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 myeloprogenitor 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)† family was first discovered in experiments investigating proteins binding to the 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 myeloprogenitor 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 hsp70/b promoter with and without stress. These experiments suggest that HSF2 is involved in the stress response, but unlike the ubiquitous HSF1, which 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 hsp70/B 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 (erythroleukemic) cell line K562, HeLa S3 human carcinoma cells, and Chinese hamster ovary CHO K1 cells 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 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 pBluescript 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).

Nuclear DNA Cloning of Human and Mouse HSF2A and HSF2B—To clone HSF2A and HSF2B from human and mouse 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 (PolyATtract 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′-CG(AAATCC)ATGAGG-AGAGTTCCA-3′, and reverse primer was 5′-AAAGTCGCA/TTC-TGCGGGATTATGACTA-3′. For mouse HSF2, forward primer was 5′-GG(AAATCC)ATGAAGGACAGAGTTCCA-3′, and reverse primer was 5′-AG(TCTGCGCAG)TGGAGGATTTAAATCT-3′. For human HSF2, forward primers consisted of EcoRI restriction site consensus sequences (in parentheses) and the reverse primers contained HSF2 insert sequences, and the reverse primers contained Sall sequences for subsequent cloning of amplified DNAs into the PGEKS prokaryotic expression vector (Amersham Biosciences). After transformation and growth of competent bacteria, colonies were screened for either total HSF2 using oligonucleotides (1764–1785 (CAGAGGCAAGTCCATAATAA) and 1786–1807 (GGCATACTACTATCCAGGAGTTG)), 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 (CATTGCGACAGAATCGT)). Oligonucleotides were end-labeled with [32P]ATP and T4 polynucleotide kinase. Plasmids derived from this species (1420–1440 (TTGTATTATTGATGTAATCT) and 1392–1412 (CATTGCGACAGAATCGT)) were recloned into the pBluescript II SK− plasmid and sequenced.

In Vitro Transcription—HSF2 species (approximately 1 μg of HSF1, 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 μg MgCl2 in a final volume of 13 μl for 30 min at 30 °C. Seven μl of nuclear extracts prepared from CHO K1 cells or 1 μl of recombinant human and mouse mRNA of ~1.6 kb (predicted sizes for the inserts were, respectively, 1.621 (hsHSF2A), 1.567 (hsHSF2B), 1.616 (mHSF2A), and 1.652 (mHSF2B) kb). The cDNAs from clones 94 and 6 are slightly retarded in the gels consistent with their slightly larger size (data not shown).
Expression of HSF2A Stimulates hsp70 Transcription

Expression of HSF2A and HSF2B fusion proteins both bound to a 32P-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 an HSF2 dilution of 1/220 (lanes 1–7). Significant differences in the extent of repression were observed between HSF2A and HSF2B, with greater repression by HSF2A (lanes 1–5)

Fig. 1. HSE binding of purified HSF2A and HSF2B. Purified GST-HSF2A and GST-HSF2B were incubated with 32P-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), 1/600 (lanes 2 and 8), 1/1200 (lanes 3 and 9), 1/600 (lanes 4 and 10), 1/220 (lanes 5 and 11), and 1/220 (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.

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Expression of HSF2A Stimulates hsp70 Transcription

We next 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 apparent 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 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 4A). 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-
pressed to high level (Fig. 4B). Relative levels of HSF2A and HSF2B in the stable transfectants are shown in Fig. 4C.

**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 5A); using pBHSF2A as a template in the reaction, a 232-bp product was observed, and, when pBH2F2B 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. 5B). HSF2A appeared to be the predominant isoform in these samples (Fig. 5B). 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. 5B), 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. 5C). 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. 5D). 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. 5D). 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. 5E and 4C). 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-1,3-dihydro(6-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 HSF2A 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. 4. Effects of heat shock on activation of the HSP70B promoter by HSF2A and HSF2B in vitro.** A, wild-type K562 cells and cells stably expressing HSF1, HSF2A, and HSF2B were transfected with pGL3.HSP70B promoter reporter construct, heat-shocked (45 min at 43 °C), and then assayed for the synthesis of luciferase at time points after healing. 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 cDNA3.1(−), pHSF1, pHSF2A or pHSF2B, and pGL3.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 μM 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.
HSF2A 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 μM 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 μM 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.
Expression of HSF2A Stimulates hsp70 Transcription

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 μM 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. 8D). 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 °C as a control or heat-shocked at 43 °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 °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 homotrimmerization 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. 4, 5, and 6). However, other 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
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/EBPβ, 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 κB 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 κB is ineffective in transcription, whereas p50 homodimers function 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 ~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-

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

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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 cycling 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 and protein vary between tissues (23, 24, 50). HSF2A and HSF2B differ in the deletion of 18 codons 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 2A-2A and 2A-2B). Products were isolated on GSH 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 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 2A-2A and 2A-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 acid wild-type HSF1 (marked 2A-178, 2A-229, 2A-279, and 2A-379). The reactions were analyzed as above. The first three lanes are from control incubations in which GST protein (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-HSF2 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.
Expression of HSF2A Stimulates hsp70 Transcription

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 activity 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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Expression of HSF2A Stimulates hsp70 Transcription
Elevated Expression of Heat Shock Factor (HSF) 2A Stimulates HSF1-induced Transcription during Stress

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