

Elevated Expression of Heat Shock Factor (HSF) 2A Stimulates HSF1-induced Transcription during Stress*

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Heat shock factor 2 (HSF2) belongs to a family of structurally related transcription factors, which share the property of binding to heat shock elements in the promoters of *hsp* molecular chaperone genes. However, unlike HSF1, which is essential for *hsp* gene transcription, the cellular functions of HSF2 are not well known. Here we show that human HSF2, although an ineffective activator of the *hsp70* promoter *in vitro* and *in vivo* in the absence of stress, participates in the activation of the *hsp70* promoter by heat shock. HSF2 was not, however, activated by heat shock in cells deficient in functional HSF1, suggesting a requirement for HSF1 in HSF2-mediated transcriptional enhancement. In addition, HSF2 regulation involves differential activity of two isoforms, HSF2A and HSF2B, which arise from alternative splicing of a common *hsf2* gene. Under basal conditions, both HSF2 isoforms are ineffective in activating the *hsp70* transcription. However, heat shock differentially activates HSF2A *in vivo*. This phenomenon appears to be physiologically significant, as human myelopoietic cells differentiating along the erythroid lineage express HSF2A *de novo* and undergo a large increase in capacity to activate the *hsp70* promoter. Our experiments further show that HSF1 is physically associated with HSF2 in the cell and that such binding is enhanced by heat shock. Our data suggest a mechanism involving the formation of heterocomplexes between HSF1 and HSF2 with enhanced activity to activate the *hsp70* promoter when compared with HSF1 or HSF2 homotrimers.

The heat shock transcription factor (HSF)¹ family was first discovered in experiments investigating proteins binding to the

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¹ The abbreviations used are: HSF, heat shock factor; *hsp70*, heat shock protein 70; HSE, heat shock element; GST, glutathione *S*-transferase; CMV, cytomegalovirus; CHO, Chinese hamster ovary; DTT, dithiothreitol; MEL, mouse erythroleukemia; PMSF, phenylmethylsulfonyl fluoride; DRB, 5,6-dichloro-1D-ribofuranosyl benzimidazole; RT, reverse transcriptase.

promoters of *hsp* genes (1–3). HSF isolated from yeast, *Drosophila*, murine, and human cells was shown to be involved in binding to the heat shock response elements (HSE) in heat shock promoters and activating transcription (3–9). Since the isolation of *hsf* from *Saccharomyces cerevisiae*, related genes have been found in a wide range of species and a *hsf* multigene family has now been described (10). Members of the *hsf* family share structural domains and functional properties including a highly conserved N-terminal DNA binding domain and a heptad repeat "leucine zipper" structure adjacent to the DNA binding domain that regulates trimerization and the ability to bind DNA (10–12). In mammalian and avian cells, there are multiple *hsf* family members and four distinct *hsf* genes have been described to date (6, 7, 13–15). These include heat shock factor 1 (*hsf1*) with a crucial role in the stress response and other, structurally related genes (*hsf2*, *hsf3*, and *hsf4*) (10, 14–19).

The cellular functions of the *hsf2* product still remain largely unknown. Some studies show that HSF2 increases in concentration and becomes activated to a DNA binding form in differentiating K-562 myelopoietic cells and that this increase correlates with an increase in transcription of HSP70 (18, 19). Although these studies suggest that increases in HSF2 expression may be involved in the activation of HSF2, others show that cells expressing high levels of HSF2 do not exhibit elevated activation of potential target genes such as *hsp70* (20, 21). Likewise, the role of heat shock in HSF2 activation is uncertain with *in vitro* studies suggesting that heat shock inhibits HSF2 binding to HSE and studies with transfected HSF2 suggesting a role for heat shock in activating HSF2 from a latent, cytoplasmic state to a nuclear form through a mechanism reminiscent of HSF1 activation by heat shock (7, 9). In yeast, human *hsf2* complements the endogenous *hsf* when the latter is disrupted, protects cells from heat stress, and activates a stress-inducible promoter in heat shocked cells (22). In the present study, we have examined the ability of HSF2 to activate the promoters of heat shock genes with the aim of determining the mechanism of activation of HSF2 and its place in the stress response. We have also compared the properties of two variant forms of human HSF2 (HSF2A and -B), inspection of whose sequences suggest that they arise from the alternative splicing of a common HSF2 gene. Closely related HSF2 isoforms arising from alternative splicing have also been isolated from murine cells (23, 24). As the region absent from the smaller transcript (HSF2B) overlaps a sequence highly conserved between HSF1 and HSF2A that encodes the powerful activation domain 1, HSF2A and HSF2B would be predicted to differ in transactivating potential, as suggested for the corresponding mouse isoforms (23, 25, 26). We have therefore investigated the ability of HSF2A and HSF2B (and HSF1) to acti-

vate stress-inducible transcription *in vitro* and *in vivo* under control and stressed conditions. We subsequently examined the differential expression of HSF2A and B in differentiating myeloprogenitor cells and investigated the ability of endogenous HSF2A and HSF2B to activate the *hsp70b* promoter with and without stress. These experiments suggest that HSF2 is involved in the stress response, but unlike the ubiquitous HSF1 operates in a cell-line specific manner through differential expression of alternatively spliced isoforms. Curiously, HSF2A could not be activated by heat shock in cells deficient in functional HSF1 and required the expression of HSF1 for heat induction of the *hsp70B* gene in cells. We next demonstrate that HSF2 is associated with HSF1 *in vitro* and *in vivo*, both before and after heat shock, and this interaction could potentially mediate the response of HSF2A to heat shock.

EXPERIMENTAL PROCEDURES

Cell Lines and Tissue Culture—The myeloprogenitor (erythroleukemia) cell line K562, HeLa S3 human carcinoma cells, and Chinese hamster ovariocytes CHO K1 were obtained from the American Type Tissue Culture Collection. K562 cells were maintained in RPMI 1640 medium, whereas HeLa and CHO K1 cells were maintained in Ham's F-12 medium. Media were supplemented with 10% fetal bovine serum and 2.0 mM L-glutamine.

Materials—Enzymes and cloning reagents were from New England Biolabs (Beverly, MA) and Invitrogen. Culture products were from Invitrogen. All other chemicals were from Sigma or Fisher. Heparin-Sepharose CL-4B and Mono-Q HR 5/5 columns were from Amersham Biosciences. Human HSF1 complementary DNA cloned into pBlue-script SK- (Stratagene, La Jolla, CA) was a gift of Dr. Carl Wu (Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD). Anti-HSF2 antibodies were a kind gift from Dr. Vincenzo Zimarino (San Raffaele Institute, Milan, Italy).

Complementary DNA Cloning of Human and Mouse HSF2A and HSF2B—To clone HSF2A and -B cDNAs, human carcinoma cells (HeLa) were either untreated or heat-shocked for 30 min at 43 °C. RNA was then isolated and messenger RNA prepared by poly(T) affinity chromatography (PolyAtract® system, Promega, Madison, WI). cDNA was then prepared from the mRNA using the avian myeloblastosis virus reverse transcriptase system (Promega), and HSF2 cDNAs were amplified using *Taq* polymerase and the polymerase chain reaction using the following primer sets:

For human HSF2, forward primer was 5'-GC(GAATCC)ATGAAGC-AGAGTTCGA-3', and reverse primer was 5'-AAA(GTCGAC)TTC-CTGGGGATTAGCTA-3'.

For murine HSF2, forward primer was 5'-GG(GAATCC)ATGAAGCAGAGTTCGAACG-3', and reverse primer was 5'-AGT(GTCGAC)TTGGGAGTTTAACTATCT-3'.

Forward primers contained *Eco*RI restriction site consensus sequences (in parentheses) and the reverse primers contained *Sal*I sequences for subsequent cloning of amplified DNAs into the pGEX5 prokaryotic expression vector (Amersham Biosciences). After transformation and growth of competent bacteria, colonies were screened for either total HSF2 using oligonucleotides (1764–1785 (CAGGAGCAAGTTCACATAAATA) and 1786–1807 (GGCATATCACTATCCAGAGGTG)) predicted to detect all forms of HSF2 or for the larger form (HSF2A) using oligonucleotides predicted to hybridize specifically with this species (1420–1440 (TTGTATTATTGATGTAATCT) and 1392–1412 (CATCTGCACAGACAGTAGTGA)). Oligonucleotides were end-labeled with [³²P]ATP and T4 polynucleotide kinase. Plasmids detected using these probes were isolated, screened for the presence of inserts, and screened for the production of HSF2-glutathione transferase fusion proteins from representative cDNAs in bacteria exposed to the inducing agent isopropyl-1-thio-β-D-galactopyranoside (Amersham Biosciences). After induction, bacterial lysates were prepared and screened by immunoblot with anti-GST antibodies (Santa Cruz Antibodies) and anti-HSF2 antibody Ab-3158. Representative clones from human HSF2A (clone 94) and HSF2B (clone 85) and murine HSF2A (clone 6) and HSF2B (clone 7) were then further analyzed by dideoxynucleotide sequencing. Restriction digestion and gel electrophoresis indicated the production of cDNAs from the human and mouse mRNA of ~1.6 kb (predicted sizes for the inserts were, respectively, 1.621 (hHSF2A), 1.567 (hHSF2B), 1.616 (mHSF2A), and 1.562 (mHSF2B) kb). The cDNAs from clones 94 and 6 are slightly retarded in the gels consistent with their slightly larger size (data not shown).

Replicate sequencing of the clones indicated conservative substitutions in two codons of hHSF2A (and hHSF2B) but identical predicted protein sequence sequences to hHSF2 (13) except that HSF2A contain the conserved sequence CTTTTCACTAGTTCTGTGCCAGATGAATCCCA-CAGATTACATCAATAATACAAAATCT present at position 1264–1318 in the published sequence of hHSF2 (13) but not present in the homologous region (1329) of the mHSF2, which is otherwise similar to the hHSF2 (23).

Purification of HSF2 Proteins—HSF2 variants were cloned into the pGEX-5 expression vector, between the *Eco*RI and *Sal*I sites and the resulting plasmids were used to transform BL21 (DE3) *Escherichia coli* bacteria. HSF2 was thus expressed as a fusion protein with glutathione *S*-transferase. All purification steps were carried out at 4 °C. Briefly, isopropyl-1-thio-β-D-galactopyranoside-induced bacteria were pelleted and dissolved in 7 M guanidine HCl in 0.1 M potassium phosphate buffer, pH 7.4, containing 50 mM DTT and 0.05% Nonidet P-40 and dialyzed against 50 mM potassium phosphate buffer containing 0.1 M KCl and 2 mM DTT. The samples were centrifuged at 2500 × *g* for 5 min and the supernatant loaded on a 20-ml volume glutathione-Sepharose column at a flow rate of 0.5 ml/min, washed extensively with buffer A (50 mM potassium phosphate buffer, pH 7.4, containing 0.1 M KCl, 0.05% Nonidet P-40, and 2 mM DTT) and eluted with buffer A containing 10 mM reduced glutathione. The elute was loaded onto a Mono-Q HR 5/5 ion exchange chromatography column at a flow rate of 0.8 ml/min and eluted with a 24-ml linear gradient from 0.1 to 1 M KCl final concentration of KCl in buffer A. Absorbance was monitored at 280 nm, and the fractions corresponding to HSF2 were assayed for binding to HSE, pooled, and concentrated with a Centricon 10 ultrafilter in the presence of 0.1 mg/ml bovine serum albumin. Relative concentrations of active HSF2 were estimated by quantitative electrophoretic mobility shift assay (EMSA) (Fig. 1). HSF1 was purified as described (27).

Nuclear Extraction and EMSA—Nuclear extracts were prepared according to Schreiber *et al.* (52). Briefly, cells were incubated for 15 min in 200–800 ml of 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.0 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2.0 mg/ml each aprotinin and leupeptin, 20 mM NaF, and 2.0 mM Na₃VO₄, pH 7.9, on ice. Cells were then lysed by addition of Nonidet P-40 to 0.6% and microcentrifuged at 12,000 × *g*. Nuclear pellets were resuspended in 25 ml of 20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1.0 mM EGTA, 1.0 mM DTT, 0.5 mM PMSF, 2.0 mg/ml aprotinin, and 2.0 mg/ml leupeptin, pH 7.9. Extracts were then aliquoted and stored at –80 °C. Each binding mixture (12 ml) for EMSA contained 2.0 ml of nuclear extract or recombinant protein, 2.0 mg of bovine serum albumin, 2.0 mg of poly(dI-dC), 0.5–1.0 ng of labeled double-stranded oligonucleotide probe, 12 mM HEPES, 12% glycerol, 0.12 mM EDTA, 0.9 mM MgCl₂, 0.6 mM DTT, 0.6 mM PMSF, and 2.0 mg/ml aprotinin and leupeptin, pH 7.9. Final concentrations of KCl in the binding mixture were defined for optimal binding of each oligonucleotide. Samples were incubated at room temperature for 15 min, then electrophoresed on 4.8% polyacrylamide, 1× TBE gels. Oligonucleotide hHSE was synthesized, annealed, and labeled by end filling with ³²P for EMSA. hHSE contains the heat shock element (HSE) from the top strand of the human *HSP70A* promoter (28): 5'-CACCTCGGCTGGAATATTCCTCCGACTGGCAGCCGA-3'. Gel supershift assays with anti-HSF1 or anti-HSF2 antibodies were carried out as described previously (29).

Polyacrylamide Gel Electrophoresis and Western Blotting—Eukaryotic cell extracts or recombinant materials were boiled in the presence of reducing electrophoresis sample buffer, analyzed by 10% SDS-PAGE, and blotted onto Immobilon membranes as described (30). The membranes were incubated in 10 mM Tris, pH 8.0, 0.15 M NaCl, 5% nonfat rehydrated milk in the presence of specific rabbit anti-HSF1 and anti-HSF2 polyclonal antibody raised against the C-terminal part of the molecules (29) (1/300 dilution), then with a secondary antibody coupled to alkaline phosphatase in 10 mM Tris, pH 8.0, 0.15 M NaCl, 5% nonfat dry milk (1/7500 dilution, Amersham Biosciences). The antigen-antibody complexes were visualized by incubating the membrane in 0.1 M Tris, pH 9.5, 0.1 M NaCl, 5 mM MgCl₂, 0.3 mg/ml nitro blue tetrazolium, and 0.16 mg/ml 5-bromo-4-chloro-3-indolyl phosphate.

In Vitro Transcription—HSF species (approximately 1 μg of HSF1, HSF2A, or HSF2B in storage buffer) were incubated in the presence of 0.25 μg of plasmid 308, which contains the human *hsp70* promoter to –120 fused to a G-less cassette of 380 bp in length and 1 mM MgCl₂ in a final volume of 13 μl for 30 min at 30 °C. Seven μl of nuclear extracts (31) purified over a P11 column (32) and previously titrated to give maximum basal transcription, 10 μCi of [α-³²P]UTP (800 Ci/mmol, NEN Life Sciences), 2 mM MgCl₂, 10–20 units of RNasin (20–40 units/μl, Promega, Madison, WI), 54 μM ATP, 54 μM CTP, 25 μM UTP, 10 μM *O*-methyl-GTP (Amersham Biosciences), 25 units of RNase T1 were

added to a final volume of 49 μ l and the transcription allowed to proceed for 1 h at 30 °C. Transcription products were subsequently extracted with phenol/chloroform, precipitated, separated on 8% urea-PAGE in 89 mM Tris, 89 mM boric acid, 2 mM EDTA and analyzed using a Phosphor-Imager (Amersham Biosciences). Phosphatased and kinased pBR322 DNA-*Msp*I digest (New England Biolabs) fragments were used as molecular weight markers.

Plasmid Constructs, Transient Transfection, and Assays for Luciferase, β -Galactosidase, and Protein Concentration—Human *hsp70b* (33) promoter construct p2500CAT was from StressGen (Victoria, British Columbia, Canada). pGL.Basic3 and pSV- β -galactosidase plasmids, Genelight luciferase, and β -galactosidase assay reagents were from Promega (Madison, WI), restriction and modifying enzymes from New England Biolabs (Beverly, MA), and sequencing reagents, Hybond-N membrane, and Rediprime kit from Amersham Biosciences. Oligonucleotides for EMSA were synthesized by Bio-synthesis (Coralville, IA). Reporter construct pGL.hsp70B was constructed by inserting the *HSP70B* gene promoter fragment from *Bgl*III site to *Hind*III site (1.44 kb) of p2500-CAT into pGL.Basic 3 at the corresponding sites. Expression construct pHSF1 was prepared by inserting the human HSF1 cDNA (6) into the pcDNA3.1(-) expression vector at the *Xho*I and *Eco*RI sites. The human HSF2A and HSF2B cDNAs were inserted into the pcDNA3.1(+) *Eco*RI and *Xho*I sites, to produce pHSF2A and pHSF2B. Then, the fragment from *Pst*I to *Vsp*I sites of pSV- β -galactosidase plasmid, containing SV40 early promoter and enhancer segments, *lacZ* coding region, and SV40 poly(A) signals, was inserted into the pHSF plasmids at *Nsi*I and *Sap*I sites to replace the neomycin resistance gene and these constructs were termed pBHSF1, pBHSF2A, and pBHSF2B, respectively. pBcontrol plasmid was derived from pBHSF1 by deleting HSF1 at the *Xho*I and *Eco*RI sites. After being blunted with Klenow, the resulting fragment was re-ligated. Constructs were confirmed by sequencing with a T7 kit (Amersham Biosciences). Double-stranded DNA templates were prepared from minipreps as described. Transient transfection was carried out using either electroporation (K562) or liposomal transfection reagent DOTAP (CHO-K1) (Roche Applied Science). For electroporation, 5×10^6 cells were mixed with appropriate plasmid DNA in 250 μ l of RPMI 1640 plus 10% fetal bovine serum and electroporated at 650 V/cm (in 0.4-cm cuvettes) and 800-microfarad capacitance and then immediately returned to culture at 37 °C. For transfection by DOTAP, cells were seeded in 24-well tissue culture plates at 1×10^5 /well 18 h before transfection according to Ref. 34. Cells were harvested 18–24 h after transfection and assayed for luciferase activity (34). To analyze the effects of endogenous HSF1 on promoter activity, cells were transfected with reporter plasmid DNA, allowed to recover for 8–10 h before heat shock, and analyzed for luciferase and β -galactosidase activity 24 h later (34). To analyze the effects of heat shock in HSF2 transfectants, reporter plasmid DNA-transfected cells were allowed to recover for 14–16 h before heat shock and luciferase activity was analyzed at the time points after heat shock. Luciferase activity was normalized to β -galactosidase activity, which was used as an internal transfection efficiency control. Analysis of variance with Tukey's multiple range test was employed for statistical analysis of luciferase values.

Stable Transfection—After transfection, cells were maintained in Ham's F-12 medium containing 10% fetal calf serum for 24 h. Neomycin (G418) was added to 300 ng/ml until surviving cells reached confluence. Surviving clones were then selected and amplified.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis of HSF2 Isoforms—We probed for the expression of HSF2A and HSF2B using RT-PCR, using β -actin expression as a constitutively active control. Primers were designed to produce amplified products from human β -actin of 340 bp and from human HSF2A and HSF2B of 232 and 178 bp, respectively. Primers were: (a) for human HSF2, forward primer (5'-AAGGTTGAGCTGTTGGA-3') and reverse primer (5'-GATTTTCTCCCTCTTC-3'), were from 1157–1173 and 1389–1373 (reverse) in the human HSF2A cDNA in clone pBHSF2A94; (b) for human β -actin, forward primer (5'-GCCAGCTCACCATGGAT-3') and reverse primer (5'-AGGGGGGCGCTCGGTGAG-3') were from 31–47 and 370–354 in the human β -actin gene. Primers were first tested for the ability to amplify the 232- and 178-bp HSF2 fragments from plasmids containing human HSF2A and HSF2B. For RT-PCR analysis of cellular RNA, cDNAs were transcribed at 42 °C from 1 μ g of total RNA isolated from either K562 or HeLa S3 cells. Reverse transcription reactions were carried out with 50 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen), 4 mM each of dATP, dCTP, dGTP, and dTTP, 10 mM dithiothreitol, 20 units of RNase inhibitor, and 2.5 μ M random hexamers (Roche Applied Science) in a 20- μ l volume. The entire 20 μ l of RT reaction mixture and 75 ng each of forward and reverse primers was

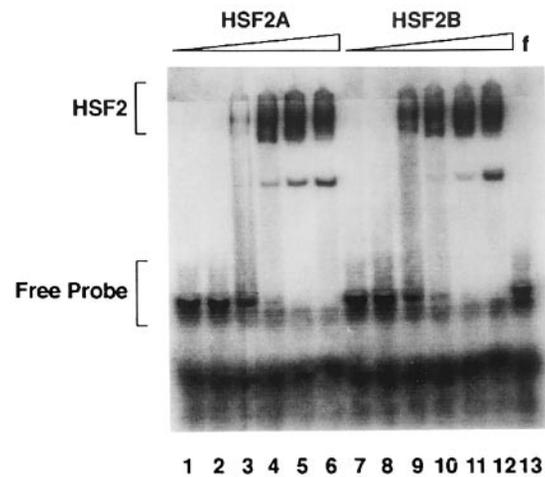


FIG. 1. HSE binding of purified HSF2A and HSF2B. Purified GST-HSF2A and GST-HSF2B were incubated with 32 P-labeled HSE at a range of dilutions and then subjected to EMSA analysis as described under "Experimental Procedures." GST-HSF2A and GST-HSF2B were diluted 1/2200 (lanes 1 and 7), 1/660 (lanes 2 and 8), 1/220 (lanes 3 and 9), 1/66 (lanes 4 and 10), 1/22 (lanes 5 and 11), and 3/22 (lanes 6 and 12). Relative migration of HSF2-HSE complexes and free probe are indicated. The incubation for lane 13 contained free probe only. Experiments were repeated three times with consistent findings.

used for PCR reactions using an AmpliTaq DNA polymerase PCR kit (PerkinElmer Life Sciences). PCR reactions were carried out, after denaturing at 94 °C for 3 min, with 25 cycles of denaturing (94 °C for 30 s), annealing (42 °C for 2 min), and primer extension (72 °C for 1 min). Finally, PCR products were extended for 5 min at 72 °C.

Northern Analysis—Total RNA was isolated from cells fractionated on 1% agarose/formaldehyde gels and transferred to membranes as described (35). Specific mRNAs were detected by hybridization with a full-length 1.6-kb HSF2A probe and a murine *HSP70.1* cDNA probe (36) radiolabeled with [α - 32 P]dCTP using a Rediprime kit (Amersham Biosciences). To probe for HSF2 in various organs, tissue blots containing 5 mg each of poly(A)⁺ RNA from a range of organs were purchased commercially (Clontech, Palo Alto, CA) and probed sequentially for total HSF2 with the intact HSF2A cDNA probe and for HSF2A mRNA with the oligonucleotide probes described earlier derived from the region present in HSF2A but absent in HSF2B mRNA.

In Vitro Protein Interaction Study Using Glutathione S-Transferase "Pull-down" Analysis—*In vitro* translated proteins (35 S-labeled) for GST pull-down were obtained by the Promega TNT Quick Coupled Transcription/Translation System, and the procedure was performed following the instructions from the manufacturer. GST fusion proteins were harvested as described below. 1.5 mg of GST protein was mixed with 50 μ l of glutathione-Sepharose 4B beads (Amersham Biosciences). The volume was brought up to 1.0 ml with NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, pH 8.0, 0.5% Nonidet P-40, 1.0 mM DTT); protease inhibitors were added freshly, including 0.5 mM PMSF and 2.0 mg/ml aprotinin and leupeptin. The mixture was rocked at 4 °C for 1 h, then washed three times with 1 ml of NETN buffer. Beads were resuspended in 500 μ l of NETN buffer, 35 μ l of 35 S-labeled protein was added, and the mixture was rocked at 4 °C for 1 h. The beads were subsequently washed thoroughly, resuspended in 30 μ l of 2 \times SDS sample buffer, and proteins then separated by 10% SDS-PAGE. Gels were dried and exposed to x-ray film (Eastman Kodak Co.).

RESULTS

HSF2 Is an Ineffective Activator of Heat Shock Transcription In Vitro and When Overexpressed In Vivo

We first investigated the activity of purified HSF2A and HSF2B GST fusion proteins *in vitro*. Purified HSF2A and HSF2B fusion proteins both bound to a 32 P-labeled probe based on the TATA box-proximal HSE from the human *HSP70A* gene (Fig. 1). Equivalent amounts of each protein were serially diluted and analyzed by gel mobility shift assay against a constant amount of probe (Fig. 1). In each case there was displacement of ~50% of free probe at a HSF2 dilution of 1/220 (lanes

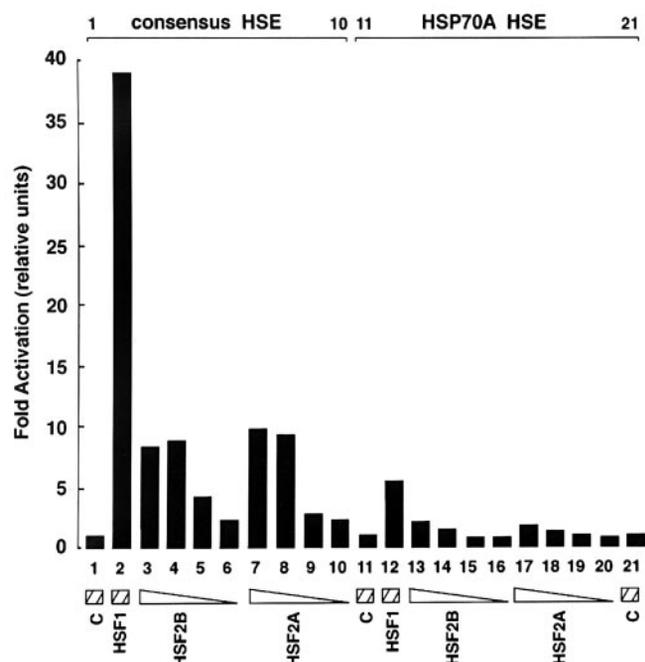


FIG. 2. Effects of purified HSF1, HSF2A, and HSF2B on *in vitro* transcription from heat-inducible promoters. Transcription from the promoter-reporter constructs p311, which contains multiple HSE consensus sequences upstream of a 380-bp G-less cassette, and p308, which contains the TATA proximal HSE from human *HSP70A*, was determined at a range of HSF2A and HSF2B concentrations. HSF2A and HSF2B were diluted 1/26 (lanes 3, 7, 13, and 17), 1/90 (lanes 4, 8, 14, and 18), 1/260 (lanes 5, 9, 15, and 19), and 1/900 (lanes 6, 10, 16, and 20). HSF1 was used at a dilution (1/26) that gave maximal activation of each reporter, and its concentration was approximately equivalent in terms of ability to complex HSE in the EMSA assay to the concentration of HSF2A and HSF2B that fully complexed the EMSA probe (Fig. 1) and gave maximal promoter activation. Experiments were repeated four times with reproducible findings.

3 and 9). We then investigated the ability of HSF2A and HSF2B to activate transcription *in vitro* from an HSE-containing promoter-reporter construct (Fig. 2). Both HSF2A and HSF2B showed apparently maximal activation of the P311 construct (Fig. 2, lanes 4 and 8), which contains a consensus HSE array that is transcribed very efficiently *in vitro*, at dilutions above 1/90, which caused complete displacement of probe in the EMSA assay (Fig. 1). HSF1 used at an equivalent concentration (lane 2) stimulated transcription with 4–5-fold more efficiency in this experiment and in all replicate experiments (Fig. 2, lane 2). Similar results were observed with the less active *HSP70A*-based P308 promoter-reporter system (Fig. 2, lanes 12–21). These experiments indicate that HSF2A and HSF2B both bind to HSE and activate the HSP70 promoter *in vitro* at an equivalent rate, indicating that the recombinant proteins have similar transcriptional activating potential *in vitro*. However, as it is possible that regulatory factors present in cells *in vivo* are lacking in the *in vitro* incubations used here, we next examined the ability of HSF2A and HSF2B to activate the promoters of two human heat shock genes when stably co-transfected into mammalian cells. We examined the activity of the promoter from human *HSP70B* in K562 human erythroleukemia cells stably transfected with HSF1 or HSF2 (Fig. 3). For these experiments, HSF2A and HSF2B were expressed from a vector that incorporates an internal transfection efficiency control (CMV- β -galactosidase) to ensure comparable transfection efficiency in experiments using the HSF2 expression plasmids. Cells were stably transfected with the HSF1 and HSF2 expression vectors and transiently transfected with pGL.hsp70B (Fig. 3). Although HSF1 overexpression strongly

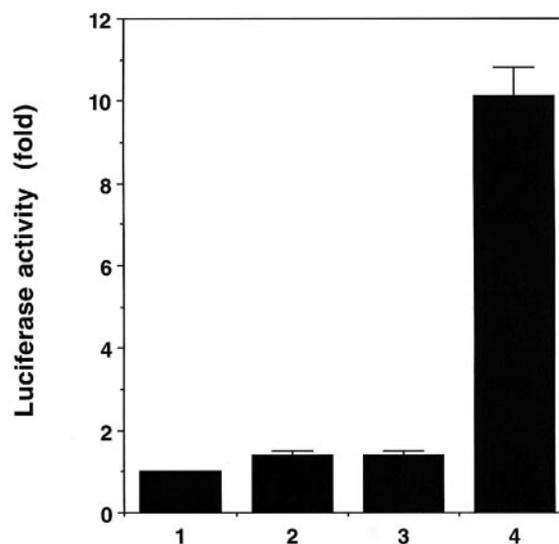


FIG. 3. Effect of high level expression of HSF1, HSF2A, and HSF2B on the activity of heat-inducible promoters in unstressed cells *in vivo*. Relative activation of the human *HSP70 B* promoter in K562 cells stably expressing empty expression plasmid pcDNA3.1(–) (lane 1), HSF2A (lane 2), HSF2B (lane 3), and HSF1 (lane 4); cells were transfected with pGL.hsp70B 24 h prior to lysis and assay for luciferase and β -galactosidase activity. Luciferase activity was indexed to β -galactosidase activity and calculated and subject to statistical analysis as described under “Experimental Procedures.” Experiments were repeated 5 times with reproducible findings.

activated the *HSP70B* promoter, we observed only marginal activation of the promoter by expression of either HSF2 isoform, and no significant difference between HSF2A and HSF2B. Similar findings were observed with another heat shock promoter-reporter construct, pGL.hsp27 (data not shown). As shown in subsequent experiments, both HSF2A and HSF2B are expressed at equivalent amounts in the stably transfected cells (Fig. 4C).

Elevated Activation of Heat Shock Promoters by Stress in Cells Overexpressing HSF2A

As previous studies indicated that HSF2 regulation involves constitutive repression through an intermolecular coiled-coil interaction between leucine zipper domains in the C terminus and the trimerization domain in the N terminus, and that such interactions are antagonized by heat shock, we next investigated the potential role of heat shock in the transcriptional activation of HSF2A and HSF2B (9). Heat shock caused a large increase in *HSP70B* reporter activity in control cells, presumably as a result of the activation of endogenous HSF1 (Fig. 4A). However, expression of HSF2A from the CMV promoter led to a large increase in response to heat shock compared with wild-type control cells, presumably because of heat-induced activation of transcriptionally latent, overexpressed HSF2A, which is able to bind HSE under these conditions (Fig. 4A). The increase in transcriptional activation of the *HSP70B* promoter by heat shock in HSF2A overexpressing cells far exceeded the additive effects of endogenous heat-induced activity and the unstimulated activity of overexpressed HSF2A (Figs. 3 and 4A). No increase in heat-induced activity was seen in the HSF2B transfectants compared with the vector-alone control despite the fact that HSF2B is overexpressed in these cells (Figs. 1 and 4 (A and C). The increase in *HSP70B* activation during heat shock by HSF2A expression also exceeded the effects of HSF1 overexpression (Fig. 4A). Similar experiments using transient co-transfection with the HSF expression vectors and *HSP70B* reporter construct further confirmed these findings, indicating that HSF2A is strongly activated by heat shock when ex-

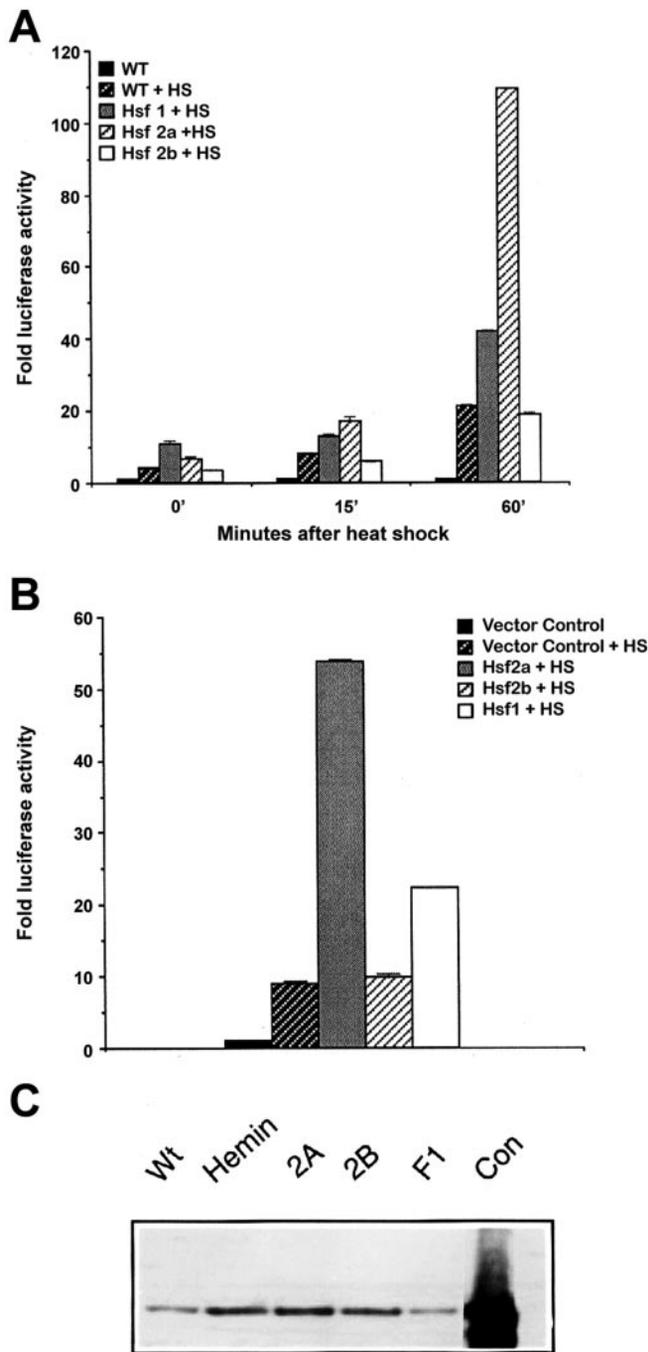


FIG. 4. Effects of heat shock on activation of the *HSP70B* promoter by HSF2A and HSF2B *in vivo*. *A*, wild-type K562 cells and cells stably expressing HSF1, HSF2A, and HSF2B were transfected with pGL.HSP70B promoter reporter construct, heat-shocked (45 min at 43 °C), and then assayed for the synthesis of luciferase at time points after heating. Luciferase activity was indexed to β -galactosidase activity and calculated as described under "Experimental Procedures." Experiments were repeated three times with reproducible findings. *B*, K562 cells were transiently co-transfected with empty expression vector pcDNA3.1(-), pHSF1, pHSF2A or pHSF2B, and pGL.HSP70B and then subjected to heat shock (45 min at 43 °C) and another 1 h recovery at 37 °C prior to lysis and assay for luciferase and β -galactosidase activity. Luciferase activity was calculated as in Fig. 3. Experiments were repeated three times with consistent findings. *C*, relative concentrations of HSF2 protein in wild type K562 cells (*Wt*) and cells stably transfected with pHSF2A (*2A*) or pHSF2B (*2B*) or in wild-type cells exposed to 30 μ M hemin (*Hemin*) for 20 h. Cell proteins were extracted, separated by 10% SDS-PAGE, and probed by immunoblot analysis with anti-HSF2 antibodies as described under "Experimental Procedures." *Con* refers to *in vitro* translated HSF2A as a positive control. Experiments were repeated twice with reproducible findings.

pressed to high level (Fig. 4*B*). Relative levels of HSF2A and HSF2B in the stable transfectants are shown in Fig. 4*C*.

Elevated Response to Stress of K562 Cells Induced to Express HSF2A by Exposure to Hemin—We next went on to examine the effect of increasing endogenous HSF2A concentrations on the activity of the transfected *HSP70B* promoter. We examined the expression of HSF2A and HSF2B in K562 myeloprogenitor cells induced along the erythroid lineage by exposure to hemin (37). RT-PCR analysis was used to probe expression of these isoforms with primers designed to produce a 232-bp transcript from HSF2A and a 178-bp transcript from HSF2B. When these primer pairs were used to amplify cDNA from plasmids containing the human HSF2 isoforms, cDNA products of the appropriate size were produced by PCR amplification (Fig. 5*A*); using pBHSF2A as a template in the reaction, a 232-bp product was observed, and, when pBHSF2B was used, a 178-bp cDNA was produced. We next examined the relative levels of HSF2A and HSF2B mRNA in hemin-treated K562 cells. HSF2A and HSF2B were at trace levels in untreated controls, increased at 6 and 20 h of hemin exposure, and declined at 68 h (Fig. 5*B*). HSF2A appeared to be the predominant isoform in these samples (Fig. 5*B*). These findings are consistent with earlier studies showing that HSF2A is induced preferentially at the protein level in K562 cells exposed to hemin (38). The level of the control cDNA (β -actin) appeared to be reduced in the 6- and 20-h samples (Fig. 5*B*), apparently because of competition for reagents in the PCR reaction, as the decrease was not observed when the reaction was carried out using actin primers alone (Fig. 5*C*). The expression of HSF2A mRNA in the hemin-treated K562 cells was in marked contrast to another cell line of human origin, HeLa S3 cells, in which only HSF2B mRNA was detected (Fig. 5*D*). HeLa cells thus preferentially express HSF2B, and this was not altered by exposing cells to heat shock at 30, 60, or 90 min at 43 °C (Fig. 5*D*). Expression of HSF2A coincided with an overall increase in HSF2 mRNA species and HSF2 protein levels in hemin induced K562 cells, as determined by, respectively, Northern analysis and immunoblot (Figs. 5*E* and 4*C*). Increased expression of HSF2A mRNA and nuclear accumulation of HSF2 in a HSE binding form as analyzed by EMSA both reached a peak at 41 h (Fig. 5, *E–G*). In addition, inhibition of the HSF2 mRNA increase in hemin-treated cells with RNA polymerase II inhibitor 5,6-dichloro-1 α -ribofuranosyl benzimidazole (DRB) prevented accumulation of HSE binding activity in these cells (Fig. 5, *E* and *F*). Thus hemin causes the accumulation of HSF2 in a DNA binding form. Previous studies have also indicated that hemin may elevate HSF2 levels in K562 cells by inhibiting proteolysis through the proteasome and HSF2 accumulation may thus be regulated at a number of levels, including both increased mRNA accumulation and decreased HSF2 degradation (39). As HSF2A is induced under these conditions, it may be assumed that hemin induces HSF2A binding to HSE, although, with currently available reagents, this cannot be proven formally. We next investigated whether HSF2 accumulating in the hemin-treated K562 cells is active in inducing the *HSP70B* promoter and whether it could be further activated by heat shock as with HSF2A overexpressed from a viral promoter. Although inducing HSF2 expression and HSF-HSE binding (Fig. 5, *E* and *F*), hemin was ineffective in activating the *HSP70B* promoter (Fig. 6). However, a combination of hemin exposure and subsequent heat shock led to a marked activation of the *HSP70B* promoter, suggesting that increased HSF2A levels in cells responding to hemin enhances the responsiveness of the *HSP70B* promoter to stress (Fig. 6). These findings are in line with earlier studies showing markedly increased heat-induced tran-

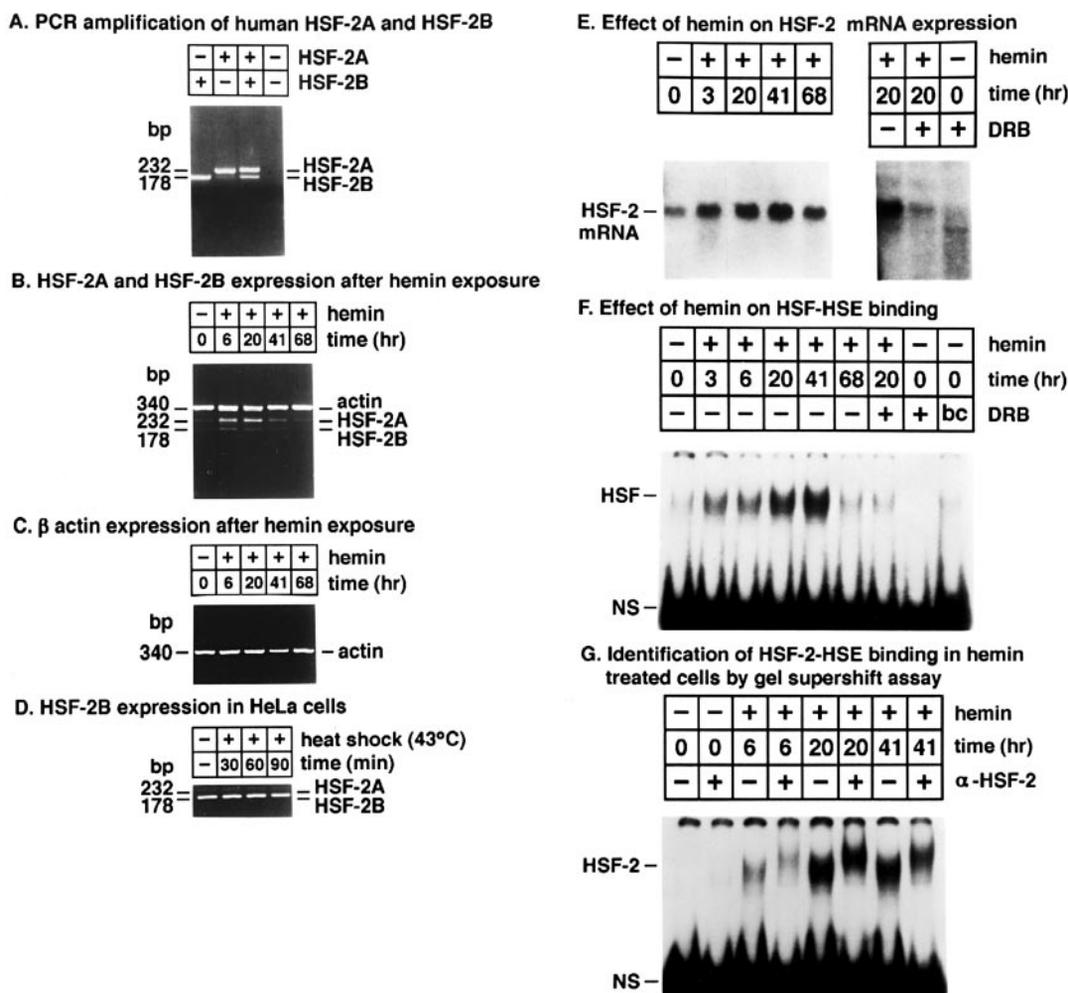


FIG. 5. Expression and transcriptional activity of HSF2A in differentiating myeloid progenitor cells under control and heat shock conditions. *A*, relative expression of HSF2A and HSF2B mRNA in human cells. The primer pair designed to amplify the 232-bp HSF2A fragment and the 178-bp HSF2B fragment were tested for specificity in reactions containing plasmid DNA containing HSF2A cDNA (lane 1), HSF2B cDNA (lane 2), a mixture of HSF2A and HSF2B cDNAs (lane 3), or empty plasmid containing no cDNA insert (lane 4). *B*, RNA extracted from K562 cells exposed to hemin for 0, 6, 20, 41, and 68 h was subjected to RT-PCR analysis for HSF2A, HSF2B, and human β -actin as in *A*. Experiments were repeated three times with reproducible findings. *C*, the reverse transcribed cDNA from *B* was re-analyzed for β -actin expression in the absence of competition from the HSF2 primers. *D*, relative expression of HSF2A and HSF2-B mRNA in HeLa cells exposed to heat shock (0, 30, 60 and 90 min at 43 °C). Experiments were repeated three times with consistent results. *E*, overall expression of HSF2 mRNA in K562 cells treated with 30 μ M hemin. Northern analysis was carried out on RNA extracted from K562 cells exposed to hemin for 0, 3, 20, 41, and 68 h. In addition, HSF2 mRNA expression in K562 cells exposed to hemin for 20 h without and with DRB and in unstimulated controls treated with DRB. Experiments were repeated three times with reproducible findings. *F*, HSF2-HSE binding activity in hemin-exposed K562 cells. Cells were exposed to 30 μ M hemin for 0, 3, 6, 20, 41, and 68 h without DRB or for 0 or 20 h with hemin and DRB prior to lysis, HSF extraction, and EMSA analysis. The last lane (marked *bc*) is from control cells not exposed to hemin but treated with DRB buffer. Experiments were repeated three times with reproducible findings. *G*, gel supershift analysis of HSF-HSE complexes with anti-HSF2 antibody Ab3105. Cells were exposed to hemin for 0, 6, 20, and 41 h either without antibody addition or with anti-HSF2 antibody. The specificity of this antibody for HSF2 was demonstrated previously (29). Experiments were repeated twice with reproducible findings.

scription of an endogenous *hsp70* gene in K562 cells treated with hemin (19).

HSF2A Fails to Respond to Heat Shock in Mouse Erythroleukemia (MEL) Cells Lacking Inducible HSF1, but Is Activated by Complementation with Human HSF1 Expression Vector—As these results are in apparent conflict with *in vitro* studies suggesting that HSF2 does not contain stress inducible activation domains (40, 41), we next examined whether HSF2A could activate the *HSP70B* promoter in a line of MEL cells shown previously to be deficient in the heat shock response and to be incapable of activating a *HSP70* reporter-promoter construct in response to heat shock (42). We prepared MEL cell lines stably expressing HSF2A. In these cells, heat shock did not activate the *HSP70B* promoter (Fig. 7*B*). However, when MEL cells were stably transfected with a HSF1 expression vector, the heat shock response was restored to the cells and the *HSP70B*

promoter was strongly activated in response to heat (Fig. 7, *A* and *B*). This indicates that the MEL cell line contains the signaling pathways required for HSF1 to respond to heat shock but contains an *hsf1* gene, which is apparently transcriptionally inert. The failure of HSF2A overexpression to stimulate heat shock activation in these cells thus appears to reflect the absence of active HSF1 and suggests that HSF1 is essential for this effect. To further test the dependence of stress-inducible HSF2A activity on HSF1, we examined the ability of transfected HSF1 to restore the heat responsiveness of HSF2A in MEL cells. We transfected pHSF1 into MEL cell lines stably expressing either HSF2A or HSF2B. Transfection of pHSF1 into the HSF2A cells permitted strong activation of the *HSP70B* promoter in response to heat shock (Fig. 7*C*). HSF2B expression, however, failed to activate *HSP70B* transcription in the presence of heat and HSF1 expression (Fig. 7*C*). HSF2A

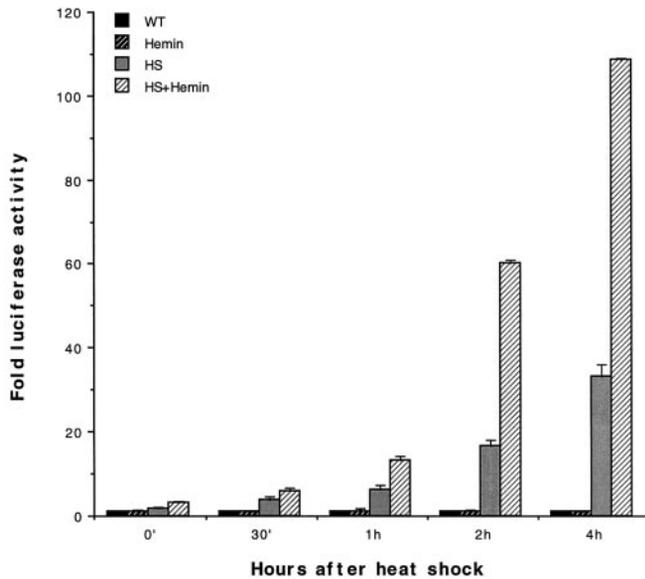


FIG. 6. Activation of a *HSP70B* gene reporter in myeloid progenitor cells induced by hemin and exposed to heat shock. The effect of heat shock on the *HSP70B* promoter was examined in K562 cells after exposure to hemin. Cells were transfected with pGL.HSP70B and then left untreated (control), exposed to 30 μ M hemin for 20 h, heat-shocked, or heat-shocked after hemin pretreatment prior to lysis and assay for luciferase and β -galactosidase activity at selected time points after recovery. Luciferase activity was indexed to β -galactosidase activity and calculated and subject to statistical analysis as described under "Experimental Procedures." Experiments were repeated four times with reproducible findings.

and HSF2B expression at the protein level was confirmed by Western analysis (Fig. 7D).

HSF1 and HSF2 Associate in Vitro and in Vivo, and Binding Is Enhanced by Heat Shock—As these experiments suggested functional interaction between HSF1 and HSF2 in cells, we next examined whether the two proteins were physically associated. We first investigated interactions between the endogenous intracellular proteins. When cell extracts were fractionated by size exclusion chromatography and the fractions probed by SDS-PAGE/immunoblot analysis, HSF1 and HSF2 were found to co-exist in the same column fractions, suggesting possible association (Fig. 8A). Examining these same fractions by immunoprecipitation with anti-HSF1 antibodies and immunoblot analysis of the immunoprecipitates with anti-HSF2 antibodies, we detected the presence of HSF2, thus providing direct evidence for physical association between the proteins (Fig. 8B). The control immunoblot (II) showed no binding, thus indicating the specificity of the HSF1-HSF2 interaction. We next went on to examine effects of stress on HSF1-HSF2 association. Cells were transfected with FLAG-tagged HSF1 to increase the recovery of HSF1 and then either maintained at 37 $^{\circ}$ C as a control or heat-shocked at 43 $^{\circ}$ C and then analyzed by immunoprecipitation with anti-FLAG antibodies. As can be seen, incubation with anti-FLAG antibody permitted co-immunoprecipitation of HSF2, and this association was increased by heat shock for 10 min at 43 $^{\circ}$ C (Fig. 8C, I). This interaction was not observed when the anti-FLAG immunoprecipitation experiment was carried out under denaturing conditions (lanes 3 and 4). The control experiment shows that the level of HSF1-FLAG remained constant in each cell condition (Fig. 8C, II, lanes 1 and 2) and that denaturing conditions, although preventing HSF2 co-precipitation, did not affect the degree of HSF1-FLAG recovery (Fig. 8C, II, lanes 3 and 4).

We next examined HSF1-HSF2 binding in an *in vitro* system, using the GST pull-down assay (Fig. 8D). Chimeric GST-HSF1

protein was initially used as bait in the assay and incubated with radiolabeled, *in vitro* translated HSF2A and HSF2B (marked 529-2A and 529-2B). Both HSF2 isoforms were retained specifically by GST-HSF1, indicating that HSF1 and HSF2 interact *in vitro* (Fig. 8D). Neither HSF2A nor HSF2B bound to the GST propeptide, as indicated in the lanes marked 2T-2A and 2T-2B, thus showing the specificity of the interaction of the two HSF2 isoforms with GST-HSF1 (Fig. 8D). In the reverse experiment, we used GST-HSF2A as bait this time to probe for interaction with HSF1. Full-length HSF1 (marked as 529 on the figure) was avidly retained by GST-HSF2A, confirming the HSF1-HSF2 interactions (Fig. 8D). In control incubations, HSF1 failed to bind to GST propeptide as indicated by lane 2T-529, indicating the specificity of the interaction of HSF1 with the GST-HSF2A fusion protein (Fig. 8D). We next investigated potential structural domains within HSF1 involved in interacting with HSF2. We examined the effect of deletion of residues from the C terminus of HSF1 on binding of the resulting truncation mutants, after *in vitro* translation, to GST-HSF2A (Fig. 8D). Mutants 1-379, 1-279, and 1-229, containing serial truncations from the C terminus of HSF1 bound avidly to HSF2A, as did wild-type HSF1 (amino acids 1-529) (Fig. 8D). However, deletion of an additional 50 amino acids produced the 1-179 fragment, which, although translated efficiently, failed to bind to GST-HSF2A (Fig. 8D). These experiments therefore suggest a requirement for sequences between residues 179 and 229 in the HSF1-HSF2 interaction (Fig. 8A) (similar results were obtained with GST-HSF2B; data not shown). As the region between amino acids 179 and 229 in HSF1 contains a section of the trimerization domain that governs homotrimerization between activated HSF1 monomers, a mechanism involving the formation of heterotrimers between HSF1 and HSF2 through the leucine zipper trimerization is plausible (7, 43).

DISCUSSION

These experiments indicate that HSF2A, in common with HSF1 cells, participates in the heat shock response. Our experiments indicate that purified HSF2 or HSF2 transcribed from transfected expression vectors only weakly activates *hsp70* gene transcription when compared with HSF1 (Figs. 2 and 3). However, earlier studies have shown that HSF2 binds with apparently similar affinity to HSE compared with HSF1, suggesting a difference in downstream trans-activating ability between the two factors (8). These differences were, however, not apparent when cells overexpressing HSF1 and HSF2 were exposed to heat shock. Heat shock caused a pronounced increase in activation of the stress-inducible *HSP70B* promoter in cells overexpressing HSF2A, as compared with HSF1 overexpressors (Fig. 4). The significance of these findings is indicated by the fact that, when HSF2A expression is increased in K562, myeloprogenitor cells induced along the erythroid lineage and *HSP70B* promoter also become more inducible by heat shock (Figs. 5 and 6). Previous studies had suggested that HSF2 is not involved in the stress response (20, 21). The present studies, however, indicate that the activity of the heat shock response may be highly sensitive to the levels of HSF2A and that the response is amplified by elevated HSF2A expression (Figs. 4 and 6). Our studies also indicate that HSF2A does not activate *hsp70* transcription independently but is dependent on the presence of active HSF1 (Fig. 7). The requirement for HSF1 for HSF2A to respond to heat shock suggests that cooperative interactions occur between the two factors during heat shock that mediate enhanced transcriptional activation (Figs. 4, 6, and 7). Such a cooperative interaction is indicated by earlier studies showing that hemin, a potent inducer of HSF2A, leads to a large increase in heat-induced *hsp70* gene transcription in K562 cells (19).

The functional interaction between HSF1 and HSF2 may

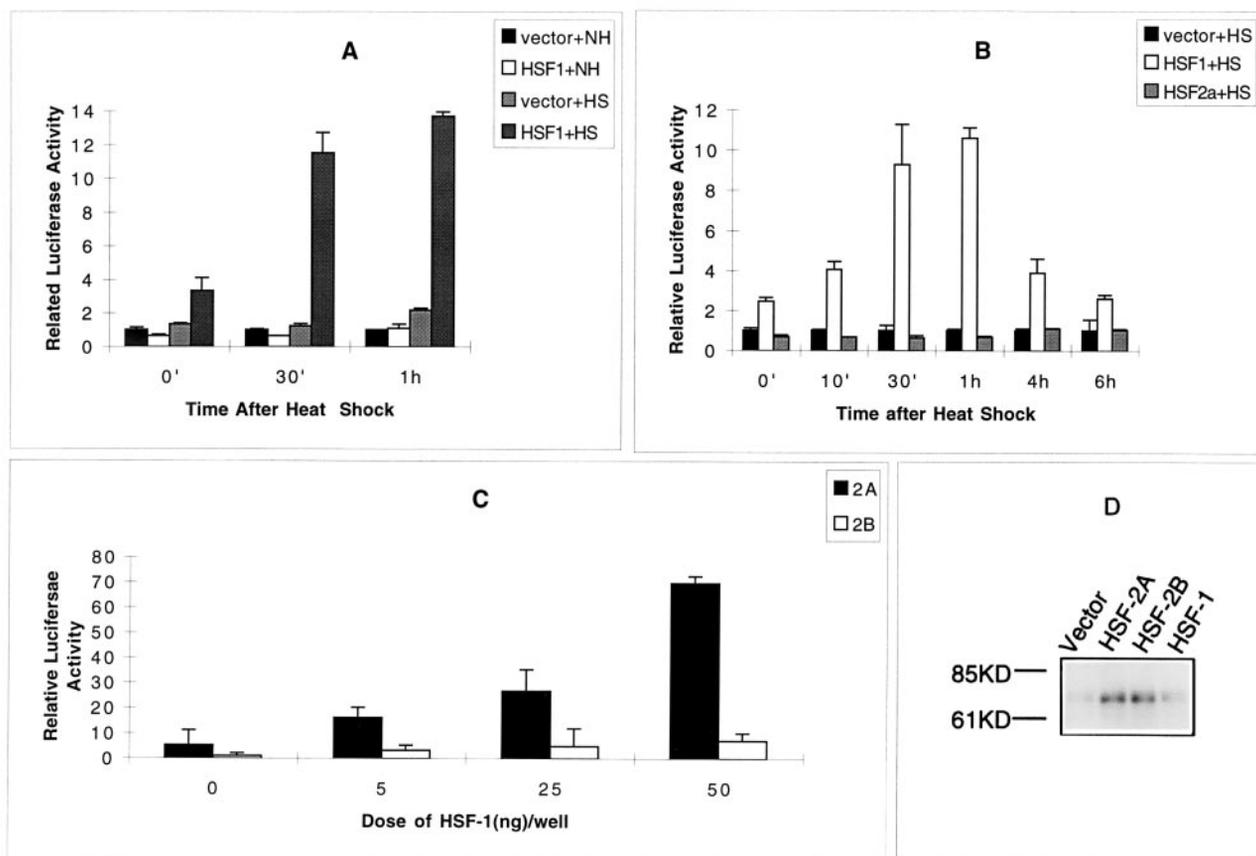


FIG. 7. Heat shock and HSF2A overexpression do not enhance *HSP70B* gene transcription in cells deficient in active HSF1. A, MEL cells stably transfected with pcDNA 3.1(-) (empty vector only) and pHSF1 were transiently transfected with pGL.HSP70B promoter reporter construct, without heating (NH) or heat-shocked (HS, 45 min at 43 °C) and then assayed for the synthesis of luciferase at time points after heating. Luciferase activity was indexed to β -galactosidase activity and calculated and subject to statistical analysis as described under "Experimental Procedures." Experiments were repeated three times with reproducible findings. B, MEL cells stably transfected with empty expression vector, pHSF1 or pHSF2A were transiently transfected with the pGL.HSP70B promoter reporter, heat-shocked (45 min at 43 °C), and assayed for luciferase activity after heating. Luciferase activity was calculated and subjected to statistical analysis as above. Experiments were carried out three times in triplicate with reproducible findings. C, MEL cells stably expressing either HSF2A or HSF2B were transiently co-transfected with pGL.HSP70B and different dosages of pHSF1 (25 ng/well containing 4×10^4 cells) prior to heat shock (45 min at 43 °C). After 1-h recovery at 37 °C, cells were lysed and assayed for luciferase activity. Corrected luciferase activity was calculated as above. Experiments were carried out three times in triplicate with reproducible findings. D, relative concentrations of HSF2 proteins in MEL cells stably transfected with pcDNA3.1(-) empty vector, HSF1, pHSF2A, or pHSF2B. Cell proteins were extracted, separated by 10% SDS-PAGE, and probed by immunoblot analysis with anti-HSF2 antibodies as described under "Experimental Procedures." Experiments were carried out reproducibly on two occasions.

reflect the ability of the proteins to interact physically and form complexes in cells before and after heat shock (Fig. 8). This interaction was observed under resting conditions and was strongly increased by heat shock, suggesting increased avidity of HSF2 binding to HSF1 during stress (Fig. 8). Remarkably, this increased HSF2-HSF1 association occurred under conditions in which overall HSF2 levels decrease in the cell because of targeted degradation through the proteasomal pathway (39). The ability to associate with HSF1 is apparently shared by both HSF2A and HSF2B, as indicated by our *in vitro* interaction studies (Fig. 8D). The domains involved in HSF1-HSF2 binding are not known. However, a role for the N-terminal leucine zipper region that is largely conserved between the HSF proteins and mediates the formation of homotrimers might be suggested (43, 44). Interestingly, this region has been shown to be involved in the binding of HSF1 to the basic zipper family protein C/EBP β , indicating that this region is involved in the binding of alternative partners by HSF1 (45, 46). When the C-terminal domain of HSF1 was deleted, leaving the N-terminal DNA binding and leucine zipper regions, HSF2 binding was retained, suggesting the possibility that leucine zipper-leucine zipper interactions are involved in HSF1-HSF2 binding (Fig. 8D). Further studies will be needed to clearly define this interaction.

These findings with HSF family proteins are reminiscent of earlier studies of the basic zipper family of activating protein-1 binding transcription factors and the nuclear factor κ B complex. Activating protein-1 factors including the Fos and Jun proteins are ineffective or weak activators of transcription when in homodimeric complexes but become powerful activators when complexed as heterodimers (47, 48). Likewise, the p65 component of nuclear factor κ B is ineffective in transcription, whereas p50 homodimers functions as repressors, in contrast to p65/p50 heterodimers, which are powerful and versatile activators of transcription (49). Our experiments suggest that similar mechanisms may be involved with HSF regulation of transcription and that HSF1-HSF2A complexes have increased potency in the activation of the *hsp70* promoter when compared with these factors acting individually (Fig. 4). The exact role of HSF2 in cellular responses to stress is currently not clear. Our unpublished studies suggest that aggregate HSF2 mRNA levels are $\sim 1/4$ the level of those of HSF1 in a series of tissue culture cells and, if this distribution is reflected at the protein level, HSF1-HSF2 interactions are likely to be far from stoichiometric.² In addition, we and others have ob-

² D. Tang and S. K. Calderwood, manuscript in preparation.

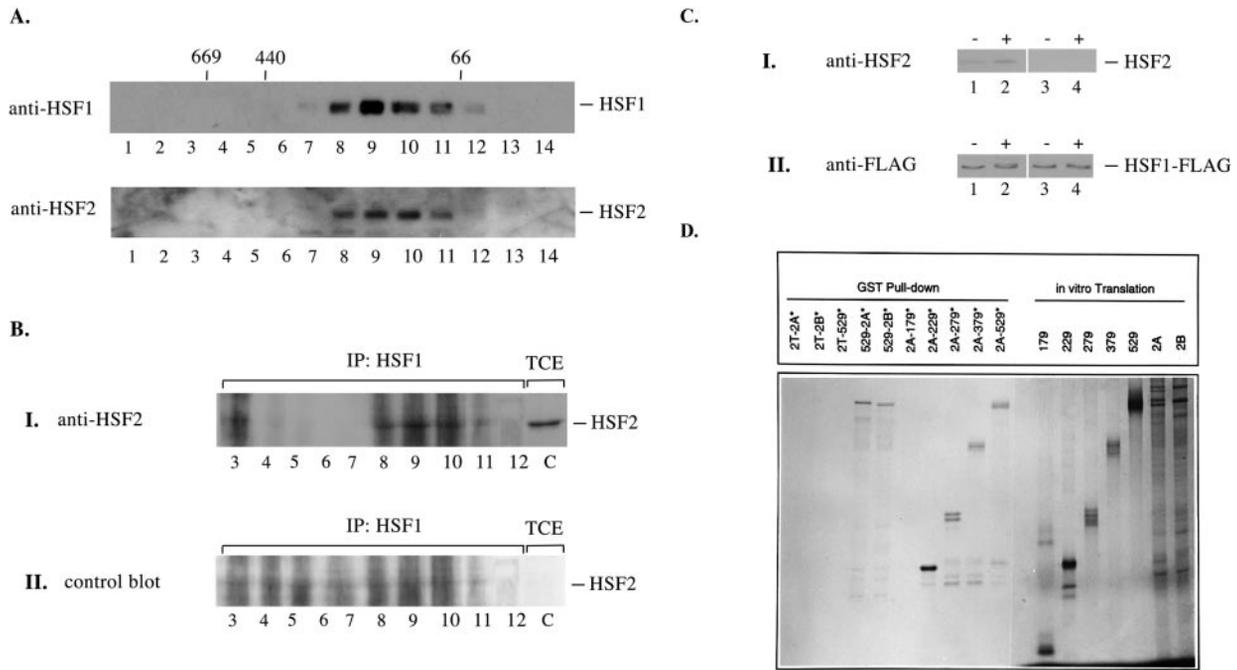


FIG. 8. Physical interactions between recombinant HSF1 and HSF2 *in vitro*. A, HSF1 and HSF2 co-fractionate in similar molecular weight complexes during gel-filtration chromatography. Cycling cells were solubilized and fractionated by gel-filtration chromatography on a Superdex 200 HR10/30 column using an FPLC system. Aliquots of the fractions were examined by SDS-PAGE followed by Western/ECL using the indicated antibodies. Molecular weight markers are indicated at the top. B, endogenous HSF1 and HSF2 co-immunoprecipitate from similar molecular weight complexes during gel-filtration chromatography. Fractions in lanes 3–12 above were subjected to anti-HSF1 immunoprecipitation and tested for associated HSF2 using immunoblot analysis with anti-HSF2 (top) or control (bottom) antibodies. Lane C, involves total cell extract (from the same fraction run in lane 9 in A), included as a blotting and molecular mass control. C, HSF2 co-immunoprecipitates with HSF1-FLAG. HSF2 was co-expressed in two subconfluent cultures along with HSF1-FLAG. One of the transfected cultures was subjected to heat shock (10 min at 43 °C), and both plates were harvested, solubilized in extraction buffer, and subjected to affinity immunoprecipitation with anti-FLAG Sepharose (lanes 1 and 2). As controls, parallel pull-downs containing 0.1% SDS, 0.5 M NaCl in the extraction and wash buffer were included (lanes 3 and 4). Following SDS-PAGE, anti-HSF2A and anti-FLAG blotting was performed to detect associated HSF2 and verify levels of HSF1-FLAG expression and recovery, respectively. Experiments were each repeated twice, and similar results were observed. D, HSF2-HSF1 interaction was examined *in vitro* using the GST pull-down assay. Recombinant GST-HSF1 and GST-HSF2 “bait” proteins were prepared as described under “Experimental Procedures” and tested for functional activity by EMSA as in Fig. 1. Radiolabeled HSF2A, HSF2B, HSF1, and HSF1 deletion mutants shown were prepared by *in vitro* transcription/translation in the presence of ³⁵S-labeled methionine and the relative efficiency of translation is shown in the autoradiograph on the right hand side of the figure. For this purpose, 2 μl of translation reaction mixture was separated by 10% SDS-PAGE and ³⁵S-labeled proteins detected by x-ray film autoradiography. For GST pull-down, 10 μl of translation mixture, containing ³⁵S-labeled target proteins, was incubated with the GST propeptide or GST fusion protein. Initially, GST-HSF1 (529) was incubated with ³⁵S-labeled HSF2A and HSF2B (fourth and fifth lanes, marked 529-2A and 529-2B). Products were isolated on GSH beads, washed, and analyzed by 10% SDS-PAGE/x-ray film autoradiography. In the next experiments, GST-HSF2A (2A) was incubated with wild-type HSF1 (529) and a series of HSF1 mutant proteins with C-terminal truncations of, respectively, 350, 300, 250, and 150 amino acids from the 529-amino acid HSF1 and marked 2A-178, 2A-229, 2A-279, and 2A-379). Initially, GST-HSF1 (529) was incubated with HSF2A and HSF2B (fourth and fifth lanes, marked 529-2A and 529-2B). Products were isolated on GSH-Sepharose beads, washed and analyzed by 10% SDS-PAGE/x-ray film autoradiography. In the next experiment, GST-HSF2A (2A) was incubated with wild-type HSF1 (529) and a series of HSF1 mutant proteins with C-terminal truncations of, respectively, 350, 300, 250 and 150 amino acids from the 529 amino acids of wild-type HSF1 (marked 2A-179, 2A-229, 2A-279, and 2A-379). The reactions were analyzed as above. The first three lanes are from control incubations in which GST propeptide (2T) was incubated with, respectively, HSF2A, HSF2B, and HSF1 (marked 2T-2A, 2T-2B, and 2T-529). All autoradiographs were quantitated by densitometry and relative density of the bands used to determine the relative retention of translated proteins by the GST-fusion proteins. Relative retention of HSF1 and HSF2B (% of input) on GST-HSF1 was, respectively, 7.7% and 6.8%. Relative retention of HSF1 polypeptides on GST-HSF2A was, respectively, 2.55 (wild-type HSF1), 5.2%, (1–379), 4.9% (1–279), and 7.2% (1–179) and undetectable for 1–179. The experiment was repeated twice with reproducible findings.

served that prolonged heat shock causes a marked decline in overall HSF2 levels (data not shown), and this has been ascribed to increased HSF2 degradation by targeted proteolysis (39). Levels of HSF1 are thus likely to far exceed HSF2 in heat-shocked cells. However, as discussed above, HSF1-HSF2 association is enhanced by heat shock, suggesting the formation of high affinity HSF1-HSF2 complexes after heat (Fig. 8). The ability of HSF2A to potentiate the activation of transcription may thus partially depend on the relative intracellular levels of HSF2A surviving after stress. Conditions that enhance HSF2A expression (such as exposure to hemin; Fig. 5) or limit HSF2 degradation (such as proteasomal inhibitors) increase HSF1-directed transcription (39). Alternatively, HSF2 degradation may be involved in the resolution of the heat shock response and may serve to attenuate *hsp* gene transcription in cells recovering from stress. Our studies do not define the cellular role of HSF2B in the heat shock response. Under the

conditions used here, elevated expression of HSF2B neither activated nor repressed heat-induced *hsp70* transcription (Figs. 1, 3, 4, and 7).

Our studies also suggest that the stress-induced activity of HSF2 depends on the relative levels of alternatively spliced HSF2 isoforms (Fig. 4). The levels of HSF2A and HSF2B mRNA and protein vary between tissues (23, 24, 50). HSF2A and HSF2B differ in the deletion of 18 codons from the HSF2B mRNA encoding a sequence from amino acids 393–411 (Fig. 9). Although the significance of deleting this region for HSF2 regulation is not clear, splicing removes sequences from a domain in HSF2 highly conserved with HSF1 that encodes activation domain 1 (Fig. 9). The N-terminal 4 amino acids of the DS domain (LFTS) overlap with the sequence in HSF2 corresponding to AD-1 in HSF1, a structural overlap that suggests a potential role for alternative splicing of the DS region in HSF2 regulation. However, on exposure to heat shock, only HSF2A

HSF1	13	SNVPAFLTKLWTLVSDPDIDALICWSPSGNSFHVFDQGGFAKEVLPKYFKHNNMASFVRQ	72
		SNVPAFL+KLWTLV + T+ I WS +G SF V D+ +FAKE+LPKYFKHNNMASFVRQ	
HSF2	5	SNVPAFLSKLWTLVEETHNEFIITWSQNGQSFVLVDEQRFKEILPKYFKHNNMASFVRQ	64
		<u>SNVPAFLSKLWTLVEETHNEFIITWSQNGQSFVLVDEQRFKEILPKYFKHNNMASFVRQ</u>	
	73	LNMYGFRKVVHIEQGGLVKPERD-DTEFQHPFCFLRGQEQLLENIKRKVTSVSTLKSEDIK	131
		LNMYGFRKVVHI+ G+VK ERD EFQHP F +GQ+ LLENIKRK VS+ K E+ K	
	65	LNMYGFRKVVHID-SGIVKQERDGPVEFQHPYFKQGDDLLENIKRK---VSSSKPEENK	120
		<u>LNMYGFRKVVHID-SGIVKQERDGPVEFQHPYFKQGDDLLENIKRK</u>	
	132	IRQDSVTKLLTDVQLMKGKQECMDSKLLAMKHENEALWREVASLRQKHAQQQKVVNKLIQ	191
		IRQ+ +TK+++ Q ++ KQE ++S+L +K ENE+LW+EV+ LR KHAQQQ+V+ K++Q	
	121	<u>IRQEDLTKIISSAQKVQIKQETIESRSLSELKSENESSLWKEVSELRAKHAQQQVIRKIVQ</u>	180
		<u>IRQEDLTKIISSAQKVQIKQETIESRSLSELKSENESSLWKEVSELRAKHAQQQVIRKIVQ</u>	
	192	FLISLVQSNRILGVKRIPLMLNDSGS--AHSMPKYSRQFSLEHVHXXXXXXXXXXXXXX	249
		F+++LVQ+N+++ +KRK PL+LN +G+ + + + H H	
	181	<u>FIVTLVQNNQLVSLKRRPRLLLNTNGAQKKNLFQHVIVKEPTDNHHHKVPHSRTEGLKPRE</u>	240
		<u>FIVTLVQNNQLVSLKRRPRLLLNTNGAQKKNLFQHVIVKEPTDNHHHKVPHSRTEGLKPRE</u>	
	250	XXXXXXDAVASSGPIISDIT-----ELAPASPMASPGGSIDERPLSSSPLVRVKEEPPS	303
		S II D+T E P P + D S P ++ E+	
	241	R-----ISDDIIIVDVTDDNADEENIPVIPETNEDVISDPSNCSQYDPDIVIVEDNE	292
		<u>R-----ISDDIIIVDVTDDNADEENIPVIPETNEDVISDPSNCSQYDPDIVIVEDNE</u>	
	304	PPQSPRVEEASPRPSSVDTLSPALIDSILRESEPPASVTALTDARGHTDTEGRXXX	363
		+P ++ P+ + S S P +S L + T +	
	293	DEYAPVIQSGEQNEPARES-----LSSGSDGSSPLMSSAVQLNGSSSLTSEDPTMM	344
		<u>DEYAPVIQSGEQNEPARES-----LSSGSDGSSPLMSSAVQLNGSSSLTSEDPTMM</u>	
	364	XXXXXXXXEKCLSVACLKNEKSDHLDAMDSNLDNLQTMSSHGFSVDTALLDLFSPSVT	423
		++ L K EL D+LD++D +L++ Q MLS FS+D L+DLF+ SV	
	345	<u>DSILND-----NINLLGKVELLDYLDSDCSLEDFAQLSGRQFSIDPDLVDFLFTSSVQ</u>	399
		<u>DSILND-----NINLLGKVELLDYLDSDCSLEDFAQLSGRQFSIDPDLVDFLFTSSVQ</u>	
	424	V-PDMSLPDLSSLASIQELLSPQEPPEAENSSPDGKQLVHYTAQPLFLDLPGS	480
		+ P ++ S ++ + P E S KQL+ YTA PL G+	
	400	<u>MNPTDYINNFKSENKGLTETTKNNVVQPVSEGRKSKPKDKQLIQYTAPLLAFLDGN</u>	457
		<u>MNPTDYINNFKSENKGLTETTKNNVVQPVSEGRKSKPKDKQLIQYTAPLLAFLDGN</u>	

— DNA binding — Trimerization — LZ4 — DS

FIG. 9. Amino acid sequence comparison between human HSF1 and HSF2. We have compared the 629-amino acid sequence of HSF1 with the 636-amino acid sequence of HSF2 using BLAST2 analysis. The HSF1 sequence is shown above and the HSF2 sequence below, with conserved amino acids indicated in the intervening line. Functional domains including the DNA binding domain, the trimerization domain and leucine zipper 4 (LZ4) are assigned as in Ref. 10 and indicated by *underlining* the sequence. The differentially spliced sequence present in HSF2A, determined by our molecular cloning studies described under "Experimental Procedures," which is identical to the differentially spliced sequence in murine HSF2 (23), is indicated as *DS*. Minimal homology was found between the remainder of the two protein sequences outside of the regions shown here, and these sequences have not been included here.

stimulates *trans*-activation of the *HSP70B* promoter, suggesting a role for the DS domain in stress-inducible transcription (Fig. 4, A and B). This may be a result of the fact that only HSF2A contains an intact AD-1-like domain or to other residues in the DS sequence. The failure of transfected HSF2A to activate the *HSP70B* promoter in MEL cells casts doubt on the presence of stress-inducible activation domains in HSF2 as suggested previously (40, 41). One possible hypothesis to explain the role of HSF2A in the heat shock response is that transcriptional activation is conferred by HSF2 only when complexed with HSF1 and that gene induction by such complexes is the result of activation domains in HSF1. However, this domain does not activate transcription and, when fused to heterologous activation domains, functions as a repressor rather than an activator of transcription (40). The AD-1-like domain in HSF2A may thus play another role in heat shock gene transcription such as interacting with a co-activator or might possibly function as an activation domain only in the context of HSF2A-HSF1 complexes. Earlier studies in fact suggest that this site may contain a binding site for potential regulatory proteins.

Thus, HSF2A is an activator of stress-induced *hsp70* transcription and contributes to the complexity becoming evident in the eukaryotic heat shock response (15, 51). Although most studies indicate that HSF1 is the primary factor that leads to protection from stress, it is now evident that both HSF2A and HSF3 may contribute to the response (Figs. 4 and 6) (51). The more elaborate regulatory mechanisms controlling HSF2 activ-

ity suggests a cell- or tissue-specific role for HSF2 in developing embryonic and tissue stem cells that may complement the effects of the ubiquitously expressed HSF1 and take part in the protection of such cells from environmental stresses (Fig. 5) (21, 23, 24, 50).

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