino acids (aa 401 to 420) and is rich in bulky hydrophobic and acidic amino acids, and has the potential to form an α -helical structure with negative charges predominantly grouped on one face. AD2 (aa 431 to 529) is highly acidic, but probably lacks helical secondary structure due to the large number of proline and glycine residues. *Drosophila* HSF is organized in a similar manner with a transactivation domain rich in hydrophobic and acidic residues located at the extreme C-terminus (Wisniewski et al 1996).

In addition to positive acting domains, mouse and human HSF1 contain a region that exerts negative control, preventing transcriptional activation in non-heat shock conditions. This regulatory domain is located predominantly between the OD and the C-terminal activation domains (Green et al 1995; Shi et al 1995; Newton et al 1996), but also includes the C-terminal portion of the OD (Zuo et al 1995). Both AD1 and AD2 are negatively regulated by this domain in HeLa cells, but in yeast it does not appear to confer heat inducibility (Green et al 1995).

We have characterized HSF1 and HSF2 expression in HeLa cells and in yeast in order to examine conservation of regulatory mechanisms. In most of these experiments, the GAL4-DBD was fused to the N-terminus of the HSF constructs. The reporter gene for monitoring HSF activity was the GAL4 promoter joined to *lacZ* (Chevray and Nathans 1992). We have examined basal repression in both systems and have characterized AD2 of HSF1 by point mutations. Basal repression of HSF1 was shown to be independent of the DBD and the C-terminal activator, essentially confirming the previous results (Green et al 1995; Newton et al 1996). We have also identified a C-terminal activation domain for HSF2 on the basis of its activity in yeast. The potential for HSF1 and HSF2 activation domains to interact cooperatively was analyzed by co-expression of GAL-DBD fusions in HeLa

cells. In addition, the endogenous ScHSF of yeast was substituted with wild-type human HSF1 or HSF2 and monitored for growth under normal and prolonged conditions of high temperature.

MATERIALS AND METHODS

Yeast strains and methods

Yeast strain PCY2 (MATa Δ gal4 Δ gal80 URA3::GAL-lacZ lys2-801 his- Δ 200 trp1- Δ 63 leu2 ade2-101) was used to assess β -galactosidase (β -gal) activity of all constructs containing the GAL4-DBD fused to the N-terminus of the mutant HSFs (Chevray and Nathans 1992). Strain YJB341 (a/ α trp1-289 [HSE-lacZ, TRP1] ura3-52 leu2-3, 112 his3 Δ 1/his3 Δ HindIII met2/MET2 his4-519/HIS4 ade1-100/ADE1 hsf1 Δ gal⁺/gal⁺ [YE_pHSF^{RS}URA]) contains the ScHSF on a YEplac vector and was used in substitution studies with the heterologous HSFs from humans (Bonner et al 1992). This strain also contains an integrated HSE-lacZ gene for monitoring HSF activity. The plasmid pBS16M232v was grown in strain YJB341 (designated 341/BS16M232v) and contained a VP16 fusion with the N-terminus of ScHSF_{M232v} in a pDBD vector (Bonner 1991). The point mutation of methionine at residue 232 makes the HSF constitutive (Bonner et al 1992).

Yeast vectors and their derivatives

A PCR product coding for the GAL4-activation domain (AD) was subcloned into pPC97 at *Bgl* II and *Not* I sites to generate plasmid pYDA containing a translational fusion of the GAL4-DBD (N-terminal) and the GAL4-AD (C-terminal) separated by a site for insertion of HSF sequence. Plasmid pPC97 is an improved version of the vector pPC62 (P. M. Chevray, personal communication) differing only in the polylinker sequence which is identical to that in pPC86 (Chevray and Nathans 1992). Plasmid pPC86 was used as template to PCR-amplify the GAL4-AD (aa 768-881).

Plasmid YCpGAL2 was used to shuttle heterologous HSFs into yeast for HSF substitution experiments (Bonner 1991). The polylinker was modified to facilitate the cloning of HSF1 by insertion of a synthetic oligonucleotide between the *Hind* III and *Bgl* II sites to create the plasmid pYGAL2. The sequence of the new polylinker was 5'-AA,GCT,TCG,TCG,ACC,CGG,GAG,ATC,-TAA,C,TAA,G,TAG-3'. Yeast strain PCY2 and vector plasmids pPC97 and pPC86 were kindly provided by Dr P. M. Chevray (The Johns Hopkins University, USA). Yeast strain YJB341 and vector YCpGAL2 were kindly provided by Dr J. J. Bonner (Indiana University, USA).

Transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum at 37°C. The calcium phosphate precipitation method (Ausubel et al 1991) was used to transfect HeLa cells with each transfection including the following plasmid DNAs: 0.5 to 2 µg of Gal4-DBD-HSF1 fusion constructs in vector pNG-SB (derived from the pCI-neo vector from Promega Corporation), 0.5 µg of pXGH5 (growth hormone vector, Nichols Institute Diagnostics) and 2 µg of pG5Luc containing a luciferase reporter with a promoter containing five GAL4 binding sites (Patel et al 1995). Control and heat shocked cells were harvested 48 h after transfection. Heat shocked cells were incubated at 37°C for 44 h after transfection, heat stressed at 42°C for 2 h, and allowed to recover for 2 h at 37°C before harvesting. Transfection efficiency was estimated from growth hormone (hGH) production (hGH-TGES 100T Kit, Nichols Institute Diagnostics), and luciferase activity of the reporter was measured with the Luciferase Assay System from Promega Corporation. Variability of normalized activities varied no more than 10% between experiments. Expression of the Gal4-DBD/HSF1 fusion proteins was detected by Western blotting using a Gal4-DBD monoclonal antibody (Santa Cruz Biotechnology).

Construction of HSF mutants

The cDNA clones containing HSF1 and HSF2 were provided by Dr Carl Wu (National Institutes of Health, Bethesda, MD, USA) and Dr Robert Kingston (Massachusetts General Hospital, Boston, MA, USA). Terminal deletions of HSF1 and HSF2 were generated by PCR using Vent DNA polymerase (New England Biolabs) and a series of downstream and upstream primers with added *Sal* I and *Bgl* II sites. The PCR products were digested with *Sal* I and *Bgl* II and subcloned directionally into yeast expression vectors pPC97, pYDA and pYGAL2. Modified HSFs cloned into vectors pPC97 (GAL4-DBD fusions) and pYDA (GAL4-AD fusions) were transformed into yeast strain PCY2, and HSF1 and HSF2 in pYGAL2 were transformed into strain YJB341. Transformants from strain YJB341 were further selected on plates supplemented with 5-fluoro-orotic acid (5-FOA) which ensured loss of the endogenous SchSF present on the URA3 vector plasmid. Absence of the URA3 vector was confirmed by the lack of growth on media lacking uracil. Yeast strain YJB341 was grown in yeast medium supplemented with 2% galactose, and PCY2 was grown in medium containing 2% glucose.

PCR-mediated mutagenesis

Point mutations were introduced into the C-terminal domain of HSF1 by low fidelity PCR (Kassenbrock et al

1993). Two oligonucleotide primers (5'-GACTACGTC-GACGCAGCTGGTGCCTACACAG-3' and 5'-TGCAGTAGATCTCGGAGACAGTGGGGTCTTGGC-3') were used to amplify the region corresponding to amino acid residues 464 to 529 and to introduce flanking *Sal* I and *Bgl* II sites. The reaction contained 0.4 mM MnCl₂, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl at pH 8.2; and *Taq* DNA polymerase (Promega). The amplified products were cloned into pPC97 at the *Sal* I and *Bgl* II sites to form fusion proteins with the GAL4-DBD and transformed into yeast strain PCY2. Two hundred colonies were assayed for β-gal activity using liquid (Miller 1972) and filter assays (Clontech yeast two-hybrid protocol). Plasmids from colonies showing impaired activity were recovered and the DNA sequenced.

Heat shock treatment and β-galactosidase assay

Enzyme activity (Miller 1972) was determined after incubation of yeast cells at non-HS (25°C) and HS (37°C) conditions for 1 h. In experiments involving prolonged stress, temperatures of 25°C, 37°C and 40°C were used from 0 to 20 h after the OD₆₀₀ of liquid cultures reached approximately 0.5. After temperature treatments, β-gal liquid assays were performed in triplicate (Ausubel et al 1991). In order to test the survival of exogenous HSF-substituted strains under HS and cold stress conditions, the transformants selected from 5-FOA plates were grown on Leu drop-out and galactose-containing yeast medium and incubated at 15°C, 30°C or 37°C.

Gel retardation assays

Yeast cells (50 ml) were grown to an OD₆₀₀ of 1.0 at 25°C, stressed at 37°C for 1 h, and harvested for preparation of whole cell extracts according to Bram and Kornberg (1985). DNA binding reactions were conducted at 30°C for 15 min and contained 10 µg of extract protein, 1 ng of the GAL4 binding site probe (10 000 cpm; ³²P-labeled), 25 mM HEPES (pH 7.5), 5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT, 12% glycerol, 100 mM KCl, and 0.5 µg poly dI-dC in a total volume of 40 µl. The GAL4 binding site probe was formed by annealing two synthetic oligomers (5'-CTAGGAGCG-GAGGACAGTCCTCCGAAT-3' and 5'-CTAGATTCGGAG-GACTGTCTCTCCGCTC-3'), and then labeled at the 5' ends. Bound and free probe were resolved by 2.6% PAGE (acrylamide: bis = 30:1) at 4°C using prechilled 0.5 × TBE running buffer. The gel was dried and exposed to X-ray film at -70°C overnight to visualize bands.

RESULTS

Mapping the transcriptional activation domain of HSF1 and HSF2

To identify domains in HSF1 involved in transcriptional activation and maintenance of basal repression, a series

of N- and C-terminal truncations were initially evaluated in yeast cells. Activity of HSF mutants was monitored using β -gal activity generated by a GAL1-*lacZ* reporter integrated into the chromosome of yeast strain PCY2. All HSF constructs were fused with the GAL4-DBD at the N-terminus (plasmid pPC97). Since nuclear localization and DNA binding functions were provided by the GAL4-DBD, this system tolerated deletion constructs which eliminated the corresponding regions of the HSF. Mutations could also be placed in the OD since HSF trimerization was presumably not needed for binding to the GAL4 DNA binding site.

The full length HSF1 fusion protein showed activity in yeast under HS conditions and no detectable activity under normal growth conditions indicating that basal repression and heat inducible activation of HSF1 was able to occur in this heterologous system (Fig. 1A, construct 1). Removal of 119 amino acid residues from the N-terminus demonstrated that the HSF1 DBD was not involved in basal repression or transcriptional activation (construct 2). Further deletion of amino acids 120 to 174, which removed hydrophobic heptad region-A (HR-A) of the OD, resulted in both an increase in overall activity and a loss of basal repression (construct 3). Activity under both control and HS conditions nearly doubled when the remaining portion of the OD (HR-B) and the region N-terminal to HR-C was deleted in construct 4 despite low levels of protein expression (Fig. 1B). Loss of transcriptional activity was not seen until a portion of the C-terminus was deleted in construct 6. These results indicated that, when assayed in yeast cells, the transcriptional activation domain of HSF1 mapped to the 107 amino acids which are C-terminal to HR-C. The difference in β -gal activity between constructs 4 and 5 was most likely artifactual due to the extremely low amount of stable protein derived from construct 4. All other constructs showed similar levels of expression and correct protein size by western blot analysis (Fig. 1B). The C-terminal activation domain identified here in yeast corresponds to the AD2 region characterized by Shi et al (1995) and Newton et al (1996) and, based on the results from construct 6, does not include the adjacent region surrounding HR-C (AD1

domain) (Green et al 1995; Zuo et al 1995; Newton et al 1996). Substitution of AD2 (aa 423 to 529) with the acidic activation domain of GAL4 (construct 8) resulted in heat inducible activity at levels comparable to those obtained with AD2 (compare constructs 1 and 8) indicating that heat inducibility was not dependent on the natural activator domain of the HSF.

Gel retardation assays were performed using whole cell extracts from yeast containing construct 2 to determine if basal repression was due to the inability to bind DNA under control conditions. Analysis of extracts from construct 2 grown under non-HS conditions indicated that the GAL4-DBD was able to bind DNA despite the lack of transcriptional activity (Fig. 1C, lanes 4 and 5). These results indicate that the GAL4-DBD was not masked during basal repression under non-HS conditions, and suggest that the DBD of the wild-type protein may also be accessible under basal conditions. The slight reduction in DNA binding activity upon HS may reflect a partial loss of function of the GAL4-DBD. The presence of the GAL4-DBD/HSF1 protein in the DNA complexes was confirmed by the lack of complex formation by the vectorless control strain PCY2, and by removal of complexes by immunoprecipitation with antiGAL4-DBD antibody (not shown).

The C-terminal location of the activation domain of HSF2 (aa 399 to 536) is similar to HSF1, as demonstrated by constructs 9 and 10 (Fig. 1A). Activity of full length HSF2 was only evident under HS conditions and was only one-fourth that of HSF1. It is not clear if the low levels of activity observed represent true heat inducible activation of transcription, or direct temperature effects on transcriptional and β -gal enzymatic activities. As with HSF1, removal of all portions of the HSF N-terminal to HR-C resulted in a large increase in constitutive activity. The C-terminal activator (CTA) of HSF1 (AD2) appeared to be approximately eight times stronger than the HSF2 activation domain when assayed in isolation in yeast cells.

Comparison of activity in yeast and HeLa

GAL4-DBD fusions with HSF1 showed a similar pattern of expression in the two systems, with the exception of

Fig. 1 Mapping of human HSF1 and HSF2 regions involved in transcriptional activation and basal repression. (A) Transcriptional activity was monitored with the GAL1-*lacZ* reporter gene in strain PCY2 using derivatives of pPC97 for constructs 1–7 and 9–10, and pYDA for construct 8 (Chevray and Nathans 1992). Basal conditions of 25°C and HS conditions of 37°C for 1 h were used for this and subsequent figures. Boxes indicate domains involved in basal repression and transcriptional activation. HR-C includes activation domain AD1 at its C-terminus. AD2 extends from residue 422 to 529 (construct 5) and was set as the standard for relative transcription quantification. Luciferase activity from HeLa cells was normalized for transformation efficiency by monitoring human growth hormone production. Activity of construct 5 was assigned a value of 100%. HR = hydrophobic heptad region (A, B and C). (B) Western blot of human HSF constructs expressed in yeast and HeLa cells probed with antiGAL4-DBD antibodies. Arrow indicates predicted size of expressed protein for construct 4. Numbers correspond to constructs depicted in panel A. (C) Gel retardation assay of the protein derived from construct 2. The GAL4 DNA binding site was used as a probe to test the ability of construct 2 to bind DNA under control and HS conditions. Lane 1 contains free probe. Whole cell extracts from strain PCY2 containing no vector plasmid were used in gel shift assays shown in lanes 2 (non-heat shock) and 3 (heat shock), and assays using cultures containing construct 2 are shown in lanes 4 (non-heat shock) and 5 (heat shock). The presence of GAL4-DBD fusion proteins in the DNA complexes was confirmed by elimination of complex formation by immunoprecipitation of extract with antiGAL4-DBD monoclonal antibodies (not shown). F = free probe; B = bound.

Fig. 1

construct 3 where basal repression was evident in HeLa but absent in yeast (Fig. 1A). In HeLa cells, significant activity at non-HS temperatures was not observed until both HR-A and HR-B were removed in construct 4. In both yeast and HeLa very little activity was observed for construct 6 in which HSF1 was missing only AD2. AD1 appeared to be repressed under basal and HS conditions in both systems. The reduction in activity seen in comparing construct 4 with construct 5 reflects the contribution of AD1 in HeLa. The relatively low activity of construct 4 in yeast does not necessarily indicate a lack of function of AD1, but was probably due to very inefficient expression of construct 4 protein in yeast cells (Fig. 1B).

Substitution of AD2 with the GAL4 activation domain (GAL4-AD) caused no loss of basal repression or heat inducibility in yeast (construct 8), but heat inducible activity was lower in HeLa, presumably due to the inefficiency of the GAL4-AD in HeLa cells (Kakidani and Ptashne 1988). A similar substitution construct utilizing the VP16 acidic activator, and lacking AD1, AD2 and the OD, was also reported to be heat inducible in HeLa cells (Newton et al 1996).

HSF2 constructs 9 and 10 showed little activity in HeLa cells compared to yeast (Fig. 1A). Full length HSF2 had no significant activity under either basal or HS conditions, whereas in yeast, a low level of heat inducible activity was observed. Furthermore, activity of the C-terminal domain of HSF2 (construct 10) was less than 2% of HSF1 AD2 activity (construct 5), but in yeast this domain exhibited approximately 10% of AD2 activity.

Subdomains within transcriptional activation domain AD2 of HSF1

A series of C-terminal and N-terminal deletions within AD2 were constructed and analyzed in yeast in order to define more precisely the amino acid motifs involved in transcriptional activation (Fig. 2). The results shown in Figure 2A essentially confirm the results of a similar study by Newton et al (1996) conducted in HeLa cells. The organization of AD2 appears to be complex with virtually the entire region contributing to the transcriptional activation potential of HSF1. The dramatic loss of activity seen after removal of residues 422 to 463 in construct 12 indicated that the N-terminal portion of AD2 is important for activity. No detectable activity was observed when an additional 30 amino acids were removed in construct 13, which seems to indicate that residues 494 to 529 (C-terminus) made no contribution. This interpretation is contradicted, however, by the results obtained with construct 15 where deletion of these C-terminal residues caused a reduction in activity. This result suggests that the C-terminal amino acids are unable to activate transcription in isolation, but make a

Fig. 2 Detailed mapping of activation domain AD2 of human HSF1. All constructs were derived from pPC97 and were expressed in yeast strain PCY2 (Chevray and Nathans 1992). (A) Reporter gene (β -gal) activity in Miller units under basal (NHS) and HS conditions. (B) Western blots of HSF1 constructs expressed in yeast using antiGAL4-DBD antibody as probe. Numbers correspond to constructs depicted in panel A. (C) Western blots of HSF1 point mutants described in the Table within the C-terminal region of AD2 (construct 12; aa 464 to 529) and expressed in HeLa cells. NHS = non-heat shock; HS = heat shock.

strong contribution when other components of AD2 are present. This type of synergism is also seen in comparing the activities of constructs 17 and 12, which together comprise the entire AD2, where the sum of activities for each fragment was much less than the activity of the intact AD2 (construct 5). Various internal fragments showed either little or no activity consistent with the presence of weak activation elements that only function in the context of the intact AD2. Finally the loss of activity exhibited by constructs 13, 18 and 21 was not due to poor expression of these proteins in yeast as demonstrated by the Western blot presented in Figure 2B.

Point mutations were introduced into a portion of AD2 in an attempt to identify individual residues critical for transcriptional activity. Since AD2 seemed to be

Table Analysis of single and multiple point mutations of the human HSF1 activation domain AD2.

Mutant	% β-Gal (yeast)	% Luc/hGH (HeLa)	Point mutations			
Group A						
MA-1	0	6.9	D477V	D482A	L515P	P522K
MA-2	0		D477V	D482A	L515P	P521S
MA-3	< 3	6.1	A470V	F500Y	L514P	
MA-4	< 3		P489L	T516S		
Group B						
MB-1	30–50		L491Q	E508G		
MB-2	30–50		S480P	L515P	K524term	
MB-3	30–50	17.7	E508K			
MB-4	30–50	20	A470V			

Mutations were randomly placed in the terminal two thirds of AD2 (aa 464 to 529), selected in yeast, and representative mutants assayed in HeLa cells. Percentage of β -gal and luciferase activities (Luc/hGH) were determined relative to wild-type activity of HSF1-AD2 (construct 12) in yeast and HeLa cells, respectively. Mutations affecting residues showing conserved identity among HSF1 and HSF2 families in humans, mouse and chicken are in bold letters, and those affecting residues with conserved similarity are underlined. Mutants MA-3 and MA-4 were randomly selected twice from 6 clones showing < 3% activity in yeast.

comprised of multiple elements, single amino acid changes were not likely to result in large changes in activity which would facilitate screening of mutants. To reduce this problem, we used only the C-terminal portion of AD2 (construct 12) from residues 464 to 529 (AD2_{464–529}) for these studies. Although this fragment showed only 31–36 units of intact AD2 activity, the remaining N-terminal portion of AD2 had even less activity when assayed in isolation (construct 17). It is noteworthy that all areas of high conservation between class 1 and 2 HSFs in vertebrates are located in the amino acid 464 to 529 fragment.

Analysis of point mutations within AD2_{464–529} indicated the importance of conserved residues present in HSF1 and HSF2 families. Randomly positioned mutations were introduced into AD2_{464–529} of construct 12 using low fidelity PCR (Kassenbrock et al 1993). Approximately 200 yeast transformants containing cloned DNA fragments derived from PCR mutagenesis were then assayed for β -gal activity. Roughly 15% showed impairment of activity ranging from 50 to 100%. The results of DNA sequence analysis of some of the clones with impaired activity are shown in the Table. Mutations were assigned to two groups based on activity compared to the wild-type AD2_{464–529}: group A showing zero or residual activity, and group B exhibiting 30–50% activity. Mutations which resulted in partial loss of activity (group B) were the most

informative and revealed the importance of alanine 470 (mutant MB-4) and glutamic acid 508 (mutant MB-3). Alanine 470 shows identity among all members of the HSF1 and HSF2 families and is located within conserved region C-1 (Fig. 3). Amino acid residue 508 is adjacent to the conserved region C-2, and is occupied by negatively charged residues in vertebrate HSF1. The deleterious effect of removal of the negative charge at position 508 by mutations E508G and E508K, and the conservation of negative charge at position 509, suggests that both residues 508 and 509 may be required for C-2 function within the HSF1 family. Mutants MB-3 and MB-1 had roughly the same activity suggesting that mutation of L491 to Q (MB-1) had little effect. The A470V mutation within C-1 occurred twice demonstrating the critical role of alanine 470. The difference in activity between MB-4 and MA-3 indicates that either phenylalanine 500 or leucine 514 (within region C-2), or both, are also involved in transcriptional activation. In addition, the occurrence of the L515P mutation in three of the mutants suggests that this conserved residue of C-2 is also required for full activity. Assays of select mutations in HeLa cells showed similar results to the yeast expression studies, and western blots indicated that all mutants were properly expressed (Fig. 2C). It seems significant that in this limited analysis most changes involved either hydrophobic or negatively charged amino acids.

Fig. 3 Comparison of the C-terminal region (aa 463 to 529) of HSF1 activation domain AD2 with the C-terminal region of other vertebrate HSFs (Rabinدران et al 1991; Sarge et al 1991; Schuetz et al 1991; Nakai and Morimoto 1993) Amino acid sequences were compared using the CLUSTAL W(1.4) program (Thompson et al 1994). Identical residues are marked by a solid circle and those with conserved biochemical character are indicated by an open circle. Two regions of high conservation are identified in the shaded boxes C-1 and C-2. h = human; m = mouse; and ch = chicken. Amino acids that were listed as mutated in the Table are underlined. Single amino acid mutations at A470 (MB-4) and E508 (MB-3) that resulted in a clear reduction in activity are indicated.

Co-expression of C-terminal portions of HSF1 and HSF2 in HeLa cells suggests a lack of synergism between activation domains

Experiments were conducted to evaluate potential interactions between HSF1 and HSF2 activation domains since activation of the *hsp70* gene appears to be synergistic in cells simultaneously induced for HSF1 and HSF2 (Sistonen et al 1994). GAL4-DBD fusion proteins containing either HSF1 AD2 or the HSF2 C-terminal domain were co-expressed and activity monitored using the GAL4 DNA binding site reporter. As shown in Figure 4, activity of AD2 (Fig. 1 construct 5) was approximately 65-fold greater than the HSF2 C-terminal domain (Fig. 1 construct 10) when each was expressed alone. Co-expression of HSF1 and HSF2 activation domains resulted in activity levels that were intermediate to the two domains expressed separately indicating a lack of synergistic interaction. These results suggest that the synergistic response seen previously in hemin and heat induced K562 erythroleukemia cells must involve events not directly related to the C-terminal activation domains of HSF1 and HSF2. Alternatively, the lack of synergism seen here may be due to the lack of specific coactivators that may be present in K562 cells, but absent in HeLa cells.

Substitution of ScHSE with human HSE1 and HSE2

Human HSFs were substituted for the endogenous ScHSF of yeast to assess the degree of conservation in the mechanisms of basal repression and transcriptional activation. Yeast strain YJB341 has a deletion of the chromosomal ScHSF and is totally dependent on a copy of the ScHSF present on a URA3 vector plasmid (YE_pHSF^{RS}URA) (Bonner et al 1992). The heterologous HSFs were introduced into YJB341 using plasmids pY_{GAL}2 and then selected on 5-FOA plates which eliminated the URA3 plasmid containing ScHSF. Each of the HSF substitution strains maintained the pY_{GAL}2-derived plasmid after several days growth on rich medium, indicating that the heterologous HSF was vital for normal growth. HSF activity was monitored using the *HSE-lacZ* reporter gene present in yeast strain YJB341 (Fig. 5). HSF substituted strains grew optimally at 30°C and showed varying degrees of heat and cold sensitivity. Yeast substituted with HSF1 grew slower than wild-type yeast at 15°C (not shown), and its growth was drastically inhibited at 37°C (Fig. 5B). In contrast, the HSF2 substituted strain had a growth rate similar to wild-type yeast under cold or heat stress conditions. There was no obvious correlation between the activity of the HSF as monitored by the β-gal assay and the ability to maintain sustained growth at high temperature (compare Figs 5 and 6).

Fig. 4 Co-expression of GAL4-DBD fusion proteins containing the HSF1 activation domain AD2 and the C-terminal region of HSF2 (HSF2-AD). GAL4-DBD fusion proteins were expressed in HeLa cells using vector pNG-SB. DNA used in transformations: lane 1, HSF1-AD2 (0.5 µg, construct 5); lane 2, HSF2-AD (0.5 µg, construct 10); lane 3, HSF1-AD2 (0.5 µg) plus HSF2-AD (0.5 µg). Each transformation contained 0.5 µg of growth hormone vector (pXGH5) and 2 µg reporter plasmid (pG5Luc). The amount of DNA in transformations 1 and 2 was kept constant by the addition of 'empty' vector DNA (0.5 µg pNeo-NB). Relative luciferase activity was normalized using growth hormone production. Error bars were derived from three replicate experiments.

Activity of HSF1 and 2 in yeast under sustained heat shock

During prolonged heat stress, two noticeable differences were seen when human HSFs were substituted for the ScHSF: a delayed heat activation, and the lack of a clearly defined transient response. The time course of activity for human HSFs in yeast lacking the endogenous ScHSF was determined by heat stressing cells at either 37°C (normal HS), or 40°C (severe heat stress) for up to 20 h. The control strain harbored the plasmid YEpHSF^{RS} URA3 (Bonner 1991) which contained a C-terminal truncation of ScHSF (at aa 583), and was heat inducible. Under normal HS conditions, the two human HSFs exhibited delayed activation detectable after approximately 30 min (Fig. 6). The control strain showed a typical transient response (Sorger 1990) with activity peaking from 1 to 2 h after HS induction and declining to slightly less than one-half of peak activity by 4 h. In contrast, the human HSFs showed a gradual reduction in activity after a peak at 2 h. After 20 h at 37°C, ScHSF had a residual activity of approximately 1 unit, HSF2 had roughly 4 units, and HSF1 still exhibited 10 units of activity. Yeast HSF was attenuated by 96%, whereas HSF2 lost 64% of its activity, and HSF1 was only reduced by 41%. Under severe heat stress the transient nature of ScHSF expression diminished. Peak values of ScHSF activity after 2 h at 40°C

were nearly identical to those at 37°C, but attenuation during extended periods of stress was impaired. The human HSFs also showed less decline in activity under severe heat stress. The inability of the human HSFs to down modulate after 2 h of heat shock may explain the poor growth at 37°C of HSF substituted yeast cells.

DISCUSSION

Overall, the degree of conservation in the mechanisms of HSF function between yeast and human cells was adequate to support growth of yeast cells when yeast HSF was substituted by human HSF1, or HSF2. However, expression of the human HSFs in yeast showed some differences between the two systems including the degree of involvement of HR-A in basal repression, activity of HSF2, and the ability to support growth under periods of sustained heat stress.

Mechanism of basal repression

The strong activity of the isolated C-terminal portion of HSF1 under non-HS conditions is consistent with the constitutive activity shown by the isolated CTAs of *S. cerevisiae* and *K. lactis* HSFs when fused to heterologous DBDs (Nieto-Sotelo, et al 1990; Sorger 1990; Jakobsen and Pelham 1991). The true inherent activity of the HSF1 C-terminal domain is illustrated by the progressive increase in activity under non-HS conditions as residues N-terminal to the activation domains are sequentially removed (Fig. 1, constructs 1–4). Activity of these deletion mutants under basal conditions suggests that no stress specific modifications of the activation domains are required during HS expression, and lends support to models of basal repression based on masking of the HSF. These results do not, however, rule out the possibility that the activation domains must be modified posttranscriptionally; only that potential modifications are likely not stress specific.

Masking models of basal repression propose that activities of the various functional domains of HSFs are inhibited due to their inaccessibility during non-stress conditions (for review see Sorger 1991). Repression is thought to result from the conformational state of the HSF itself, or from interactions of the HSF with other proteins. Hydrophobic repeats located within the OD, HR-C, and a region within AD2 have been shown to play a role in maintaining basal repression (Nieto-Sotelo et al 1990; Jakobsen and Pelham 1991; Rabindran et al 1993; Sheldon and Kingston 1993). One model for basal repression involves folding of the HSF under non-stress conditions so that HR-C interacts with one, or more, hydrophobic repeats of the OD (Nakai and Morimoto 1993; Rabindran et al 1993; Sheldon and Kingston 1993). Our experiments do not distinguish between inter- and

Fig. 5 Substitution of ScHSF with human HSFs. (A) β -gal activity determined in triplicate assays ($< 3\%$ variability) for constructs assayed in strain YJB341 after removal of ScHSF by selection on media containing 5-FOA (Bonner et al 1992). Both constructs were derived from pYGAL2. Growth conditions: 30°C (non-heat shock), 37°C (heat shock). (B) Colonies after several days of continuous growth on plates at indicated temperatures. C1 and C2 were positive control strains containing either the heat inducible ScHSF (strain YJB341), or constitutive ScHSF_{M232V} (341/BS16_{M232V}), respectively (Bonner et al 1992).

Fig. 6 Activity of human HSF 1 and HSF 2 in yeast cells lacking endogenous ScHSF. Human HSFs were introduced into strain YJB341 (Bonner et al 1992) using pYGAL2 and substituted for the plasmid-borne ScHSF by selection on medium containing 5-FOA. The control strain was YJB341 containing the ScHSF plasmid. HSF activity was monitored by assaying β -gal enzyme activity.

intramolecular masking, but indicate that several regions of the HSF1 protein contribute to repression of activity under both basal and HS conditions. For example, removal of HR-A of the OD (aa 120 to 174) caused activity under HS conditions to increase by 3-fold, and resulted in a significant loss of basal repression in yeast and a slight loss of basal repression in HeLa (Fig. 1). The

large differences in activity between the isolated activation domains (constructs 4 and 5) and the full length protein (construct 1) indicate that a substantial amount of inhibitory potential exists in the full length protein, even during HS.

In these experiments, DNA binding was conferred by the GAL4-DBD and presumably is not dependent on the oligomerization state of the HSF as indicated by the binding of construct 2 to the GAL4 DNA binding site under basal conditions (Fig. 1C). The clear accessibility of the GAL4-DBD to DNA under non-HS conditions suggests that the masking thought to be responsible for basal repression does not directly involve the DBD. Since DNA binding by the native HSF1 can be inhibited indirectly by the prevention of trimerization and nuclear localization, there may be no additional requirement to block DNA access by protein folding under conditions of basal repression.

Negative regulation of transcriptional activation domains

The lack of AD1 function after removal of AD2 by C-terminal deletion (Fig. 1, construct 6) was unexpected since similar experiments by Green et al (1995) and Newton et al (1996) showed from 5- to 11-fold heat induction. In both of these previous studies, the OD was not present in the GAL4- or LEXA-DBD fusions, whereas in our construct 6 the entire protein was present except for AD2. In the previous studies, the 201 to 370 fragment conferred heat inducible activity in HeLa and nonregulated, high activity in yeast (LEXA-DBD fusion). The lack of AD1 activity in our experiments may indicate that negative regulation is in effect under all conditions with a defect in the mechanism for the release of negative control under HS conditions. This failure to release repression during heat stress may be related to the presence of the OD, which has been associated with negative regulation in previous studies (Zuo et al 1995) and by the results

shown in Figure 1. Another possibility is that the presence of LEXA- and GAL4-DBDs may have differential effects on expression in the yeast system.

HSF2 expression

The GAL4-DBD fusion with full length human HSF2 (construct 9) was weakly expressed in yeast and showed little activity in HeLa cells (Fig. 1). In addition, the isolated C-terminus of HSF2 (aa 397 to 536; construct 10) exhibited activities of about 10% of HSF1 AD2 (construct 5) in yeast under both basal and HS conditions. This release of activity observed upon removal of portions of HSF2 N-terminal to the C-terminal activator (Fig. 1A) is similar to the results obtained with the removal of the negative regulatory domain from HSF1 (Fig. 1A, constructs 2–5) and raises the possibility that a similar domain of negative control is present in HSF2. In HeLa cells, the full length HSF2 fusion protein, and the isolated C-terminal domain showed less than 2% of the activity of AD2 of HSF1. Furthermore, the residual activity of the C-terminus in HeLa was even less after HS. It is noteworthy that in mammals HSF2 is largely involved in non-stress-induced *hsp* gene expression during development and is active in hemin induced differentiation of mouse embryonal carcinoma cells, in heart and brain cells, and during specific stages of spermatogenesis; whereas, HSF1 is predominantly stress responsive (Sistonen et al 1992; Sarge et al 1994; Goodson et al 1995). Perhaps the poor expression of HSF2 in yeast and HeLa cells reflects the lack of cellular conditions that mimic the developmental state that may exist when HSF2 is normally expressed.

The co-expression of GAL4-DBD fusions of HSF1 AD2 and the C-terminal domain of HSF2 resulted in non-synergistic activation of the reporter gene in HeLa (Fig. 4). This result suggests that the synergistic response in *hsp70* expression seen previously in human K562 cells that were induced with both hemin and heat stress (Sistonen et al 1994) was not due to synergism between transcriptional activation domains of HSF1 and HSF2. In our studies, we have assumed that opportunities for cooperative interactions between activation domains of HSF1 and HSF2 were provided by the formation of both homo- and heterodimers of the GAL4-DBD fusion proteins bound to the same promoter. In view of the lack of synergism between HSF1 and HSF2 activation domains, it may be that co-expression in doubly-induced human K562 cells facilitates the binding of HSF1 to HS promoters since HSF1 has more potential than HSF2 for transcriptional activation. It has been reported that during HS, HSF binds to over 150 sites scattered over the genome of *Drosophila* that are non-HS genes (Westwood et al 1991). It is also known that HSF1 binding to HSE elements is much more cooperative than HSF2 binding

(Kroeger and Morimoto 1994). Perhaps single sites within non-HS genes are preferentially occupied by HSF2 during conditions of co-expression, making more HSF1 available for synergistic binding to HS promoters which contain multiple HSEs. An alternative explanation for the lack of synergism would be that regions of the HSFs essential for synergism were missing in the activation domains used here. For example, HSF1 AD1 was not present in construct 5 used in this study, and the C-terminal activation domain of HSF2 has not been rigorously defined.

It is also possible that the combination of hemin treatment and high temperature in K562 cells may produce a more severe stress than either treatment alone resulting in a more robust induction of HSF1 and/or HSF2. In this case, the synergism would occur at the physiological level and not directly involve interactions between HSF1 and HSF2.

In summary, our functional analysis of HSF1 and HSF2 in yeast cells indicated that these types of heterologous expression studies have limited usefulness, perhaps due to differences in regulation between yeast and human cells. These differences are most apparent when studying the mechanism of basal repression and negative control by the intrapeptide negative regulatory domain. However, the yeast expression system can be useful in special situations (Gallo et al 1993; Green et al 1995) and is best illustrated here in the identification of the C-terminal transcriptional activation domain of HSF2 and in the screening of point mutations within AD2 of HSF1.

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