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Human Heat Shock Factors 1 and 2 Are Differentially Activated and Can Synergistically Induce hsp70 Gene Transcription

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Two members of the heat shock transcription factor (HSF) family, HSF1 and HSF2, both function as transcriptional activators of heat shock gene expression. However, the inducible DNA-binding activities of these two factors are regulated by distinct pathways. HSF1 is activated by heat shock and other forms of stress, whereas HSF2 is activated during hemin-induced differentiation of human K562 erythroleukemia cells, suggesting a role for HSF2 in regulating heat shock gene expression under nonstress conditions such as differentiation and development. To understand the distinct regulatory pathways controlling HSF2 and HSF1 activities, we have examined the biochemical and physical properties of the control and activated states of HSF2 and compared these with the properties of HSF1. Our results reveal that the inactive, non-DNA-binding forms of HSF2 and HSF1 exist primarily in the cytoplasm of untreated K562 cells as a dimer and monomer, respectively. This difference in the control oligomeric states suggests that the mechanisms used to control the DNA-binding activities of HSF2 and HSF1 are distinct. Upon activation, both factors acquire DNA-binding activity, oligomerize to a trimeric state, and translocate into the nucleus. Interestingly, we find that simultaneous activation of both HSF2 and HSF1 in K562 cells subjected to hemin treatment followed by heat shock results in the synergistic induction of hsp70 gene transcription, suggesting a novel level of complex regulation of heat shock gene expression.

The classical heat shock response occurs via transcriptional induction of heat shock genes mediated by activation of heat shock transcription factor (HSF) (for reviews, see references 21, 24, 26, and 49). HSF binds to a heat shock element (HSE), which consists of contiguous arrays of the alternately oriented pentanucleotide unit 5′-NGAAN-3′ found in the promoter regions of heat shock genes (5, 31, 32, 59). In addition to physiological stress, such as exposure to oxidants, heavy metals, amino acid analogs, and elevated temperatures, the hsp70 gene and other heat shock genes have long been known to be transcriptionally activated under a large number of circumstances, including early development and differentiation, bacterial and viral infections, and oncogenic activation (for reviews, see reference 25 and references therein). In part, some of this transcriptional regulation can be attributed to a complex array of basal promoter elements, which are responsible for growth-regulated and oncogene-activated transcription of the human hsp70 gene (4, 23, 51, 56, 58). Furthermore, the recent identification of a family of HSFs in larger eukaryotes, such as humans, mice, chickens, and tomato plants, suggests that the complexity of transcriptional regulation of heat shock genes may be attributable to the differential activation of distinct HSF family members (30, 35, 41, 42, 44).

In mammalian cells, the transcription factors HSF1 and HSF2 have an overall 38% identity and contain highly conserved amino acid sequences corresponding to the DNA-binding and oligomerization domains (35, 41, 44). Although the genes encoding HSF1 and HSF2 are constitutively expressed in most cell lines and tissues, both factors are kept in a latent, non-DNA-binding state under normal growth conditions, indicating that the DNA-binding activity of both HSF1 and HSF2 is negatively regulated. A fundamental distinction between these factors is that they are regulated by different signaling pathways. Previous studies have identified HSF1 as the mediator of heat shock gene transcription in response to stress conditions, whereas HSF2 DNA-binding activity is induced during hemin treatment of human K562 erythroleukemia cells (9, 36, 39, 48). Activation of HSF2 in hemin-treated K562 cells is characterized by slower kinetics and a slightly different DNA-binding pattern of the hsp70 promoter in vivo compared with those of heat shock-induced HSF1. In addition, transcription of the hsp70 gene is induced to a lesser extent in hemin-treated K562 cells relative to heat-shocked cells (48). These observations point to the fundamental questions that must be addressed in order to understand how HSF1 and HSF2, which both regulate heat shock gene expression, are activated by distinct pathways.

In this study, we explored the differential regulation of human HSF1 and HSF2 by comparing the biochemical and physical properties of these two factors in both the control non-DNA-binding state and activated DNA-binding state in heat-shocked and hemin-treated K562 cells, respectively. Thus, although the emphasis of this study was to characterize human HSF2, we chose to compare the properties of HSF2 with those of HSF1 to gain an understanding of how these two transcription factors respond to distinct activation conditions. Our results reveal an important difference in the sizes of the native complexes for HSF1 and HSF2 in the non-DNA-binding state. In addition, experiments in which HSF1 and HSF2 are simultaneously activated by heat shocking K562 cells in the presence

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of hemin indicate that these two factors are able to act synergistically to induce heat shock gene transcription.

MATERIALS AND METHODS

Cell culture, hemin treatment, heat shock conditions, and preparation of cell extracts. Human K562 erythroleukemia cells (22) (provided by S. Weil, Northwestern Medical School, Chicago, Ill.) were maintained in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics in a humidified 7% CO_2 atmosphere at 37°C. Cells were seeded at 5×10^6 cells per 10-cm-diameter plate prior to addition of hemin to a final concentration of 15 μM as described previously (52). For heat shock, plates were sealed with Parafilm and immersed in a 42°C water bath. Whole-cell, nuclear, and cytoplasmic extracts were prepared as described previously (15, 27), and the protein concentration was estimated with an assay kit (Bio-Rad). To further test whether nuclear extracts contained proper amounts of protein, we reprobed the Western immunoblots with antibodies for lamin B (a kind gift of Robert Moir), detecting a nuclear membrane-associated protein.

Western immunoblot analysis and chemical cross-linking. Cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose filters by using a Bio-Rad semidry transfer apparatus as specified by the manufacturer. After blocking for 90 min with phosphate-buffered saline (PBS) containing 3% nonfat dry milk at room temperature, filters were incubated for 60 min with a 1:10,000 dilution of the rabbit polyclonal HSF1 or HSF2 antiserum (39). After being washed three times for 10 min each with PBS–0.01% Tween 20, the filters were incubated with a 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibodies (Promega) and washed three times for 10 min each with PBS–0.01% Tween 20 prior to detection by the ECL system (Amersham). Cross-linking of HSF1 and HSF2 by incubating the cell extracts with ethylene glycol bis(succinimidylsuccinate) (EGS) was performed as described previously (39).

Gel mobility shift assay. HSF DNA-binding activity in vitro was analyzed by incubating cell extracts with γ-^{32}P-labeled HSE oligonucleotide derived from the human hsp70 promoter, and the HSE-protein complexes were analyzed on a native 4% PAGE gel as described previously (27). The intensities of radioactive signals indicating the levels of HSF-HSE complexes were quantitated with a Molecular Dynamics 400A PhosphorImager.

Gel filtration. The Stokes radii of inactive and active HSF1 and HSF2 were determined by gel filtration chromatography on a Superdex 200 column (Pharmacia) with a Pharmacia fast protein liquid chromatography system. Whole-cell extracts (500 μg) in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–20% (vol/vol) glycerol–0.1 M KCl–0.2 mM EDTA–0.5 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride–2 μM leupeptin–1.5 μM pepstatin A were applied to the column equilibrated in 20 mM HEPES (pH 7.2)–5 mM $MgCl_2$–100 mM NaCl at 4°C. The samples were eluted at 0.25 ml/min, and 25 0.5-ml fractions were collected once the void volume of the column had passed. To determine which fractions contained HSF1 or HSF2, 12 μl of each fraction was mixed with gel sample buffer (1× gel sample buffer is 50 mM Tris [pH 6.8], 2% SDS, 100 mM dithiothreitol, 10% glycerol, and 0.1% bromphenol blue), boiled, and analyzed by SDS-PAGE and Western immunoblotting. Standard curves for the molecular mass and Stokes radius were generated by using globular proteins of known molecular masses and Stokes radii (thyroglobulin, 669 kDa, 85 Å [1 Å = 0.1 nm]; apoferritin, 443 kDa; β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa, 45 Å; apotransferrin, 81 kDa; ovalbumin, 43 kDa; cytochrome *c*, 12.4 kDa, 16.4 Å) (46). A least-squares algorithm (KaleidaGraph) was used to fit a line through the standard datum points, and a correlation coefficient of $r = 0.99$ was obtained for both curves.

Sedimentation analysis. $s_{20,w}$ values of HSF1 and HSF2 were determined by centrifugation through 10 to 40% glycerol gradients. Linear glycerol gradients were prepared by using a gradient maker (SG 15; Hoefer Scientific Instruments) and mixing equal volumes (2.5 ml) of 10 and 40% glycerol buffer containing 20 mM HEPES (pH 7.9), 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, and 1 mM dithiothreitol. The gradients were chilled for approximately 4 h at 4°C before being loaded with 500 μg of whole-cell extracts or a mixture of protein standards (alcohol dehydrogenase, 7.4S, bovine serum albumin, 4.3S; cytochrome *c*, 1.9S) (46) diluted to 200 μl with 10% glycerol buffer and centrifuged in a Beckman SW50.1 rotor for 36 h at 40,000 rpm and 4°C. The gradients were fractionated from the top into 250-μl fractions, and 12 μl of each fraction was mixed with gel sample buffer, boiled, and analyzed by SDS-PAGE and Western immunoblotting with HSF1 and HSF2 antisera. The sedimentation profile of the standard markers was determined by analyzing a portion of each fraction by SDS-PAGE and staining with Coomassie blue.

Transcriptional run-on analysis. Run-on transcription reactions were performed with isolated nuclei (approximately 7×10^6 nuclei per reaction) in the presence of 100 μCi of [α-^{32}P]UTP (3,000 Ci/mmol; Amersham) as previously described (11). Radioactive RNA was hybridized to nitrocellulose filters on which the following plasmids had been immobilized: pGEM2 (vector; Promega), pH2.3 (human hsp70) (57), pUCHS801 (human hsp90/89a) (18), pHG23.1.2 (human grp78/BiP) (54), pHA7.6 (human hsc70/p72), pGAPDH (rat glyceraldehyde-3-phosphate dehydrogenase) (16), pC9 (mouse HSF2) (41), and pC12A (mouse HSF1) (41). The hybridization and washing conditions were as described previously (48). The intensities of radioactive signals were quantitated with a Molecular Dynamics 400A PhosphorImager.

Genomic footprinting. For in vivo footprinting of the human hsp70 promoter, 2×10^7 cells were harvested and treated for 5 min with 0.2% dimethyl sulfate at room temperature. Genomic DNA was isolated, digested with *Eco*RI, and cleaved with piperidine (12). The methylation pattern of naked DNA was obtained by using deproteinized DNA which was treated with dimethyl sulfate in vitro. Genomic footprinting analysis was performed by using a ligation-mediated PCR method (28). Primers used for footprinting of the coding and noncoding strands of the HSE regions of the human hsp70 promoter have been described previously (2).

RESULTS

Acquisition of HSF2 DNA-binding activity is accompanied by a change in oligomeric state. To examine the basis for the differences in regulation of HSF1 and HSF2, we compared the native size of HSF2 in untreated and hemin-treated K562 cells with that of HSF1 in untreated and heat-shocked cells. We used gel filtration chromatography and glycerol gradient sedimentation analysis to examine the hydrodynamic properties of inactive and active HSF2 and HSF1 in order to determine their oligomeric state and shape in extracts isolated from control, hemin-treated, and heat-shocked K562 cells. Extracts were fractionated by gel filtration chromatography on a Superdex 200 column and by centrifugation through 10 to 40% glycerol gradients, and the locations of HSF2 and HSF1 were analyzed

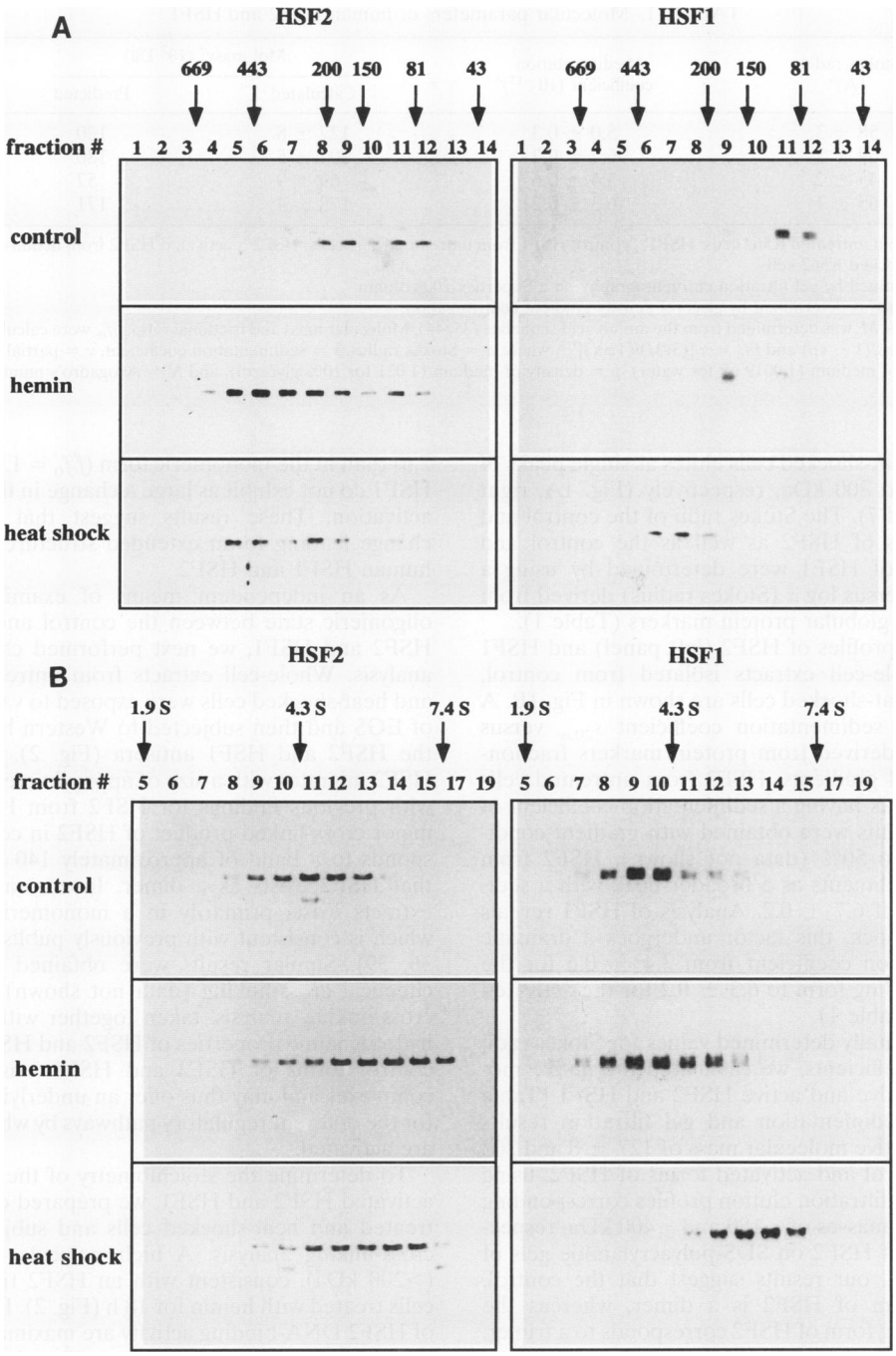

FIG. 1. Analysis of hydrodynamic properties of HSF2 and HSF1. (A) Gel filtration analysis of HSF2 and HSF1. Whole-cell extracts of untreated (control), hemin-treated (20 h), or heat-shocked (1 h at 42°C) cells were subjected to chromatography on a Superdex 200 column, and the positions of HSF2 and HSF1 proteins were visualized by Western blot analysis with HSF2 or HSF1 antiserum, respectively. The approximate elution positions of protein standards are indicated (ovalbumin, 43 kDa; apotransferrin, 81 kDa; alcohol dehydrogenase, 150 kDa; β-amylase, 200 kDa; apoferritin, 443 kDa; thyroglobulin, 669 kDa). (B) Glycerol density gradient analysis of HSF2 and HSF1. The same extracts described for panel A were separated by centrifugation through 10 to 40% glycerol gradients, and the positions of HSF2 and HSF1 proteins were visualized by Western blot analysis. The sedimentation positions of protein standards are indicated (cytochrome *c*, 1.9S; bovine serum albumin, 4.3S; alcohol dehydrogenase, 7.4S).

by Western blot analysis with antibodies specific to HSF2 and HSF1. HSF2 in untreated cells elutes from the gel filtration column as two peaks with apparent molecular masses of approximately 100 and 180 kDa (Fig. 1A, left panel; fractions 9 and 11), and this elution profile was reproducible in three independent experiments. HSF2 from hemin-treated cells elutes in a relatively broad peak corresponding to a size of approximately 400 kDa (Fig. 1A, left panel, fraction 6). HSF1

TABLE 1. Molecular parameters of human HSF2 and HSF1

Species[a]	Stokes radius (Å)[b]	Sedimentation coefficient (10^{-13})[c]	Mol mass[d] (10^3 Da)		Frictional ratio (f/f_0)[d]
			Calculated	Predicted	
$HSF2^C$	58 ± 3	5.0 ± 0.3	127 ± 8	120	1.75
$HSF2^A$	69 ± 2	6.7 ± 0.2	202 ± 5	180	1.78
$HSF1^C$	45 ± 2	3.4 ± 0.6	69 ± 9	57	1.67
$HSF1^A$	65 ± 1	6.3 ± 0.2	178 ± 4	171	1.74

[a] $HSF2^C$, control HSF2 from untreated K562 cells; $HSF1^C$, control HSF1 from untreated K562 cells; $HSF2^A$, activated HSF2 from hemin-treated K562 cells; $HSF1^A$, activated HSF from heat-shocked K562 cells.

[b] Stokes radius was determined by gel filtration chromatography on a Superdex 200 column.

[c] Sedimentation coefficient was determined by glycerol density gradient centrifugation.

[d] Predicted molecular mass, M, was determined from the amino acid sequence (35, 44). Molecular mass and frictional ratio, f/f_0, were calculated as described by Siegel and Monty (46): $M = 6\pi\eta Nas/(1 - \nu\rho)$ and $f/f_0 = a/[(3\nu M)/(4\pi N)]^{1/3}$, where a = Stokes radius, s = sedimentation coefficient, ν = partial specific volume (assuming 0.725 cm^3/g), η = viscosity of medium (1.0019 cP for water), ρ = density of medium (1.021 for 10% glycerol), and N = Avogadro's number.

from untreated and heat-shocked cells elutes as single peaks of approximately 100 and 300 kDa, respectively (Fig. 1A, right panel, fractions 11 and 7). The Stokes radii of the control and hemin-activated forms of HSF2 as well as the control and heat-activated forms of HSF1 were determined by using a standard plot of K_{av} versus log a (Stokes radius) derived from the elution volume of globular protein markers (Table 1).

The sedimentation profiles of HSF2 (left panel) and HSF1 (right panel) in whole-cell extracts isolated from control, hemin-treated, and heat-shocked cells are shown in Fig. 1B. A standard plot of the sedimentation coefficient $s_{20,w}$ versus gradient volume was derived from protein markers fractionated through identical gradients. HSF2 from untreated cells elutes with a single peak having a sedimentation coefficient of 5.0 ± 0.3. Similar results were obtained with gradient conditions of 10% to 30 to 50% (data not shown). HSF2 from hemin-treated cells sediments as a broader peak with a sedimentation coefficient of 6.7 ± 0.2. Analysis of HSF1 reveals that following heat shock, this factor undergoes a dramatic change in sedimentation coefficient from 3.4 ± 0.6 for the control non-DNA-binding form to 6.3 ± 0.2 for the activated DNA-binding form (Table 1).

From our experimentally determined values for Stokes radii and sedimentation coefficients, we calculated the native molecular masses of inactive and active HSF2 and HSF1 (Table 1). Analysis of the sedimentation and gel filtration results reveals a calculated native molecular mass of 127 ± 8 and 202 ± 5 kDa for the control and activated forms of HSF2, using the peaks from the gel filtration elution profiles corresponding to apparent molecular masses of ~180 and ~400 kDa, respectively. From the size of HSF2 on SDS-polyacrylamide gels of approximately 70 kDa, our results suggest that the control, non-DNA-binding form of HSF2 is a dimer, whereas the activated, DNA-binding form of HSF2 corresponds to a trimer. At this point, our data do not establish whether the native form of HSF2 in control cells is a homodimeric or heterodimeric species; however, the difference in molecular size of the native and denatured forms of HSF2 reveals that it is in a complex with another protein of approximately 70 kDa. Likewise, the estimates of the native molecular mass for the control and activated forms of HSF1 are 69 ± 9 and 178 ± 4 kDa, respectively. This result is entirely consistent with previous observations that HSF1 undergoes a transition from a monomer to a trimer upon activation (9, 36, 39). The calculated frictional ratios (f/f_0) range from 1.67 to 1.78, suggesting that both inactive and active forms of HSF2 and HSF1 are elongated, similar to the findings reported for *Drosophila* HSF (55). However, unlike in *Drosophila* HSF, in which the degree of asymmetry is significantly greater in the trimeric form (f/f_0 = 2.6) than in the monomeric form (f/f_0 = 1.9), human HSF2 and HSF1 do not exhibit as large a change in their axial ratios upon activation. These results suggest that the conformational change leading to an extended structure is less dramatic for human HSF1 and HSF2.

As an independent means of examining the change in oligomeric state between the control and activated forms of HSF2 and HSF1, we next performed chemical cross-linking analysis. Whole-cell extracts from untreated, hemin-treated, and heat-shocked cells were exposed to various concentrations of EGS and then subjected to Western blot analysis by using the HSF2 and HSF1 antisera (Fig. 2). The un-cross-linked HSF2 migrates with a size of approximately 70 kDa, consistent with previous findings for HSF2 from HeLa cells (39). The major cross-linked product of HSF2 in control extracts corresponds to a band of approximately 140 kDa, which suggests that HSF2 exists as a dimer. In contrast, HSF1 in control extracts exists primarily in a monomeric form (~70 kDa), which is consistent with previously published observations (9, 36, 39). Similar results were obtained with glutaraldehyde chemical cross-linking (data not shown). The results of the cross-linking analysis, taken together with our studies on the hydrodynamic properties of HSF2 and HSF1, indicate that the control forms of HSF2 and HSF1 exist as distinct native complexes and may thus offer an underlying mechanistic basis for the different regulatory pathways by which these two factors are activated.

To determine the stoichiometry of the oligomeric states of activated HSF2 and HSF1, we prepared extracts from hemin-treated and heat-shocked cells and subjected them to EGS cross-linking analysis. A high-molecular-mass HSF2 species (>200 kDa), consistent with an HSF2 trimer, is detected in cells treated with hemin for 18 h (Fig. 2). In addition, the levels of HSF2 DNA-binding activity are maximal between 18 and 24 h of hemin treatment (reference 48 and data not shown) and correlate closely with the appearance of the HSF2 trimer. These data suggest a positive correlation between oligomerization and acquisition of HSF2 DNA-binding activity in hemin-treated K562 cells. Similarly, the heat-shocked form of HSF1, which is known to bind DNA, also appears as a trimer (Fig. 2) (9, 36, 39). Thus, oligomerization appears to be a common characteristic of the activation pathways of both HSF2 and HSF1.

An important question for the regulation of heat shock gene transcription relates to the apparent specificity of the pathways by which HSF1 and HSF2 are activated. HSF1 is activated by heat shock, whereas HSF2 is activated by hemin treatment. These points were demonstrated by the use of polyclonal antisera specific to either HSF1 or HSF2 in antibody pertur-

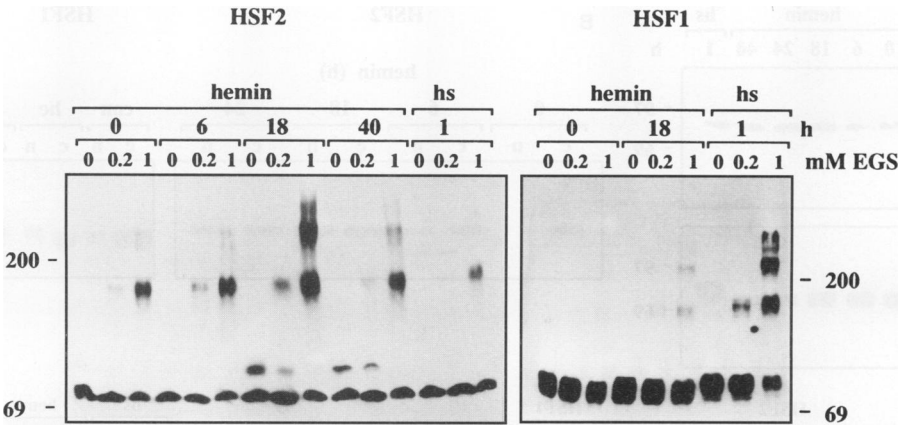

FIG. 2. Determination of oligomeric structure of HSF2 and HSF1 by chemical cross-linking. Whole-cell extracts from K562 cells exposed to hemin or heat shock treatment for the indicated periods were cross-linked with EGS at the indicated concentrations. Cross-linked extracts were run on SDS–5% polyacrylamide gels and subjected to Western blot analysis with HSF2 or HSF1 antiserum. Molecular mass markers are bovine serum albumin (69 kDa) and myosin (200 kDa).

bation analysis for the identification of specific band shift complexes (39, 48). To investigate further the apparent specificity of the HSF1 and HSF2 activation pathways, we examined whether heat shock or hemin treatment could partially activate HSF2 or HSF1, respectively, as measured by changes in the oligomeric state of these two factors. As shown in Fig. 2, EGS cross-linking of HSF2 in heat-shocked cells reveals primarily a dimeric form with very little trimeric form, consistent with a lack of HSF2 DNA-binding activity in heat-shocked cells (48). In addition, HSF1 in hemin-treated cells exists in a monomeric form similar to that in control cells, which is consistent with the finding that no HSF1 DNA-binding activity can be detected in hemin-treated K562 cells (48). However, analysis of HSF2 and HSF1 by hydrodynamic measurements reveals that both heat shock and hemin treatment have an effect on the oligomeric state or conformation of HSF2 and HSF1, respectively. Gel filtration and sedimentation profiles of HSF1 from control and hemin-treated cells show a shift toward the higher molecular weight so that part of HSF1 cosediments with HSF2 from the control cells (Fig. 1). Similarly, the gel filtration and sedimentation profiles of HSF2 from heat-shocked cells show a shift toward, but not identical with, the HSF2 profiles from hemin-treated cells (Fig. 1). Support for the idea that heat shock may cause changes in the properties of HSF2 comes from recent work of the Kingston laboratory, which used transfection of epitope-tagged HSF2 constructs to show that putative nuclear localization sequences are required for the nuclear translocation of HSF2 after heat shock (45). However, because our analysis has failed to detect heat-induced HSF2 DNA-binding activity or trimeric HSF2 by cross-linking (Fig. 2), and because Western blot analysis of nuclear and cytoplasmic fractions does not show significant translocation of HSF2 into the nucleus after heat shock (data not shown), the functional significance of HSF2 activation upon heat shock remains unclear.

Translocation of the HSF2 trimer to the nuclei of hemin-treated K562 cells. To examine the levels and subcellular localization of HSF2 in untreated and hemin-treated K562 cells, we performed Western blot analysis of whole-cell extracts isolated at various time points during continuous hemin treatment. As shown in Fig. 3A, the mobility of HSF2 on an SDS-polyacrylamide gel is identical in extracts isolated from control, hemin-treated, and heat-shocked cells (upper panel), whereas the size of HSF1 in heat-shocked cells increases as a result of phosphorylation (lower panel), as previously shown (39). During exposure of K562 cells to hemin, the amount of HSF2 is gradually increased, reaching maximum levels (three- to fivefold increase from the control level) at 18 to 24 h of treatment and declining to the control levels by 40 h. The increase in HSF2 levels correlates with the increase in DNA-binding activity and the amount of HSF2 present in the trimeric state (Fig. 2) (48). Hemin treatment neither induces HSF2 gene transcription (see Fig. 6A) nor increases the HSF2 mRNA levels (data not shown), suggesting that the increase in HSF2 protein level may be due to enhanced protein synthesis or stability. In contrast to an increase in the levels of HSF2 detected during hemin treatment, the amount of HSF1 appears to decrease, especially during later time points of hemin treatment, but the significance and mechanism of this decrease are unclear.

With biochemical fractionation of cytoplasmic and nuclear components followed by Western blot analysis, HSF2 is detected primarily in the cytoplasmic fraction of the untreated cells, although a low level of HSF2 is also found in the nuclear fraction (Fig. 3B, left panel). To control for the quality of biochemical fractionation, the Western blots containing cytoplasmic and nuclear fractions were reprobed with antibodies to lamin B (data not shown). However, since it is possible that soluble nuclear proteins leak into the cytosolic fraction during fractionation procedures, we independently assessed the subcellular localization of HSF2 by indirect immunofluorescence and confocal microscopy and found that the majority of HSF2 is in the cytoplasm of control K562 cells (data not shown). During the time course of hemin treatment, the HSF2 level in the cytoplasmic fraction remains fairly constant, whereas the amount of HSF2 present in the nuclear fraction increases significantly. Therefore, it appears that most of the increase in overall HSF2 protein levels is found in the nuclear fraction of hemin-treated cells. As a control, the subcellular localization of HSF1 was examined. HSF1 from untreated cells is present in both the cytoplasmic and nuclear fractions, although the major proportion of HSF1 is present in the cytoplasmic fraction. These results were also independently corroborated by indirect immunofluorescence and confocal microscopy (data not shown). The distribution of HSF1 is not affected by hemin treatment. In heat-shocked cells, however, HSF1 is found primarily in the nuclear fraction and exhibits an altered

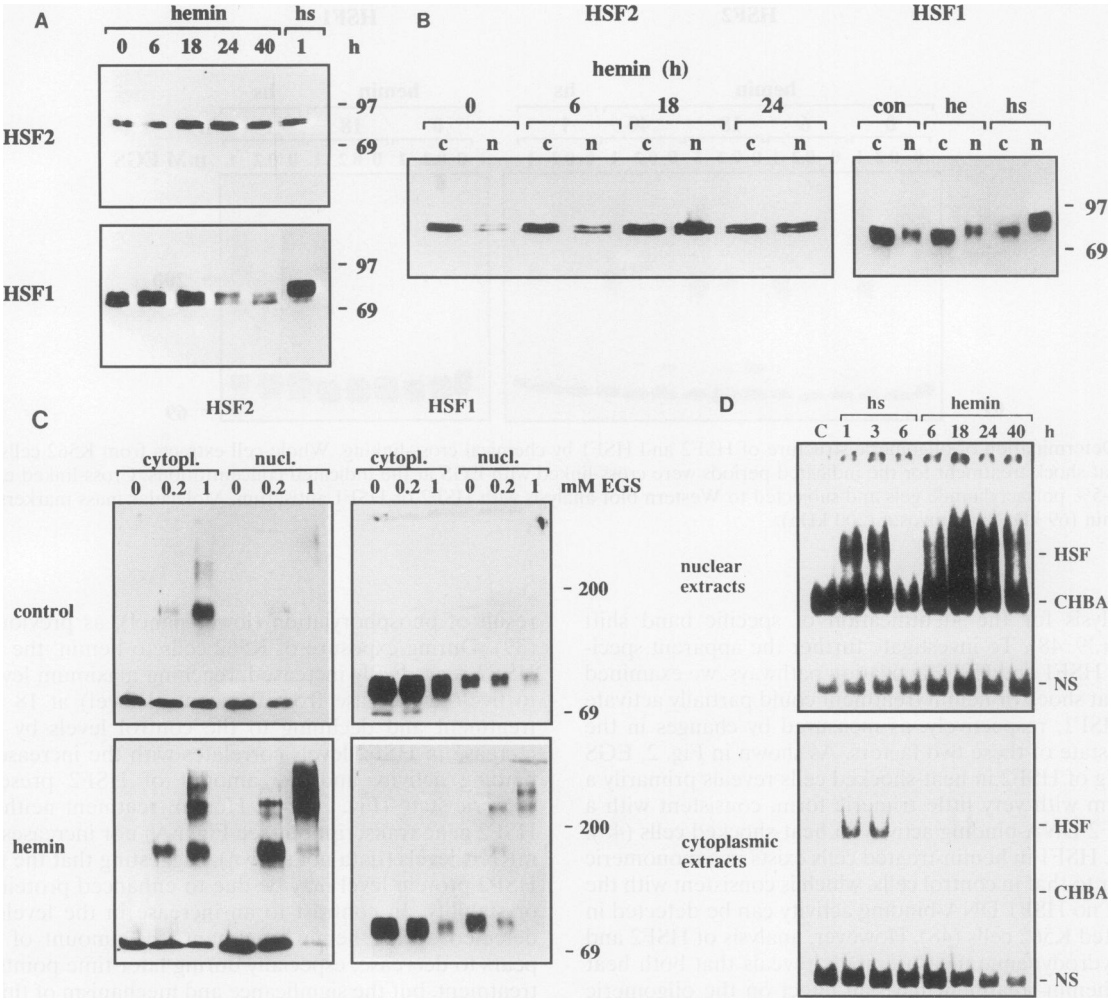

FIG. 3. Analysis of the size and subcellular localization of HSF2 and HSF1 in hemin-treated or heat-shocked K562 cells. (A) Analysis of HSF2 and HSF1 polypeptides. Whole-cell extracts from K562 cells exposed to 15 μM hemin or heat shock at 42°C for the indicated periods were run on SDS–8% polyacrylamide gels and subjected to Western blot analysis with HSF1 or HSF2 antiserum. Molecular mass markers are bovine serum albumin (69 kDa) and phosphorylase *b* (97 kDa). (B) Biochemical fractionation of HSF2 and HSF1. Cytoplasmic (lanes c) and nuclear (lanes n) extracts from untreated (lanes 0 and con), hemin-treated (he; 18 h of hemin treatment), and heat-shocked (hs; 1 h at 42°C) K562 cells were subjected to Western blot analysis. (C) EGS cross-linking of HSF2 and HSF1 in cytoplasmic and nuclear extracts isolated from untreated and hemin-treated (18 h) K562 cells. The molecular mass markers are as in Fig. 2. (D) DNA-binding analysis of HSF1 (heat shock) and HSF2 (hemin). Nuclear and cytoplasmic extracts from untreated cells (lane C) and cells exposed to either heat shock (hs) or hemin treatment for the indicated periods were examined by native gel shift assay. HSF-HSE complexes are indicated by HSF; CHBA denotes the constitutive HSE-binding activity reported previously (27); NS denotes nonspecific DNA-protein complexes.

electrophoretic mobility as a result of phosphorylation (Fig. 3B, right panel). These results confirm the previous report showing by indirect immunofluorescence and biochemical fractionation that the phosphorylated, heat-activated form of HSF1 is localized to the nucleus (9, 39).

We next examined the oligomeric state of the cytoplasmic and nuclear localized HSF2. Figure 3C (left panel) shows the results of EGS cross-linking of HSF2 in cytoplasmic and nuclear extracts isolated from K562 cells that were treated with hemin for 0 and 18 h. The cytoplasmic control form of HSF2 is a dimer, whereas in hemin-treated cells the cytoplasmic fraction contains both dimeric and trimeric forms of HSF2 and the nuclear fraction contains predominantly trimeric HSF2. In untreated K562 cells, HSF1 exists primarily as a monomer in both the cytoplasmic and nuclear fractions, whereas in hemin-treated cells the cytoplasmic HSF1 is entirely monomeric but a small amount of nuclear HSF1 is in the trimeric form (Fig. 3C, right panel). To determine whether subcellular compartmentalization of HSF1 and HSF2 correlates with their DNA-binding activities, we analyzed the cytoplasmic and nuclear extracts from heat-shocked and hemin-treated cells by gel mobility shift assay. HSE-binding activity induced by heat shock (HSF1) and hemin treatment (HSF2) is detected primarily in the nuclear extracts (Fig. 3D). Thus, although an appreciable amount of cytoplasmic HSF2 is detected by Western blot analysis (Fig. 3B), this form of HSF2 lacks DNA-binding activity.

In summary, these observations support our conclusion that the inactive non-DNA-binding form of HSF2 is located primarily in the cytoplasm as a dimer and, upon activation during

hemin treatment, undergoes trimerization, acquires DNA-binding activity, and accumulates in the nucleus.

Simultaneous treatment of K562 cells with hemin and heat shock leads to coactivation of HSF2 and HSF1 and synergistic induction of hsp70 gene transcription. The finding that HSF1 and HSF2 can be activated in K562 cells by two different treatments, heat shock and hemin, respectively, prompted us to examine whether these factors can be activated simultaneously and, if so, what the effect of coactivation is on heat shock gene transcription. Therefore, K562 cells were treated with 15 μM hemin for 20 h and then subjected to heat shock at 42°C for various periods, after which whole-cell extracts were analyzed by gel mobility shift analysis. As shown in Fig. 4A and quantitated in Fig. 4B, the combined treatment with hemin and heat shock results in a twofold increase in HSE-binding activity relative to that in cells treated with hemin alone. As is the case in cells exposed to heat shock alone, the HSE-binding activity attenuates during the continuous heat shock at 42°C and returns to the pre-heat-shock level by 4 h. These results show that despite the presence of high constitutive levels of HSF2 DNA-binding activity in hemin-treated cells, heat shock further induces the levels of HSE-binding activity.

To test whether the increase in HSE-binding activity is due to simultaneous activation of both HSF1 and HSF2 or to the effects of heat shock on HSF2, we examined the subcellular localization, oligomeric state, and DNA-binding activity of HSF1 and HSF2 in K562 cells exposed to combined treatment by hemin and heat shock. As shown by Western blot analysis of HSF2 and HSF1 in cytoplasmic and nuclear extracts, both factors are localized predominantly to the nucleus of hemin-pretreated heat-shocked cells (Fig. 5A). Furthermore, HSF1 is phosphorylated in hemin-pretreated heat-shocked cells, as has been observed in cells subjected to heat shock alone (Fig. 3A and 5A). EGS cross-linking of HSF2 and HSF1 in cytoplasmic and nuclear extracts isolated from hemin-pretreated heat-shocked cells reveals that both factors exist in a trimeric state in the nucleus (Fig. 5B). In addition, analysis of HSF1 and HSF2 DNA-binding activity by antibody perturbation assay suggests that both factors exist in the active, DNA-binding state in cells exposed to hemin and heat shock (data not shown). These results indicate that when K562 cells are subjected to hemin treatment followed by heat shock, both HSF1 and HSF2 are activated and display properties identical to those observed for HSF1 and HSF2 in cells exposed to either heat shock or hemin treatment, respectively.

We next examined whether the coactivation of HSF1 and HSF2 has any effect on the transcription of heat shock genes relative to activation by either HSF1 or HSF2 alone. Heat-shocked cells exhibit a strong and transient transcriptional activation of the hsp70 and hsp90 genes (Fig. 6A), and, as shown quantitatively in Fig. 6B, hsp70 gene transcription is maximally induced about 12-fold at 20 min, while hemin treatment results in only a 5-fold induction. These results confirm our previous observations that the hemin-mediated activation of HSF2 leads to a lower level of hsp70 transcription than that which occurs following the activation of HSF1 by heat shock (48). Surprisingly, when hemin-treated K562 cells are subsequently subjected to heat shock, the rate of hsp70 gene transcription is stimulated over 50-fold. These results indicate that coactivation of HSF1 and HSF2 in hemin-treated heat-shocked cells causes a synergistic induction of heat shock gene transcription, because the >50-fold activation observed is three times higher than the 17-fold induction which would be expected from an additive effect. Other characteristic features of the heat shock response are maintained in the hemin-pretreated heat-shocked cells; for example, the attenuation of

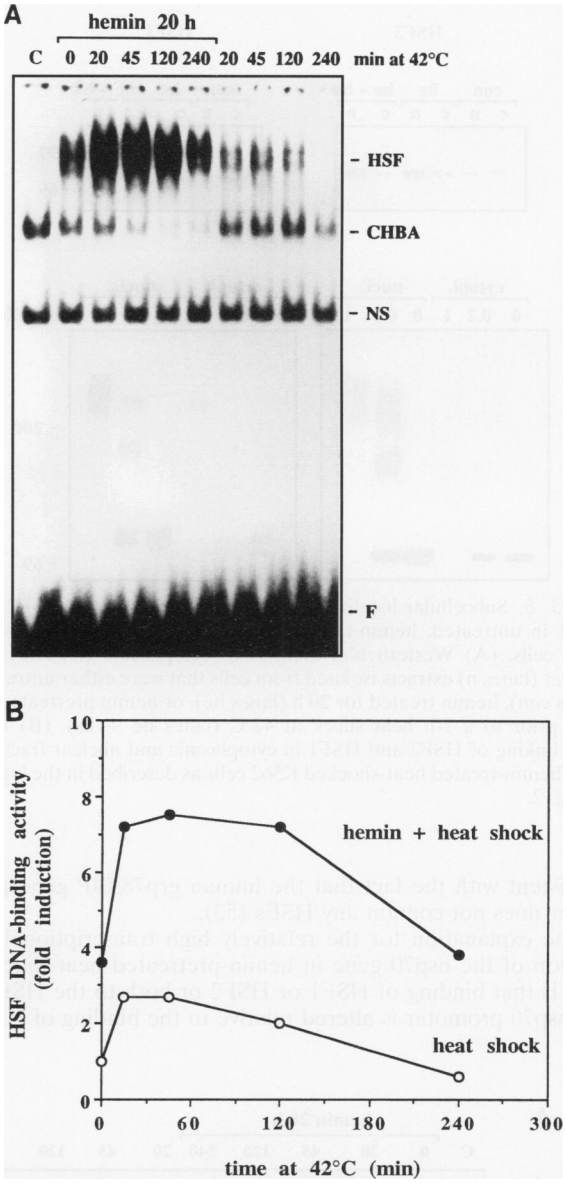

FIG. 4. (A) Induction of HSF DNA-binding activity by hemin, heat shock, and combined treatment with hemin followed by heat shock. The levels of HSF-HSE complex (marked HSF) were analyzed by gel mobility shift assay with whole-cell extracts prepared from K562 cells that were either untreated (lane C), hemin treated for 20 h (0), heat shocked, or pretreated with hemin for 20 h and then heat shocked for the indicated periods. CHBA and NS are as indicated in the legend to Fig. 3D. F denotes the free HSE oligonucleotide. (B) Graphic comparison of the levels of HSF DNA-binding activity between hemin-treated heat-shocked cells and cells exposed to heat shock alone. HSF-HSE levels were quantitated by using a Molecular Dynamics 400A PhosphorImager, and the HSF values were normalized against the respective NS values, which were presumed to be unaffected by the various treatments. Values for the fold induction of HSF levels are shown relative to the control level, which was arbitrarily assigned a fold induction value of 1.

hsp70 transcription during later time points of heat shock can still be observed, although it is delayed relative to that in non-hemin-treated cells. Transcription of the grp78/BiP gene, which is strongly induced by hemin treatment, does not show further enhancement during the entire period of heat shock,

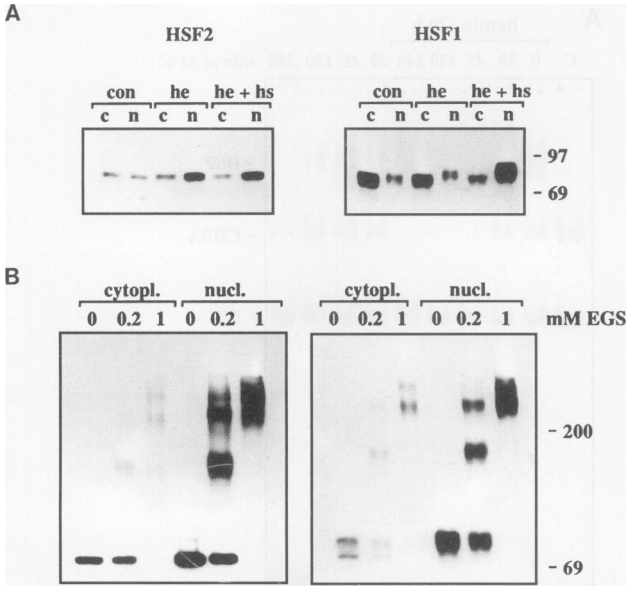

FIG. 5. Subcellular localization and oligomeric state of HSF2 and HSF1 in untreated, hemin-treated, and hemin-treated heat-shocked K562 cells. (A) Western blot analysis of cytoplasmic (lanes c) and nuclear (lanes n) extracts isolated from cells that were either untreated (lanes con), hemin treated for 20 h (lanes he), or hemin pretreated for 20 h prior to a 1-h heat shock at 42°C (lanes he + hs). (B) EGS cross-linking of HSF2 and HSF1 in cytoplasmic and nuclear fractions from hemin-treated heat-shocked K562 cells as described in the legend to Fig. 2.

consistent with the fact that the human grp78/BiP gene promoter does not contain any HSEs (53).

One explanation for the relatively high transcriptional induction of the hsp70 gene in hemin-pretreated heat-shocked cells is that binding of HSF1 or HSF2 or both to the HSE of the hsp70 promoter is altered relative to the binding of HSF1 in heat-shocked cells and to the binding of HSF2 in hemin-treated cells. To investigate this, we compared HSF-HSE interactions in hemin-treated, heat-shocked cells with those in either hemin-treated or heat-shocked cells by using in vivo genomic footprinting. The human hsp70 promoter contains two HSEs; the proximal HSE is composed of three perfect and two imperfect inverted repeats of the consensus sequence 5′-NGAAN-3′, and the distal HSE contains two perfect and four imperfect 5′-NGAAN-3′ sites. It has been previously shown by in vivo genomic footprinting that prior to heat shock, the HSEs of the hsp70 promoter are not occupied by protein and that following heat shock, all binding sites become occupied (2, 3, 48) (Fig. 7, lanes −). In hemin-treated cells, however, no protein-DNA interaction can be detected at site 1 of the proximal HSE (48) (Fig. 7, lanes 0/+), suggesting that HSF1 and HSF2, despite the ability of both factors to induce the transcription of classical heat shock genes, exhibit readily distinguishable footprints and thus do not bind to the proximal HSE in an identical manner. This difference in binding at site 1 of the proximal HSE has been confirmed in vitro by DNase I footprinting with purified recombinant mouse HSF1 and HSF2 proteins (20). On the basis of our earlier and present studies, we suggest that the diminished transcriptional activity of the hemin-induced HSF2 may be due to differences in its DNA-binding pattern in vivo.

A comparison of the methylation patterns of DNA from hemin-treated and heat-shocked cells (Fig. 7, lanes +) with those of the non-hemin-treated heat-shocked cells (Fig. 7, lanes −) reveals that the protection of G residues within the HSEs is more prominent at every time point in the samples from the hemin-pretreated cells. Moreover, the protections, now also observed at site 1, persist through 180 min of heat shock in samples from the hemin-pretreated cells, which is consistent with the slower attenuation of hsp70 transcription in these cells (Fig. 6A). Thus, the synergistic induction of hsp70 transcription during combined treatment of hemin and heat shock is correlated with alterations in the DNA-binding pattern.

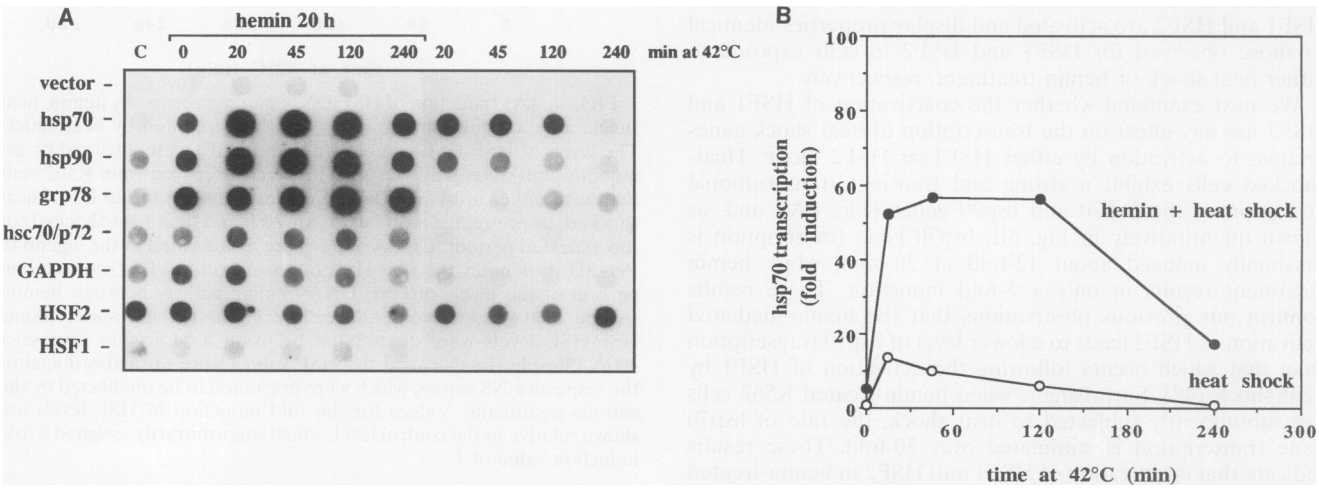

FIG. 6. Transcriptional activation of heat shock genes after exposure to hemin treatment, heat shock, or combined treatment of hemin and heat shock. (A) Nuclear run-on analysis of hsp70, hsp90/89a, grp78/BiP, hsc70/p72, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HSF2, and HSF1 gene transcription prior to (lane C) and 20 h after addition of hemin (lane 0) and/or exposure to 42°C for the indicated times. (B) Quantitative comparison of hsp70 gene transcription in hemin-treated heat-shocked cells and cells that were exposed to heat shock alone. The intensities of hybridization signals were determined by using a Molecular Dynamics 400A PhosphorImager, and the values for the fold induction of hsp70 gene transcription were normalized to the hsp70 transcription level in untreated cells (value set equal to 1).

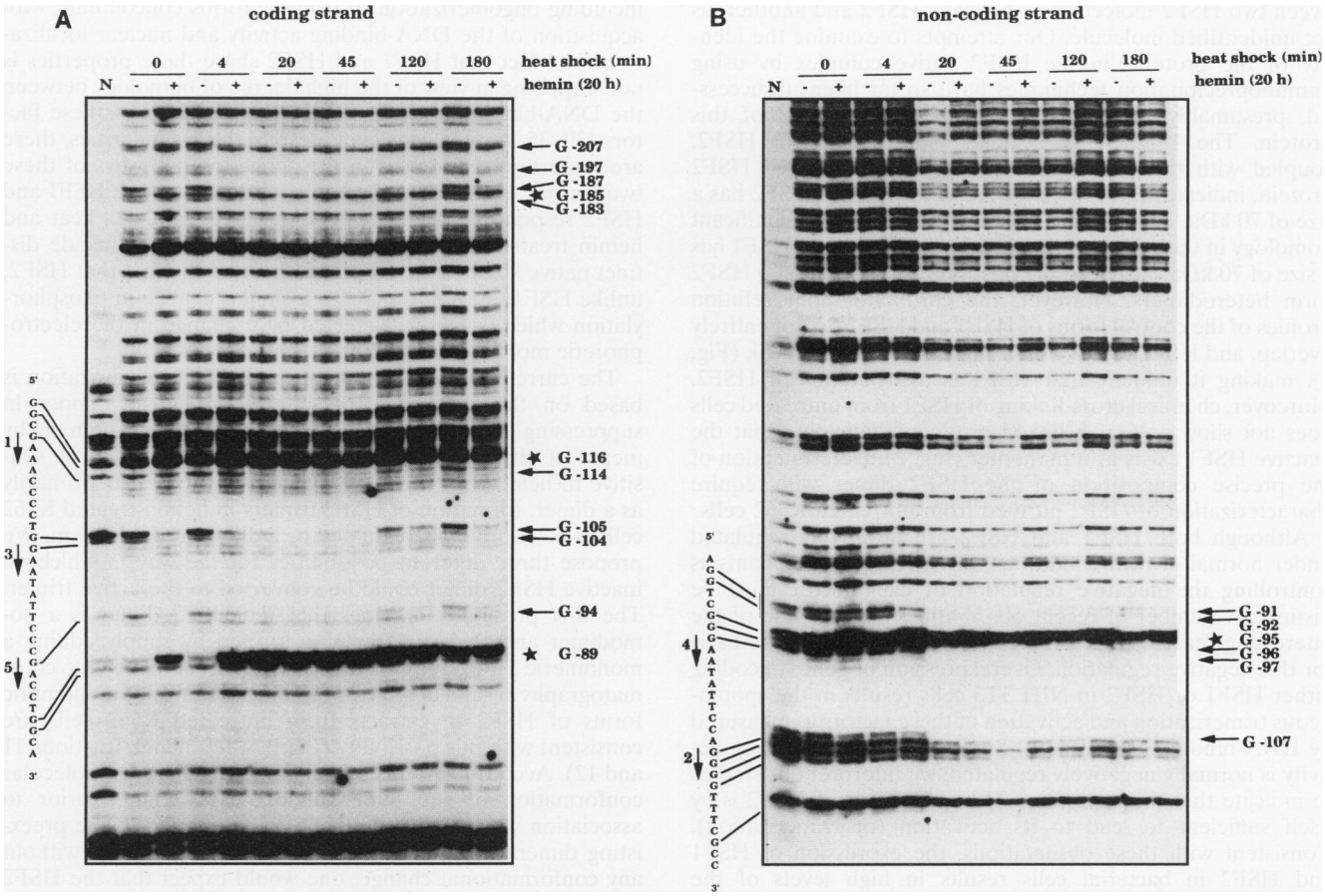

FIG. 7. In vivo footprinting of the human hsp70 promoter showing methylation patterns of the guanine (G) residues within the HSE regions for the coding (A) and noncoding (B) strands. Genomic DNA was analyzed from untreated control cells (lanes 0/−) and from cells that were either hemin treated for 20 h (lanes 0/+), heat shocked for the indicated times (lanes −), or hemin pretreated for 20 h and then exposed to heat shock for the indicated times (lanes +). Prior to DNA purification, the cells were treated with dimethyl sulfoxide in vivo except the naked DNA (N), which was treated with dimethyl sulfoxide in vitro. The sequences of the HSF-binding sites in the proximal HSE regions (around −100) are indicated and numbered to the left of each gel. Arrows indicate the G residues that are protected from methylation, and stars indicate the G residues that are hypersensitive to methylation.

DISCUSSION

One of the most important questions regarding the regulation of heat shock gene expression is the nature of the molecular mechanisms by which HSFs are converted to their active, DNA-binding forms capable of inducing heat shock gene transcription. In human cells two HSFs, HSF1 and HSF2, which bind to the same DNA recognition sequence have been identified. However, these factors are activated by distinct stimuli; HSF1 is responsive to classical stress signals, such as heat, heavy metals, and oxidative reagents, whereas HSF2 is activated during hemin-mediated differentiation of human K562 cells (9, 36, 39, 48). To elucidate the underlying mechanistic basis for the activation of these factors, we examined a number of biochemical and physical properties of both inactive and active forms of human HSF1 and HSF2. Among our findings, two observations stand out. First, our results demonstrate that the inactive, non-DNA-binding forms of HSF1 and HSF2 are distinct in their oligomeric states; the control form of HSF2 is dimeric, whereas the control form of HSF1 is monomeric. Second, HSF1 and HSF2 can be activated simultaneously in K562 cells, which results in a synergistic induction of heat shock gene transcription.

Native size of control HSF2. Using several methods, we show that HSF2 exists primarily as a dimer in untreated K562 cells. However, our results do not reveal whether HSF2 exists as a homodimer or a heterodimer. Determination of the precise nature of the HSF2 control form will be important for understanding the mechanism by which HSF2 is negatively regulated and kept in the inactive state under normal growth conditions. Nevertheless, the finding that HSF2 exists as a dimer in the cytoplasm of untreated cells, whereas the control form of HSF1 is monomeric, indicates that these two factors are kept in the inactive state by distinct mechanisms. Rabindran et al. (36) have recently identified sequences in HSF1 which are involved in the maintenance of this factor in the inactive, monomeric state. According to their model, a carboxyl-terminal leucine zipper associates with leucine zippers located in the amino-terminal region, which have been shown previously to function in the oligomerization of HSF to form trimers (33, 50). These intramolecular interactions are thought to mask the amino-terminal leucine zippers and suppress the trimerization of HSF1 which accompanies acquisition of DNA-binding activity (see below).

In contrast to HSF1, our results support a model for regulation of HSF2 activity in which this factor is maintained in the inactive, dimeric state by intermolecular interactions be-

tween two HSF2 molecules or between HSF2 and another, as yet unidentified molecule. Our attempts to examine the identity of the proteins in the HSF2 native complex by using immunoprecipitation techniques have so far been unsuccessful, presumably because of the low synthesis rate of this protein. The 140-kDa cross-linked size of control HSF2, coupled with the 70-kDa size of the un-cross-linked HSF2 protein, indicates that the protein associated with HSF2 has a size of 70 kDa. The fact that HSF2 and HSF1 share significant homology in their oligomerization domains and that HSF1 has a size of 70 kDa suggested the possibility that HSF1 and HSF2 form heterodimers. However, the chromatographic elution profiles of the control forms of HSF1 and HSF2 do not entirely overlap, and HSF1 elutes with a single well-defined peak (Fig. 1), making it unlikely that HSF1 is associated with HSF2. Moreover, chemical cross-linking of HSF1 from untreated cells does not show any cross-linked products, indicating that the inactive HSF1 exists as a monomer (Fig. 2). Determination of the precise composition of the HSF2 dimer will require characterization of HSF2 purified from untreated K562 cells.

Although both HSF1 and HSF2 are negatively regulated under normal growth conditions, the underlying mechanisms controlling the negative regulation of each factor must be distinct. A number of recent observations reveal some of the interesting features and problems in understanding the basis for this negative regulation. Overexpression of genes encoding either HSF1 or HSF2 in NIH 3T3 cells results in the spontaneous trimerization and activation of these factors as measured by DNA-binding activity (39). Since HSF2 DNA-binding activity is normally negatively regulated, we interpret our studies to indicate that the expression of higher levels of HSF2 is by itself sufficient to lead to its activation (or trimerization). Consistent with these observations, the expression of HSF1 and HSF2 in bacterial cells results in high levels of the functional trimeric states of each factor (20, 35, 44). Even when translated in reticulocyte lysates, HSF2 exhibits constitutive DNA-binding activity whereas the DNA-binding activity of HSF1 is inducibly regulated (30, 41). HSF1 exists as a monomer, yet HSF1 DNA-binding activity appears to be negatively regulated by heat shock proteins in vitro, perhaps through transient interactions (1). In addition, hsp70 has been shown to associate with activated HSF1 trimers, suggesting a further role for hsp70 in attenuation of the heat shock response (1, 10).

The native dimeric form of control HSF2 is reminiscent of another, well-studied transcription factor, NF-κB, which exists in the inactive form as a complex with an inhibitory protein, IκB, localized in the cytoplasm (8). Activation of NF-κB in response to extracellular signals, such as phorbol esters, inflammatory cytokines, UV light, and viral and bacterial proteins, involves disruption of the IκB–NF-κB complex, translocation of the NF-κB heterodimer composed of p50 and p65 subunits into the nucleus, and acquisition of DNA-binding activity (for reviews, see references 7 and 43). Another example of inhibitory mechanisms of transcriptional activators is provided by regulation of AP-1 activity. AP-1 is a leucine zipper protein whose constituents belong to the Jun and Fos protein families (for a review, see reference 6). The Fos proteins form heterodimers with the various Jun proteins, which are more stable than Jun homodimers and therefore exhibit higher DNA-binding activity. Deng and Karin (14) have recently shown that JunB can interact with c-Jun to form heterodimers, and in the absence of c-Fos, JunB acts as a repressor of AP-1 activity by attenuating transactivation of c-Jun.

Comparison of the activated forms of HSF1 and HSF2. Activation of HSF1 and that of HSF2 share several features, including oligomerization to trimeric forms concomitant with acquisition of the DNA-binding activity and nuclear localization. The fact that HSF1 and HSF2 share these properties is not surprising in view of the high degree of homology between the DNA-binding and oligomerization domains of these factors (30, 35, 41, 42). However, despite these similarities, there are important differences in the activation pathways of these two factors. The most fundamental difference is that HSF1 and HSF2 respond to distinct physiological signals, i.e., heat and hemin treatment, respectively. Other differences include distinct native sizes of the control forms and the fact that HSF2, unlike HSF1, does not undergo a similar change in phosphorylation which is readily detected by a change in the electrophoretic mobility of HSF1.

The current model for regulation of HSF1 trimerization is based on the role of carboxyl-terminal leucine zipper in suppressing the function of the oligomerization domain by means of intramolecular coiled-coil interactions that are sensitive to heat shock (36). Since inactive HSF2 exists primarily as a dimer, formation of HSF2 trimers in hemin-treated K562 cells is most probably regulated by a different mechanism. We propose three different possibilities for the way in which an inactive HSF2 dimer could be converted to the active trimer. The first possibility assumes that the HSF2 dimer is a homodimer and that the trimer is formed by simply adding a monomeric HSF2 subunit to the dimeric complex. Our chromatography results demonstrating the existence of monomeric forms of HSF2 in extracts from untreated K562 cells are consistent with this possibility (Fig. 1A, left panel, fractions 11 and 12). According to this model, it is likely that the molecular conformation of the dimer undergoes a change prior to association with an HSF2 monomer. Otherwise, if the preexisting dimers were able to associate with monomers without any conformational change, one would expect that the HSF2 dimers could undergo trimerization spontaneously in the absence of activating signals. However, our hydrodynamic studies reveal that the axial ratios of dimeric and trimeric HSF2 are quite similar, indicating that no dramatic conformational change occurs during the dimer-to-trimer transition, which makes this possibility less likely. The second possibility is that upon activation the HSF2 homodimer dissociates and the new pool of monomeric subunits associate to form trimers in a fashion similar to that suggested for HSF1 (36). The third alternative is based on an assumption that the HSF2 dimer is a heterodimer composed of HSF2 in association with an inhibitory protein. On activation the inhibitory molecule dissociates from the complex and releases the HSF2 monomer to interact with other HSF2 monomers, leading to formation of active trimeric complexes. To distinguish between these possibilities, we will need to characterize the dimeric and trimeric forms of HSF2 purified from untreated and hemin-treated K562 cells, respectively.

Sequestration of transcription factors in the cytoplasm of control cells followed by nuclear translocation on activation provides a control mechanism of transcription factor activity in eukaryotic cells. One of the best examples of this kind of regulation is the Rel-related family of transcription factors, which includes NF-κB, c-Rel, RelB and *Drosophila* dorsal (for a review, see reference 43). The inactive forms of these proteins are held in the cytoplasm, and in response to an exogenous stimulus they are activated and translocated into the nucleus. As mentioned above, NF-κB is retained in the cytoplasm in an inactive complex with the inhibitory protein IκB and cannot bind DNA. In response to various stimuli, IκB is phosphorylated and dissociated from NF-κB, and subsequently NF-κB DNA-binding activity is detected in the nu-

cleus. Recent studies indicate that the precursor for the p50 NF-κB subunit exhibits IκB-like activity by intramolecular masking of the nuclear localization signal (NLS) and dimerization domain of NF-κB (17, 37; for a review, see reference 13). An example of regulation of transactivation by intermolecular masking of NLS is provided by glucocorticoid receptor. The NLSs within the glucocorticoid receptor are nonfunctional in the absence of the activating hormone, because they are masked by the heat shock protein hsp90 (34, 38; for a review, see reference 47). Both HSF1 and HSF2 exist in the cytoplasm of untreated cells and, on activation, are translocated into the nucleus, where they bind to DNA, but the mechanism for nuclear translocation of these factors is unknown. Recently, Sheldon and Kingston (45) have demonstrated putative NLSs that are necessary for nuclear transport of HSF2 following activation and sequences within the amino-terminal leucine zippers that are required for cytoplasmic retention of the inactive HSF2. Since transfection of epitope-tagged HSF2 constructs was used for their study, it remains to be shown whether these sequences are functional in the activation process of endogeneous human HSF2. However, the model proposed in their work assumes that the control form of HSF2 is monomeric, similar to HSF1, and that the oligomerization domains are occupied through intramolecular interaction similar to that proposed for HSF1. Our results reveal, however, that HSF2 exists as a dimer in the control state, suggesting that the oligomerization domains may instead be shielded by intermolecular interactions. Future studies are needed to elucidate the mechanism by which the inactive form of HSF2 is held in the cytoplasm and translocated into the nucleus on exposure to the activation signal.

Our experiments demonstrate that the levels of HSF2 protein increase during hemin treatment, while HSF1 levels are unaffected by heat shock, indicating that HSF2 activity may be controlled at yet another level in hemin-treated K562 cells. The levels of AP-1, a heterodimer of the Fos and Jun proteins, increase in response to various growth-promoting stimuli as a result of increased transcription of *fos* and *jun* genes (for a review, see reference 19). In contrast, the increase in HSF2 protein levels is not correlated with induction of HSF2 gene transcription. Furthermore, HSF2 mRNA levels are not increased during hemin treatment, suggesting that the increase in HSF2 levels is regulated at the level of protein synthesis or stability or both. Interestingly, increases in HSF2 protein levels are observed primarily in the nuclear fraction, whereas the amount of cytoplasmic HSF2 remains nearly constant. We show also that only the HSF2 localized in the nucleus exhibits DNA-binding activity and exists in the trimeric state. These results, together with our previous studies showing that overexpression of HSF2 in transfected culture cells leads to constitutive DNA-binding activity (39), raise the possibility that the increase in HSF2 levels is a contributing factor in the HSF2 activation process.

Coactivation of HSF1 and HSF2 results in synergistic induction of hsp70 gene transcription. The fact that HSF1 and HSF2 can be activated in the same cells by distinct stimuli, i.e., when K562 cells are exposed to either heat shock or hemin, respectively, offers a unique model system in which the activation pathways of these two factors in the same cell type can be studied in vivo. Using this model system, we have demonstrated that HSF1 and HSF2 can be activated simultaneously and that this coactivation results in a synergistic induction of hsp70 gene transcription. These results reveal yet another level of complex regulation of heat shock gene expression and suggest that HSF1 and HSF2 can act together to induce transcription from the hsp70 gene promoter.

The in vivo footprinting of the HSE of the human hsp70 promoter shows that HSF1 and HSF2 interact with DNA with a slightly different pattern. The most striking difference is the lack of protection of site 1 of the proximal HSE in hemin-treated cells, whereas in heat-shocked cells all NGAAN sites within the proximal and distal HSEs are occupied. These results are consistent with results of in vitro DNase I footprinting analysis in which purified mouse HSF1 and HSF2 proteins were used and suggest that HSF1 and HSF2 have different affinities for the NGAAN repeats within the HSE of hsp70 promoter (20). Interestingly, HSF2 appears to be a weaker transcriptional activator both in vivo and in vitro (20, 48). Analysis of in vivo footprints from cells that were exposed to the combined treatment of hemin and heat shock reveals that the HSF-HSE interactions in these cells are modulated relative to the patterns found in cells exposed to either heat shock or hemin treatment. The pattern of the in vivo footprint in hemin-treated heat-shocked cells mimics that of HSF1 from cells exposed to heat shock alone, suggesting that the HSF2 bound to the HSEs of hemin-treated cells may be replaced by HSF1. However, the increased intensity and persistence of protections of the consensus G residues over long periods of heat shock indicate that the HSEs may be occupied by both HSF1 and HSF2 or by another, as yet unidentified factor. One possible explanation of the synergistic induction of hsp70 gene transcription is that HSF1 and HSF2 trimers simultaneously occupy the hsp70 promoter, in contrast to the situation in heat-shocked or hemin-treated cells in which the hsp70 promoter is occupied by two trimers of either HSF1 or HSF2, respectively (2, 20, 32, 48, 60). Alternatively, HSF1 and HSF2 subunits may associate to form mixed trimers with enhanced transcriptional properties relative to pure HSF1 or HSF2 trimers. A third possibility is that another, as yet unidentified factor interacts with either HSF1 or HSF2 trimers and modulates their transcriptional abilities.

The finding that HSF1 and HSF2 are each activated by distinct signals offers a rationale for the existence of a family of HSF proteins. Different HSFs allow cells to regulate heat shock gene expression not only in response to classical stress inducers but also under nonstress conditions. Although we do not yet know whether HSF2 activation during hemin treatment of K562 cells is directly involved in the differentiation process of these cells, HSF2 DNA-binding activity has recently been observed in a number of other biological systems, such as in certain mouse embryonic carcinoma cell lines and during spermatogenesis (29, 40). Interestingly, the common thread in all of these observations is that the cells exhibiting HSF2 DNA-binding activity are undergoing or are capable of undergoing differentiation, supporting the hypothesis that HSF2 is indeed involved in transcriptional regulation of heat shock genes or other genes during the process of differentiation or development. One of the surprising results of this study is that the distinct pathways of heat shock gene regulation can operate independently of each other, so that regulation of gene expression by HSF2 in a certain cell would not interfere with the ability of this cell to mount a heat shock response via activation of HSF1, which is critical for the ability of cells to survive physiological stress. A fundamental future goal is to determine the mechanistic features of these distinct regulatory pathways and to better understand their complementary roles in the regulation of heat shock gene expression.

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