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Regulatory Domain of Human Heat Shock Transcription Factor-2 Is Not Regulated by Hemin or Heat Shock

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Abstract Heat shock transcription factor 2 (HSF-2) activates transcription of heat shock proteins in response to hemin in the human erythroleukemia cell line, K562. To understand the regulation of HSF-2 activation, a series of deletion mutants of HSF-2 fused to the GAL-4 DNA binding domain were generated. We have found that human HSF-2 has a regulatory domain located in the carboxyl-terminal portion of the protein which represses the activity of its activation domain under normal physiological conditions. The repressive effects of this domain can be eliminated by its deletion in GAL4-HSF-2 fusion constructs. The regulatory domain of HSF-2 can also repress a heterologous chimeric activator that contains a portion of the VP16 activation domain. The activation domain of HSF-2 is a segment of approximately 77 amino acids located proximal to the carboxyl-terminal hydrophobic heptad repeat (leucine zipper 4) of the molecule. Interestingly, the GAL4-HSF-2 fusion protein and the 77 amino acids activation domain are inactive and are not activated by pretreatment of cells with either hemin or elevated temperature. Our data suggest that regulation of HSF-2 differs from HSF-1 in that its regulatory domain is not responsive to hemin or heat directly. J. Cell. Biochem. 73:56–69, 1999.

Key words: HSF-2; hemin; heat shock

Mammalian cells respond to heat shock and other stresses by upregulating transcription of genes encoding heat shock proteins (HSPs). This increase in transcription of heat shock genes is due to the activation of heat shock transcription factors (HSFs). In eukaryotes, HSFs bind to conserved regulatory sequences known as heat shock elements (HSEs) and control the expression of HSPs in response to stress [Abravaya, 1991; Larson, 1988; Mivechi, 1992; Sorger, 1987; Wu, 1984; Zimarino, 1987]. The HSE consists of two or more adjacent inverted repeats of AGAAN. HSFs bind to HSEs as trimers, with each monomer contacting an individual AGAAN sequence [Wu, 1995].

In eukaryotes, four distinctive genes for HSFs have been cloned, namely HSF-1, HSF-2, HSF-3, and HSF-4 [Nakai, 1993, 1997; Rabindran, 1991; Sarge, 1991; Schuetz, 1991]. HSF-1 responds to environmental stresses such as heat shock, amino acid analogues and heavy metals [Baler, 1993; Mivechi, 1992; Sarge, 1993]. Studies with chimeric GAL4-HSF-1 fusion proteins indicate that HSF-1 contains a heat shock responsive, negative regulatory domain which, when deleted, exposes a constitutive transcriptional activator located in the carboxyl-terminal end of the molecule [Newton, 1996; Shi, 1995; Zuo, 1995]. HSF-3 has recently been shown to be activated via interaction with c-myb in the absence of cellular stress with a slower kinetics than HSF-1 [Kanei-Ishii, 1997]. Deletion of the HSF-3 gene in chicken cells shows a reduction in heat-induced HSPs as well as absence of any HSF-1 binding ability after heat shock, suggesting that HSF-3, which so far only has been found in chicken, may regulate HSF-1 activity [Tanabe, 1998]. Studies with chimeric GAL4-HSF-4 fusion proteins suggests that HSF-4 lacks the carboxyl-terminal hydrophobic repeat and a transcriptional activation domain and is believed to be involved in the negative regulation of DNA binding of other HSFs [Nakai, 1997].

HSF-2 is unique in its activation pattern as it does not respond to heat stress efficiently but it

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activates transcription of HSPs in response to hemin in human K562 cells [Mivechi, 1994; Sistonen, 1992]. K562 cells are promyelocytic leukemia cells that nonterminally differentiate into erythroblasts in the presence of hemin [Dean, 1981; Lozzio, 1977]. Hemoglobin accumulation following treatment of K562 cells with hemin can be detected within 6 h. During this time, HSF-2 activation can also be observed. The DNA binding activity of HSF-2 can be detected as early as 1–2 h following pretreatment of cells with hemin [Mivechi, 1994; Sistonen, 1992]. In addition, HSF-2 is constitutively active in mouse embryonic carcinoma cells and expressed during mouse embryogenesis, suggesting that HSF-2 activity may be important during differentiation and development [Murphy, 1994; Sarge, 1994]. The signaling pathway leading to HSF-2 activation is not understood. HSF-2 may not be regulated directly by phosphorylation, since phosphorylated forms of HSF-2 have not been detected. Furthermore, the regulation of HSF-2 transcriptional activity is not yet known. As with other HSFs, HSF-2 contains a conserved DNA binding domain and 4 leucine zippers. Leucine zippers 1–3 regulate the trimerization that is required for DNA binding [Wu, 1995]. As with HSF-1, HSF-2 contains two isoforms, a longer species that is called HSF-2α and a shorter species called HSF-2β [Fiorenza, 1995; Goodson, 1995; Leppa, 1997]. The differential expression of these forms has been suggested to play a role in regulation of the activity of this protein. More recently, it has been shown that HSF-2β may regulate the binding activity of the HSF-2α isoform [Leppa, 1997].

Differences in the regulation of various HSFs prompted us to examine whether HSF-2 protein is regulated by hemin or heat shock. In these studies, we analyzed functional domains of HSF-2 by using a series of GAL4 fusion constructs containing HSF-2 deletion mutants. Results indicate that HSF-2 contains a regulatory domain that inhibits the activity of its activation domain. Further, unlike the HSF-1 central regulatory domain which is responsive to heat shock, the HSF-2 regulatory domain does not respond to hemin or heat shock. In addition, the 18 amino acids located proximal to the carboxyl-terminal leucine zipper 4 present in the HSF-2α isoform is repressive to the transcriptional activation domain.

MATERIALS AND METHODS

Plasmid Constructs

Deletion mutants. All GAL4-HSF-2 expression plasmids were based on the parent vector pSG424 that was kindly provided by Dr. M. Ptashne (Harvard University, MA). The pSG424 contains the first 147 amino acids of the yeast protein GAL4 (GAL4 1–147) followed by a multiple cloning site. All deletion mutants were constructed using PCR primers that incorporated appropriate restriction enzyme sites. EcoR I was designed into Forward Primers, while Sac I was incorporated into all Reverse Primers. The PCR primers for various HSF-2 deletion mutants were as follows; Forward Primers: F1: GGAATTCATGAAGCAGAGTT CGAAC; F83: GGAATTCCAA GAAAGAGATGGTCCT; F113: GGAATTCTCTTCAAAACCAGAAGAAAATAAAATTCG; F161: GGAATTCGTGTCAGAATTACGAGCAAAGCATG; F207:GGAATTCGCCCAAAAAAGAACCTGTTTCAGC; F281: GGAATTCTACCCTGATATT GTCATCGTTGAAG; F355: GGAATTCTTGGGAAAGGTTGAGCTGTTGG; F377: GGAATT CATGCTATCAGGAAGA CAA; F393: ATCGGAATTCCTTTTCACTA GTTCTGTG; F399: GGAATTCCAGATGAATCCCACAGATTACATC; F419: GGAATTCACCAAGAACAATGT AGTT; F439: GGAATTCGATAAGCAGCTTATCCAG; F459: GGAATTCGCTTCTTCTGTT GAACAG; F489: GGA ATTCAGCAGCCTAGACCCAGAA; F509: ATCGGAATTCGCTGAA GCTAGTGAAGCT. The numbers for each primer indicated above represent the location of the amino acid in the HSF-2 protein that is fused to the GAL4 DNA binding domain. Reverse Primers: R354: ATCGGAGCTCAAGATTGATGTTATCATT; R392: ATCGGAACTCATCAAC CAGGAGATCTGG; R418: ATCGGAACTCAGTTTCTAATCCTT TATT; R516: ATCGGAAC TCCAGTGTAGCTTCACTAGC; R536: ATCGGAGCTCTTAGCTATCTAAAAGTGG. For the reverse primers, the numbers indicated above represent the amino acid at the carboxyl-terminal end of the protein. PCR was performed using PFU polymerase (Stratagene, La Jolla, CA) according to the manufacturer's manual. PCR products were digested with EcoR I and Sac I and subcloned in frame into pSG424 following the GAL4 (1–147).

The plasmid pSGVP16 was also the gift of Dr. M. Ptashne. pSGVP16 contains amino acids 413–490 of the human herpes simplex virus type I. The HSF-2 deletion mutants were gener-

ated as described above. The VP16 was amplified from pSGVP16 plasmids by PCR using primers that incorporated the appropriate restriction enzyme sites (Sac I for the amino-terminal primers and Xba I for the carboxyl-terminal primers). VP16 primers used were: VP16-F413: ACTGGAGCTCGCCC CCCCGAC-CGATGTC; VP16-R490: ATGCTCTAGACGT-GCCGC-CGAATGGGTT. After PCR, the amplified fragments were digested with the appropriate restriction enzymes and subcloned into EcoR I and Xba I sites of pSG424.

Reporter plasmids. Two reporter plasmids were used in these studies. G5BCAT containing 5 GAL4 DNA binding sites and a basal promoter driving the expression of the chloramphenicol acetyl transferase gene was the gift of Dr. M. Ptachne. The internal reference plasmid was pRL-CMV in which a CMV promoter drives constitutive expression of Renilla luciferase (Promega, Madison, WI).

Cells and Transient Transfection Assays

K562 cells [Lozzio, 1977] were used in all experiments. Cells were maintained in Iscove's Minimal Essential Medium (IMDM) supplemented with antibiotics and 15% fetal calf serum (FCS). Transient transfection studies were performed using electroporation (GenePulser, Bio-Rad, Richmond, CA). Cells were diluted to 0.6×10^6 cells per milliliter one day before the onset of the experiment. Electroporation was carried out with 5 μg of GAL4-HSF-2 mutant expression plasmids, 5 μg of reporter plasmids, and 0.02 μg of pRL-CMV (Renilla luciferase). 5×10^6 cells were added to a cuvette (diameter 0.4 cm) containing 0.8 milliliter of serum-free growth medium. After electroporation (350 V, 975 μF), cells were resuspended in 6 milliliter of growth medium plus 15% FCS and was incubated at 37°C. Reporter gene activity was examined 40 to 48 h posttransfection. For hemin treatment, 19.56 mg of hemin was dissolved in 0.3 milliliter of 1 N NaOH, 0.3 milliliter of 0.5 M Tris-base, and 2.85 milliliter of IMDM. Hemin was added to cultures approximately 24 h after transfection to a final concentration of 30 μM and cultures were incubated at 37°C for 16 h. This concentration and treatment time was sufficient to increase hemoglobin levels in K562 cells, to activate HSF-2, and to induce expression of HSPs [Mivechi, 1994; Sistonen, 1992].

All transient transfection experiments were performed at least two times, and results were consistent. In each case a representative experiment is shown. The differences in transfection frequencies obtained between groups have been adjusted during quantitation of CAT assays and are shown in Figures 2C–6C.

Cholarmphenicol Acetyl Transferase (CAT) Assays

Forty-eight h after transfection, cells were rinsed with PBS and cell pellets frozen at −70°C until assayed. To determine CAT activity, cell pellets were lysed in 50–80 μl of 0.25 M Tris (pH 7.8) and freeze-thawed for three cycles. Samples were microfuged for 2 min to remove cell debris and the amount of protein estimated using bicinchoninic acid (Pierce Biochemicals, Rockford, IL). Twenty to 80 μg of protein was then incubated with acetyl Coenzyme A and ^{14}C-Chloramphenicol at 37°C for 16 h. The samples were extracted with ethyl acetate, dried, and dissolved in 30 μl ethyl acetate, spotted onto chromatographic paper, and resolved in methanol: chloroform (5:95 v/v) for 1 h. The membrane was then dried and exposed to X-ray film and quantitated using a PhosphorImager.

Electrophoretic Mobility Shift Analysis

Electrophoretic mobility shift analysis using whole cell extracts has been described in detail previously [Mivechi, 1995a,b; Zimarino, 1987]. Briefly, after various treatments, cells were rinsed with PBS and lysed by homogenization in 100 μl of extraction buffer (10 mM HEPES, pH 7.9; 0.4 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 5% glycerol, 0.5 mM PMSF). The protein concentration of each sample was estimated by the bicinchoninic acid (Pierce Biochemicals). Equal amounts of protein (15 μg) in extraction buffer (volume not exceeding 15 μl) was added to 4 μl of binding buffer (37.5 mM NaCl, 15 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.5 mM DTT, 5% glycerol) that also contained 1 μl of yeast tRNA (10 μg), 1 μg of sheared *E. coli* DNA, 10 μg of poly dI-dC and 1 ng of ^{32}P-labeled oligonucleotide. This mixture was incubated at 25°C for 15 min and was analyzed by electrophoresis on a nondenaturing 4.5% polyacrylamide gel. After electrophoresis, gels were fixed in 7% (v/v) acetic acid for 5 min, rinsed once in distilled water, dried under vacuum, and exposed to X-ray film. The double stranded oligonucleotide sequence containing a GAL4 binding site was as follows; 5'-GATCTCGGAGTACTGTC-CTCCGA-3' and 3'-AGCCTCATGACAGGAG-GCTCTAGG-5'. The oligonucleotide sequence containing a HSE binding site was 5'-GTCGAC-

GGATCCGA GCGCCTCGAATGTTCTAGAA-AAGG-3' [Mivechi, 1995b; Zimarino, 1987]. These oligonucleotides were labeled using Klenow fragment of DNA polymerase I, deoxynucleotide triphosphates, and α-^{32}P-dCTP. The antibody to HSF-2 used in supershift experiments was the gift of Dr. R. Morimoto (Northwestern University, IL).

RESULTS

Activation of HSF-2 in Response to Hemin in K562 Cells

K562 cells are promyelocytic leukemia cells that, when exposed to hemin, differentiate into erythroblasts and accumulate large amounts of fetal hemoglobin as well as several species of heat shock proteins (HSPs) [Mivechi, 1994; Sistonen, 1992]. Under these conditions, the accumulation of HSPs are driven by the activation of the heat shock transcription factor-2 (HSF-2) [Sistonen, 1992]. Thus, K562 cells treated with 30 μM of hemin show activation of HSF-2 within 1–2 h; the DNA binding activity of HSF-2 to an HSE containing DNA fragment remains elevated as long as hemin is present, as shown in the electrophoretic mobility shift assay in Figure 1. Addition of anti-HSF-2 antibody to the reaction mixture reduces the amount of DNA binding activity observed in extracts of cells treated with hemin for 4 and 6 h indicating the specificity of the HSF-2/HSE interactions (compare lanes 4 and 5 with lanes 6 and 7). However, preincubation of extracts of heated cells with anti-HSF-2 antibody have no effect on the amount of DNA binding activity observed (compare lane 8 with lane 9), suggesting that the majority of the binding observed is due to activated HSF-1 rather than HSF-2 as expected [Sistonen, 1992]. Cell extracts heated or pretreated with hemin and then exposed to control nonspecific serum show no effect on HSF-2 DNA binding ability, documenting the specificity of anti-HSF-2 antibody (compare lanes 4, 5, and 8 with lanes 10–12). Addition of unlabelled, competitor DNA fragment abolished DNA binding, providing further evidence for specificity of the reaction.

Regulatory Domain of HSF-2 Represses the Activity of its Activation Domain

Previous studies using GAL4-HSF-1 chimeric proteins suggested that the central regu-

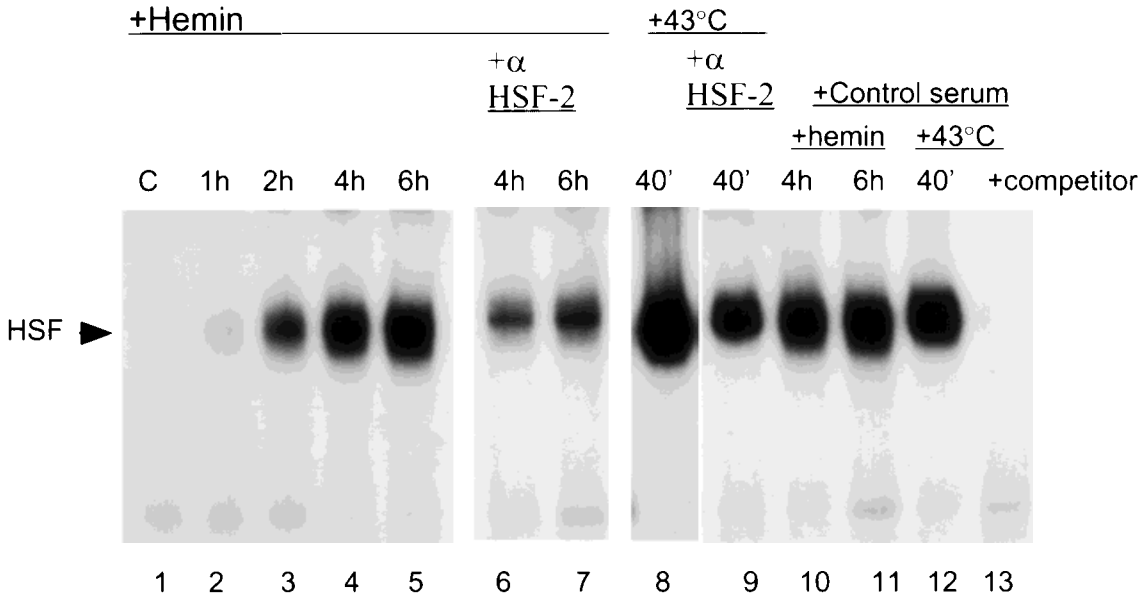

Fig. 1. Activation of HSF-2 during differentiation of K562 cells. K562 cells were treated with hemin for up to 6 h (**lanes 1–5**) or heated at 43°C for 40 min (**lanes 8,9**). Cell extracts were prepared and equal amounts of protein were analyzed by electrophoretic mobility shift assays using ^{32}P-labeled HSE containing DNA fragment. **Lanes 6,7:** Cells were pretreated with hemin for 4 or 6 h. Cell extracts were then incubated with anti-HSF-2 antibody to show specific interaction of HSF-2 with heat shock element (HSE). Cells were heated at 43°C for 40 min (in the absence of hemin). Cell extracts were supplemented with (lane 9) or without (lane 8) anti-HSF-2 antibody and were assayed. Lanes 10–12: cells were treated for 4 or 6 h with hemin (**lanes 10, 11**) or heat treated (**lane 12**), and cell extracts were exposed to nonspecific control serum and were assayed. **Lane 13:** Extract same as lane 4, but 200-fold excess unlabelled probe added to reaction to show specificity of HSF-2/HSE interaction.

latory domain of HSF-1, which can be activated by treatment of cells with heat shock or other stresses such as cadmium [Newton, 1996; Shi, 1995], represses the activity of its activation domain. In the case of HSF-2 however, it is unknown how the protein is regulated by hemin or heat shock. To analyze the HSF-2 transcriptional activation and to identify the regulatory domain and the effect of heat or hemin on HSF-2, a series of GAL4-HSF-2 chimeric genes was constructed. These constructs contained the GAL4 DNA binding domain (amino acids 1–147 of the yeast GAL4 protein) to which a series of HSF-2 fragments lacking the HSF-2 DNA binding domain (residues 1 to 112) were fused (Fig. 2A). These constructs were then cotransfected with G5BCAT reporter gene and pRL-CMV (Renilla luciferase) into K562 cells. At 48 h after transfection, cells were analyzed for the presence of CAT activity (Fig. 2B,C). As

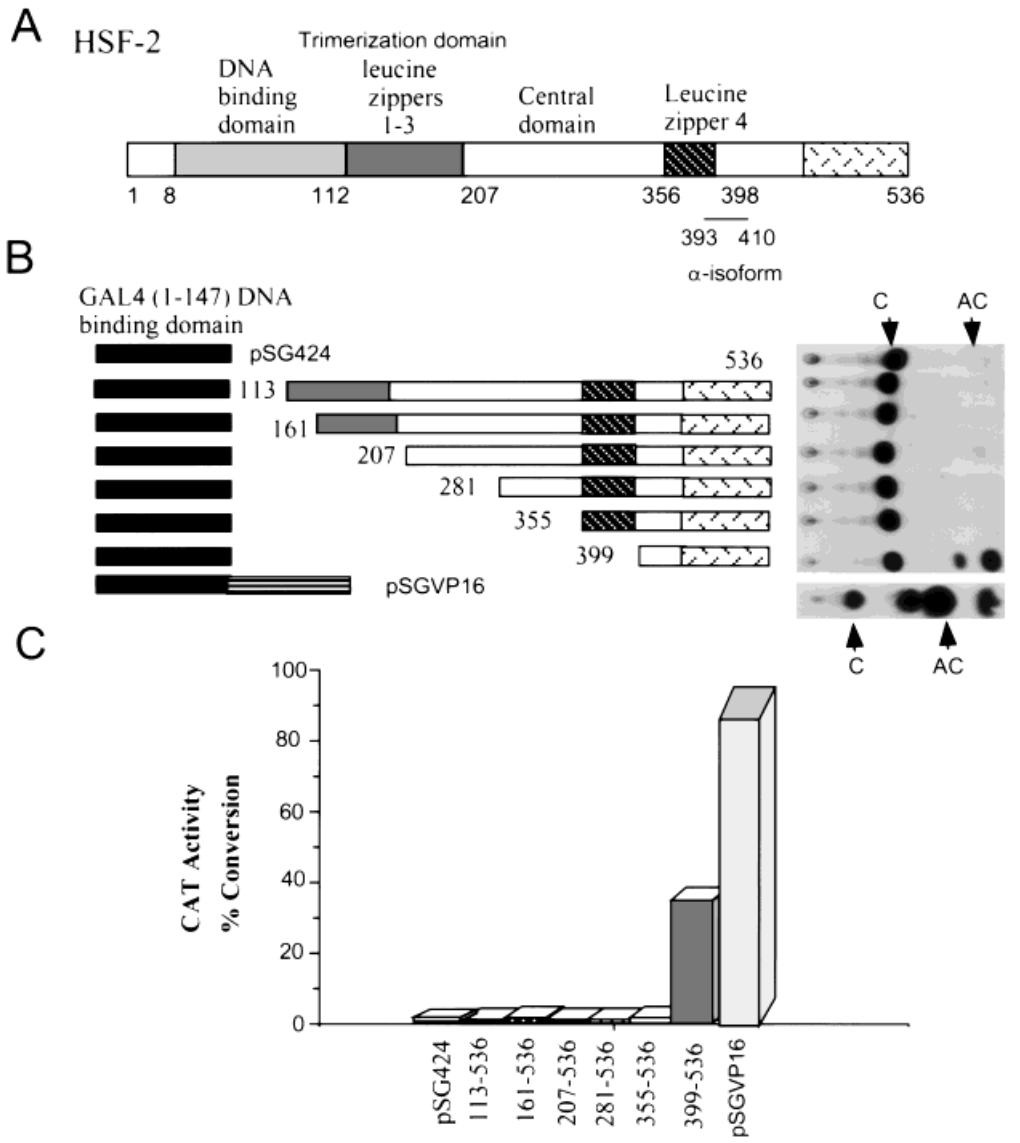

Fig. 2. Regulatory domain of HSF-2 represses its transcriptional activation domain. **A:** Functional domains of the HSF-2 protein: DNA binding domain (residues 8 to 112), N-terminal leucine zippers (residues 112 to 207), central domain (residues 207 to 356), C-terminal leucine zipper 4 (residues 356 to 398). α isoform contains amino acids 393 to 410 which is spliced out of isoform β. **B:** Deletion mutants of HSF-2 were fused to DNA binding domain of GAL4 (1–147 amino acids). Constructs were cotransfected into K562 cells with reporter construct G5BCAT. K562 cells were lysed 48 h posttransfection and CAT activity present in 80 μg of protein was determined for each sample. **C:** The quantitation of CAT activity shown in (B) using PhosphorImager analysis. C is position of chloramphenicol and AC is position of the major acetylated product of chloramphenicol.

a negative control, K562 cells were transfected with plasmids pSG424 which contains the first 147 amino acids of the yeast protein GAL4 DNA binding domain (GAL4 1–147) and as a positive control, cells were transfected with plasmid pSGVP16 which contains GAL4 DNA binding domain (GAL4 1–147) fused to amino acids 413–490 of the human herpes simplex virus type I.

A GAL4-HSF-2 fusion construct containing HSF-2 residues 113 to 536, which includes the trimerization, central and leucine zipper 4 domains, did not express the CAT reporter gene (Fig. 2B,C). Similar results were obtained when portions of the trimerization domain were deleted (constructs 161–536 and 207–536) and when the central domain was deleted (constructs 207–536, 281–536, and 355–536). However, deletion of residues 355 to 398 relieved the repressor activity since CAT activity was detected for 399–536 construct. The lack of transcriptional activity in the first 5 constructs is not due to their inability to bind to DNA, as the DNA binding activity of all mutants was demonstrated by electrophoretic mobility shift analysis shown in the latter part of the Results (see Fig. 7).

To more precisely locate the negative regulatory domain, we constructed a second series of mutants with progressive deletions in the carboxyl-terminal leucine zipper 4 region (Fig. 3). CAT reporter activity was seen with the progressive deletion of residues 377–398, 399–418, 419–458, and 460–488. However, further deletion past residue 489 resulted in a sharp reduction in CAT reporter activity, suggesting that the transcriptional activation domain resides between residues 459 and 536. These data suggest that the negative regulatory domain lies within amino acid residues 355 to 419. A large portion of the 77 amino acids in the 459 to 536 construct is required for transcriptional activation, as further deletions from either end (constructs 489 to 536 and 459 to 516) abolish transcriptional activity, suggesting that the transcriptional activation domain extends almost to the carboxyl-terminal end of the HSF-2 protein (Fig. 3A–C).

HSF-2 Regulatory Domain is not Responsive to Hemin or Heat Shock

The regulatory domain of HSF-1 has recently been analyzed in detail and results indicate that the regulatory domain inhibits the transcriptional activation domain but responds to heat shock or other forms of stress. That is, fusion of the central regulatory domain of HSF-1 to the GAL-4 DNA binding domain represses CAT activity of the G5BCAT reporter gene, but heat shock relieves this repression [Newton, 1996; Shi, 1995]. To investigate whether the regulatory domain of HSF-2 protein also responds to hemin or heat shock treatment, mutant constructs were cotransfected into K562 cells with G5BCAT in the absence or presence of hemin or heat shock (Figs. 4A and 5A). Cells were left as untreated controls or were either treated at 24 h after transfection with 30 μM of hemin for 16 h (Fig. 4B,C) or at 36 h after transfection heated at 43°C for 40 min and allowed to recover at 37°C for 6 h (Fig. 5B,C). Cells were lysed and CAT activity was determined. The results indicate that neither treatment with hemin nor heat shock relieves the repression that the regulatory domain of HSF-2 exerts on its activation domain. Thus, the HSF-2 protein is not regulated by hemin or heat shock.

Regulatory Domain of HSF-2 Represses the Heterologous Transcriptional Activator

To confirm that the regulatory domain of HSF-2 represses the activity of its activation domain, several deletion constructs containing various portions of the HSF-2 central domain were fused to the VP16 transcriptional activation domain, generating the fusion constructs shown in Figure 6A. These constructs were individually cotransfected with the G5BCAT reporter construct into K562 cells and CAT activities were measured (Fig. 6B,C). The results indicate that a construct containing HSF-2 residues 1 to 399, which includes the carboxyl-terminal hydrophobic repeats, inhibits the transcriptional activity of VP16 by 98%. The construct containing HSF-2 residues 113 to 392 resulted in a 4.2-fold reduction of CAT activity when compared to cells transfected with pSGVP16 alone while the construct containing residues 113 to 354 resulted in a 1.4-fold reduction. The construct containing only residues 355 to 418 which encode the carboxyl-terminal hydrophobic repeats (leucine zipper 4) and the carboxyl-terminal 18 amino acids of the α isoform of HSF-2, showed the highest inhibitory effect (7.3-fold). The deletion of the carboxyl-

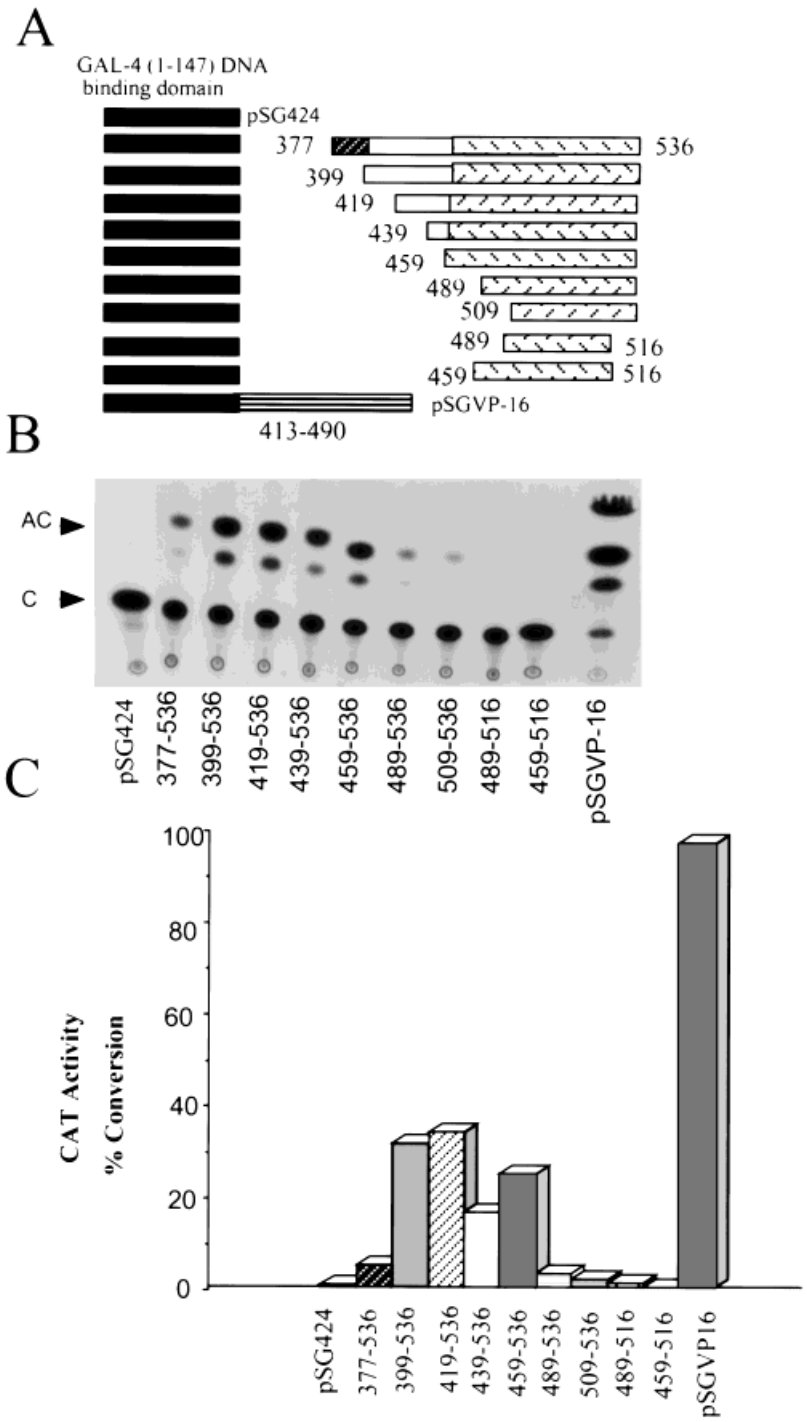

Fig. 3. Progressive deletion of the regulatory domain of HSF-2 reveals a C-terminal transcriptional activation domain that locates between residues 459–536. **A:** Various deletion mutants of HSF-2 were fused to the DNA binding domain of GAL4 (1–147 amino acids). The bottom construct is pSGVP-16 that contains amino acids 413–490 of the human herpes simplex virus type I. **B:** Constructs shown in (A) were cotransfected into K562 cells with the reporter construct G5BCAT. K562 cells were lysed 48 h posttransfection and CAT activity in 80 µg of protein per sample was determined. **C:** The quantitation of CAT activity shown in (B) using PhosphorImager analysis. C is position of chloramphenicol and AC is position of the major acetylated product of chloramphenicol.

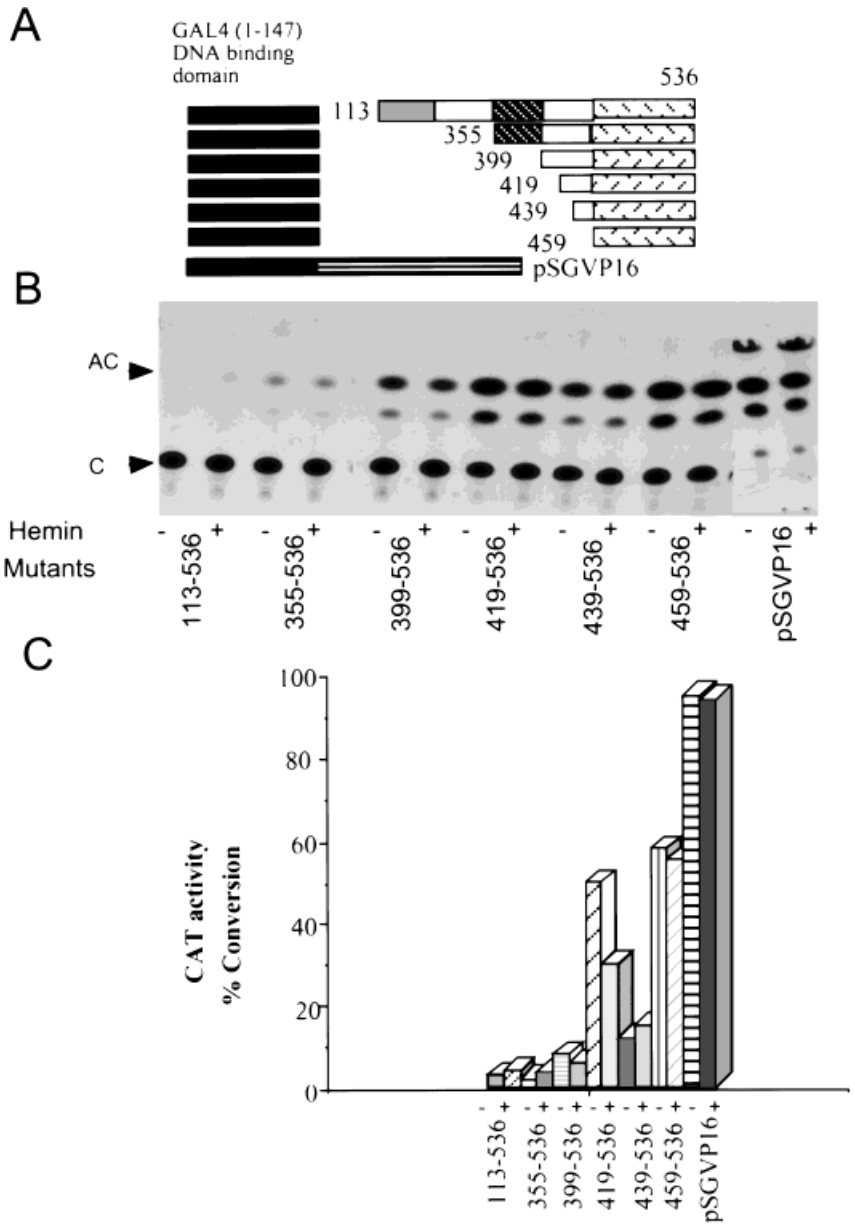

Fig. 4. HSF-2 regulatory domain is not hemin responsive. **A:** Various deletion mutants of HSF-2 were fused to the DNA binding domain of GAL4 (1–147 amino acids). **B:** Constructs shown in (A) were cotransfected into K562 cells with the reporter construct G5BCAT. Cells from each culture were split into two groups 36 hours after transfection. One group remained as untreated control (indicated as -) and the other group was treated with 30 μM of hemin (indicated as +) for 16 h. K562 cells were lysed and CAT activity in 80 μg of protein from each sample was determined. **C:** The quantitation of CAT activity shown in (B) using PhosphorImager analysis. C is position of chloramphenicol and AC is position of the major acetylated product of chloramphenicol.

terminal hydrophobic repeats, leaving only the 18 amino acids present in the α isoform of HSF-2, inhibited the VP16 transcriptional activity by 3.4-fold, suggesting that the leucine zipper 4 and the 18 amino acid segments negatively regulate HSF-2 to an equal extent.

Taken together, these results suggest that the fragment containing residues 113 to 354 that encode the central region of HSF-2 protein has a limited negative regulatory effect on the VP16 transcriptional activator. However, the region containing both the carboxyl-terminal leucine zipper 4 and the 18 amino acids present in α isoform of HSF-2 exerts strong negative regulation on the transcriptional activation domain of HSF-2.

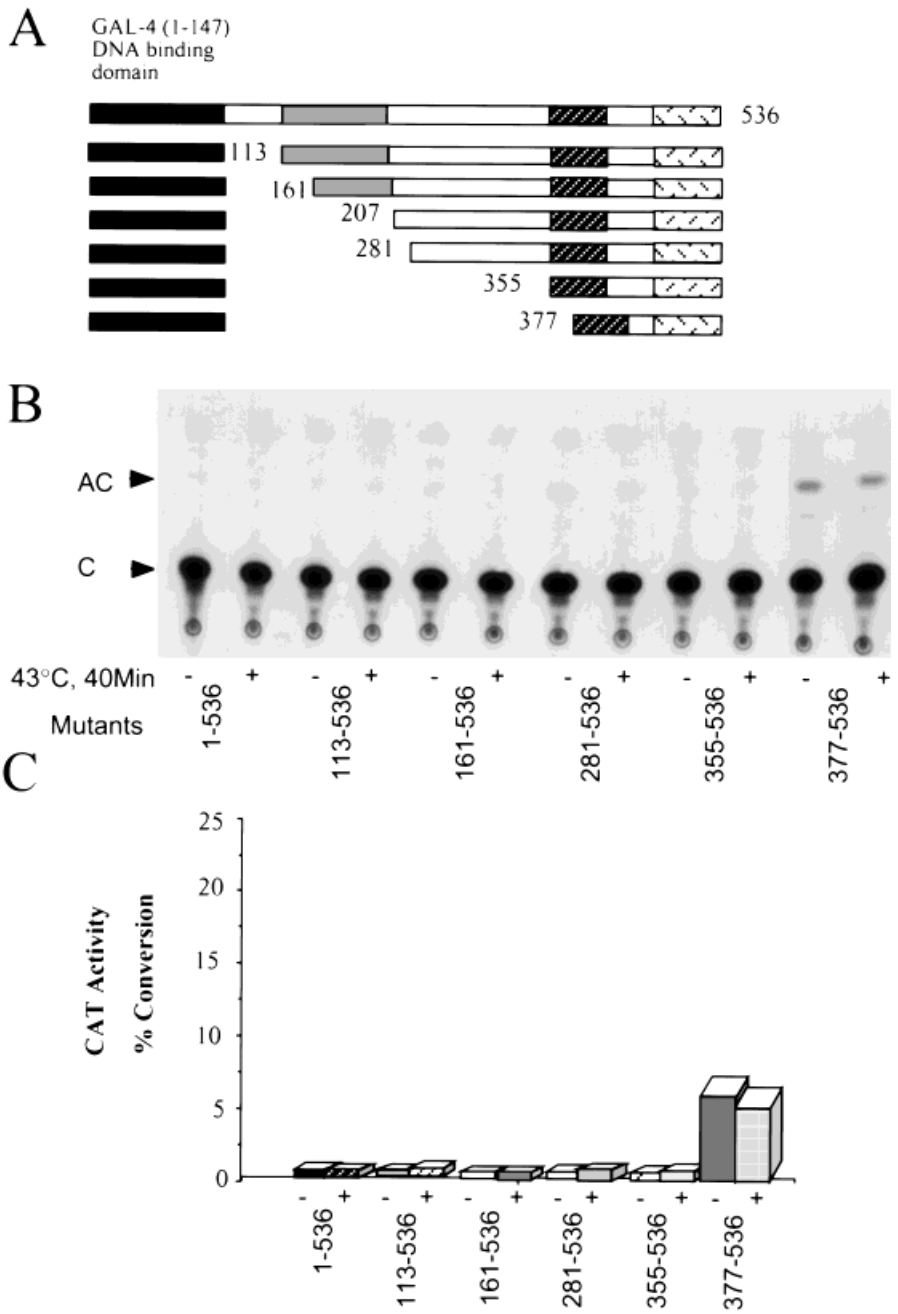

Fig. 5. HSF-2 regulatory domain does not respond to heat shock. **A:** Various deletion mutants of HSF-2 were fused to the DNA binding domain of GAL4 (1–147 amino acids). **B:** Constructs shown in (A) were cotransfected into K562 cells with the reporter construct G5BCAT. Cells from each culture were split into two groups 48 h after transfection. One group remained as an untreated control (indicated as -) and the other group was heated at 43°C for 40 min (indicated as +) followed by incubation at 37°C for 6 h to allow for the expression of the CAT reporter gene. K562 cells were lysed and CAT activity in 80 µg of protein from each sample was determined. **C:** The quantitation of CAT activity shown in (B) using PhosphorImager analysis. C is position of chloramphenicol and AC is position of the major acetylated product of chloramphenicol.

GAL4-HSF-2 Chimeric Proteins Have GAL4 DNA Binding Activity

To ensure that the various fragments of HSF-2 fused to the GAL-4 DNA binding domain are not inhibitory to GAL4 DNA binding activity, the binding activity of all constructs to the GAL4 binding site was determined by using electrophoretic mobility shift assays (Fig. 7). The data suggest that all constructs were expressed and were capable of binding to the GAL4 binding site. Pretreatment of transfected cells with

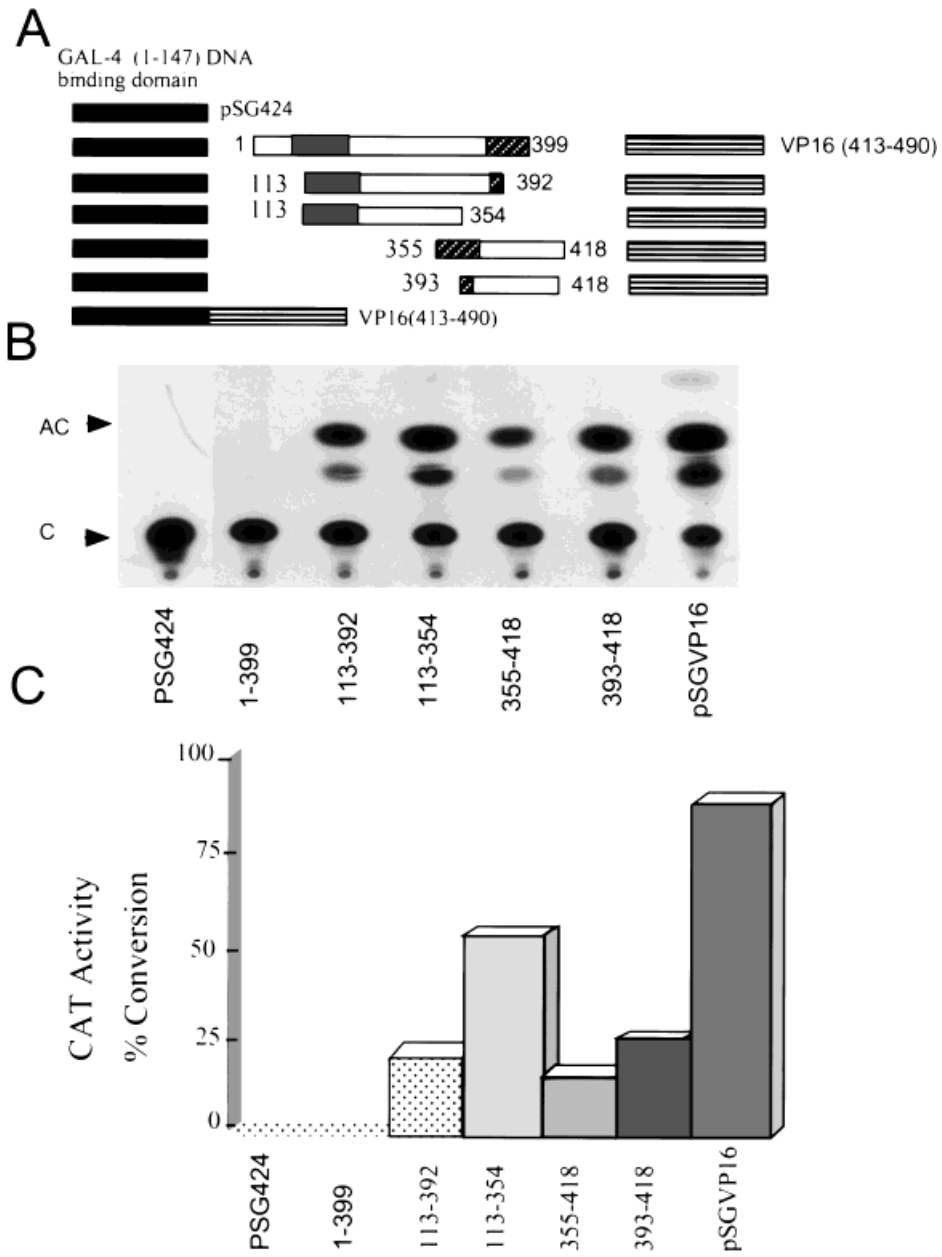

Fig. 6. HSF-2 regulatory domain represses the transcriptional activator VP16. **A:** Various deletion mutants of HSF-2 were fused to the DNA binding domain of GAL4 (1–147 amino acids) and the VP16 transcriptional activator. **B:** Constructs shown in (A) were cotransfected into K562 cells with the reporter construct G5BCAT. K562 cells were lysed 48 h posttransfection and CAT activity in 40 µg of protein from each sample was determined. **C:** Quantitation of CAT activity shown in (B) using PhosphorImager analysis. C is position of chloramphenicol and AC is position of the major acetylated product of chloramphenicol.

hemin or heat shock did not have any effect on GAL4 DNA binding ability (data not shown).

DISCUSSION

The mode of activation of HSF-2 differs from the other HSF family members in several respects. Although HSF-2 is present in many cell lines tested, it is under negative regulation and its activity has not been demonstrated under normal physiological conditions. One system where HSF-2 activity can be detected is during hemin-induced K562 cell differentiation into erythroblasts [Mivechi, 1994; Sistonen, 1992]. The signaling pathway that is induced by hemin and leads to HSF-2 activation is not yet understood. HSF-2 activation and accumulation of HSPs after treatment of cells with hemin may be required for differentiation, or the

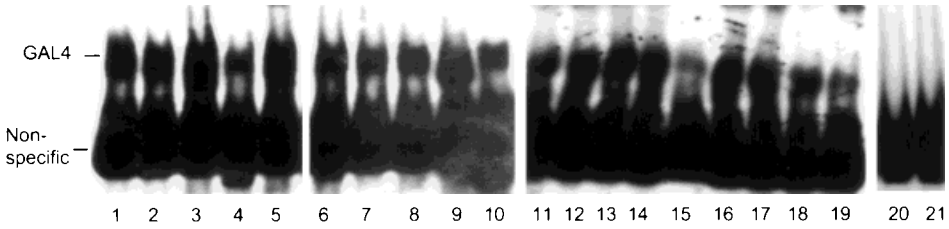

Fig. 7. Electrophoretic mobility shift assays showing expression constructs containing the GAL4 DNA binding domain can bind to GAL4 DNA binding site. Expression constructs were individually cotransfected into K562 cells with pBabe plasmids encoding a puromycin resistance marker. One µg/ml of puromycin was added to cultures 48 h posttransfection and cells were grown for an additional 10 days to enrich for cells expressing the transfected constructs. K562 cells were then lysed and the DNA binding activity in 20 µg of protein was determined using electrophoretic mobility shift assays with a GAL4 consensus binding sequence, as described under Materials and Methods. Numbers 1–19 indicate the DNA binding of the following expression vectors; 1. 113–536, 2. 161–536, 3. 207–536, 4. 281–536, 5. 355–536, 6. 377-536, 7. 399–536, 8. 419–536, 9. 439–536, 10. 459–536, 11. 489–536, 12. 509–536, 13. 489–516, 14. 489–536, 15. 459–536, 16. 1–399-VP-16, 17. 113–392-VP-16, 18. 113–354-VP-16, 19. 1–536, 20 and 21 are groups 10 and 16 plus 200-fold excess unlabelled probe as competitor.

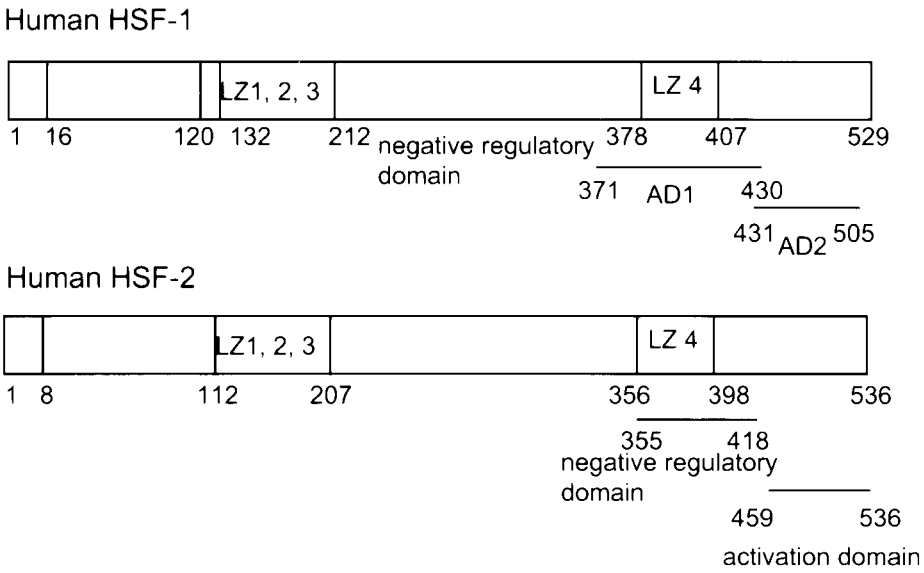

Fig. 8. Comparison of human HSF-1 and HSF-2 functional domains. Numbers indicate position of the amino acids. LZ 1, 2, 3, and 4 are leucine zippers 1–4. AD1 and AD2 are activation domains 1 and 2.

ability of hemin to induce peroxidation reactions may lead to HSF-2 activation by a secondary event. Previous studies using antisense oligonucleotides to HSP-70 or overexpression of the HSF-2β isoform inhibit hemin induced differentiation, suggesting a role for HSPs during K562 cell differentiation [Leppa, 1997; Mivechi, 1994]. Unlike the heat induction of HSF-1 activation which occurs within minutes, the hemin induced HSF-2 activation is a slow process but leads to increases in the levels of HSF-2 DNA binding activity within 1—2 h and elevated synthesis of most HSPs that have been measured (Fig. 1) [Mivechi, 1994]. Similarly to HSF-1, HSF-2 binds to HSE containing oligonucleotides. In vivo, however, both HSF-1 and HSF-2 do not normally bind DNA and stress or other stimuli are required to activate the DNA binding activity of these factors [Wu, 1995].

The studies presented here and the GAL4-HSF-1 fusion constructs previously reported [Newton, 1996; Shi, 1995] indicate some differences between activation of the human HSF-1 and HSF-2 proteins. HSF-1 appears to contain two activation domains, AD1 that is located between residues 371 to 430, and AD2 located between residues 431 to 505. The relative positions of these activation domains is shown in Figure 8. AD1 and AD2 of HSF-1 are located proximal to the carboxyl-terminal hydrophobic region. However, the HSF-2 activation domain is located between residues 459 and 536 after the carboxyl-terminal hydrophobic leucine zip-

per 4. Deletion of 20 amino acids near the carboxyl terminal end, abolishes the activity of this activation domain.

As with the human HSF-1 protein, HSF-2 also contains a negative regulatory domain that represses the activity of its activation domain. In fact, human HSF-1 contains 2 negative regulatory domains located between residues 201 and 370 [Newton, 1996]. We have shown for human HSF-2, the central domain exerts only a weak repressive effect on the HSF-2 activation domain. The fusion of this segment (residues 113 to 354) to the VP16 activation domain reduces the ability of VP16 to activate transcription of CAT by only 1.4-fold. The major negative regulatory domain of HSF-2 is located in the leucine zipper 4 and the 18 amino acids of the alternative splice form, HSF-2α. Although, our studies do not address which specific amino acids in this region confers this negative regulatory effect. Deletion of the carboxyl-terminal hydrophobic region and removal of these 18 amino acids further relieve the negative regulatory effect exerted by these regions. It is interesting to note that the shorter version of HSF-2, that is the HSF-2β isoform, had been presumed to be inhibitory to the activity of HSF-2 [Leppa, 1997]. Furthermore, overexpression of HSF-2β appears to inhibit HSF-2α activity by inhibiting its binding to the HSE [Leppa, 1997]. Our results indicate that the regulation of HSF-2α isoform by HSF-2β isoform is more complex than previously believed as the HSF-2β is somewhat more transcriptionally active than the HSF-2α.

The surprising finding is that, unlike the central regulatory domains of both human and mouse GAL4-HSF-1 chimeras that has previously been shown to respond to heat shock [Newton, 1996; Shi, 1995], the regulatory domain of HSF-2 does not respond to either heat shock or hemin. The implication of these results is that HSF-2 activation by hemin may be at the level of its binding to the HSE, which, in these GAL4-HSF-2 chimeras has been replaced by the GAL-4 DNA binding domain; thus, binding occurs at the GAL-4 binding site. This is strengthened by the fact that GAL4 DNA binding domain fused to the whole HSF-1 protein still failed to be activated by hemin (Fig. 5). Whether HSF-2 activation by hemin requires HSE warrants further investigation. A model for HSF-2 activation that reflects our results is shown in Figure 9. This model suggests that HSF-2 is present as a dimer in the cytoplasm [Sheldon, 1993] and is bound by an inhibitory protein that is hemin responsive. Upon hemin stimulation, the inhibitory protein is released,

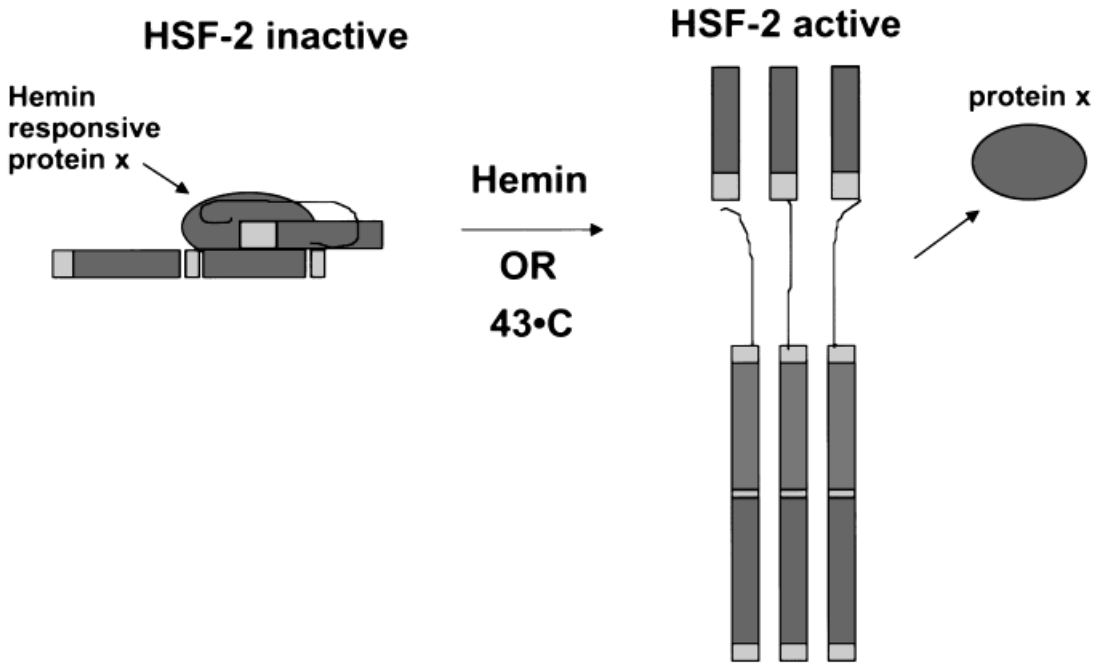

Fig. 9. Hypothetical model representing HSF-2 activation by hemin or heat. A hemin or heat responsive protein (x) may be bound to HSF-2 to keep it inactive under normal physiological growth conditions. Upon treatment of cells with heat or hemin, protein x is released allowing HSF-2 activation.

and HSF-2 forms trimers, which increases its ability to bind to DNA. There is no evidence as to the identity of the protein x. This protein may be a member of the molecular chaperones and may be released from HSF-2 when cells are treated with heat or hemin by some unknown mechanism.

Other differences between HSF-1 and HSF-2 regulation are that HSF-1 is phosphorylated under normal physiological growth conditions and hyperphosphorylated upon heat shock [He, 1998; Kim, 1997; Mivechi, 1995a; Sarge, 1993]. Constitutive phosphorylation of serines 303 and 307 negatively regulates HSF-1 activity and mutation of these two serine residues to alanine causes constitutive activation of HSF-1 [Shi, 1995]. However, phosphorylation of other sites may also be involved in down-regulation of HSF-1. The sites for hyperphosphorylation of HSF-1 after heat shock have not been clearly mapped. Recent reports suggest that HSF-1 activity is inhibited by HSP-70 and other molecular chaperones such as HdJ 1 [Shi, 1998]. HSF-2 however, does not appear to be phosphorylated and thus, its regulation differs in that respect from HSF-1. In conclusion, our data indicate that the activation domain of HSF-2 is located between amino acids 459 and 536 and the regulatory domain of HSF-2 that is located between amino acids 355 to 419 inhibits the activity of its activation domain and furthermore, the HSF-2 protein is not hemin or heat shock responsive.

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