The heat shock response is a defense reaction activated by proteotoxic damage induced by physiological or environmental stress. Cells respond to the proteotoxic damage by elevated expression of heat shock proteins (Hsps) that function as molecular chaperones and maintain the vital homeostasis of protein folds. Heat shock factors (HSFs) are the main transcriptional regulators of the stress-induced expression of hsp genes. Mammalian HSF1 was originally identified as the transcriptional regulator of the heat shock response, whereas HSF2 has not been implicated a role in the stress response. Previously, we and others have demonstrated that HSF1 and HSF2 interact through their trimerization domains, but the functional consequence of this interaction remained unclear. We have now demonstrated on chromatin that both HSF1 and HSF2 were able to bind the hsp70 promoter not only in response to heat shock but also during hemin-induced differentiation of K562 erythroleukemia cells. In both cases an intact HSF1 was required in order to reach maximal levels of promoter occupancy, suggesting that HSF1 influences the DNA binding activity of HSF2. The functional consequence of the HSF1-HSF2 interplay was demonstrated by real-time reverse transcription-PCR analyses, which showed that HSF2 was able to modulate the HSF1-mediated expression of major hsp genes. Our results reveal, contrary to the predominant model, that HSF2 indeed participates in the transcriptional regulation of the heat shock response.

The heat shock response enables the cell to cope with the deleterious effects of protein-damaging stresses, e.g. heat, heavy metals, and viral and bacterial infections. Characteristic of the heat shock response is a down-regulation of gene transcription and protein synthesis in general, whereas the transcription of a specific subset of genes called the heat shock genes is induced (1, 2). The classical heat shock genes include the hsp genes, but genome-wide analysis has revealed numerous other genes to be activated by heat and other stresses (3). During heat shock, the Hsps function as molecular chaperones that bind to and aid the folding of damaged proteins, thereby preventing protein aggregation under stressful conditions (1, 4, 5). The induction of Hsps is mainly regulated by a family of heat shock transcription factors (HSFs), which bind to the heat shock elements (HSEs) on heat shock genes and other target genes (6–9).

Four HSFs (HSF1–4) exist in vertebrates. HSF1 and HSF2 are the most studied factors because of their co-expression in most tissues and cell lines (9). HSF3 has been found only in avian species, and HSF4 is the most recently identified member in mammals and expressed predominantly in lens and brain (10–15). Mammalian HSF1 corresponds to the single HSF in yeast, nematode, and fruit fly (16–19) and is considered the bona fide stress-activated transcription factor. Mice lacking HSF1 and fibroblasts derived from these animals are sensitive to stress and do not develop thermotolerance or display induction of Hsps upon heat shock (20–22). HSF2 has been regarded as functionally distinct from the stress-activated HSF1, because no stress-related role for HSF2 was identified in the initial studies, where this role was attributed to HSF1 (23–25). The early studies utilized antibody supershift electrophoretic mobility shift assay (EMSA) to identify the factor that binds to the hsp70 HSE in response to different treatments; HSF1 was found to be the main factor responsible for the HSF-HSE signal upon heat shock, whereas HSF2 was more prominently activated upon hemin treatment (23–25). These results were the basis for the presumption that HSF1 is the sole stress-responsive factor, and HSF2 activity has been studied mainly during hemin-induced erythroid differentiation of K562 cells and during mouse embryonic development (23, 25–33).

HSF1 is activated within minutes of an increase in temperature. The activation is a multistep process, including monomer-to-trimer transition, localization to the nucleus, inducible phosphorylation, and binding to HSEs on both target gene promoters and repetitive chromosomal loci that serve as platforms for the formation of nuclear stress bodies (for reviews see Refs. 3

3 The abbreviations used are: Hsp, heat shock protein; HSE, heat shock element; HSF, heat shock factor; MEF, mouse embryonic fibroblast; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; Chip, chromatin immunoprecipitation; US, upstream; DS, downstream; RNAi, RNA interference; shRNA, short hairpin RNA; h, human; m, mouse; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

Heat Shock Factor 2 (HSF2) Contributes to Inducible Expression of hsp Genes through Interplay with HSF1*

Päivi Östling1,5, Johanna K. Björk5,6,1, Pia Roos-Mattjus1,6, Valérie Mezger1, and Lea Sistonen1,5,6

From the Departments of 1 Biochemistry & Pharmacy and 5 Turku Centre for Biotechnology, Åbo Akademi University and the 6 Laboratoire de Biologie Moléculaire du Stress, CNRS UMR8541, Ecole Normale Supérieure, 75005 Paris, France

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6, 7, 9, and 34). Similar to HSF1, HSF2 has been shown to translocate to punctuated structures in the nucleus of heat-shocked HeLa cells (35). Subsequently, we observed that during heat shock, HSF1 and HSF2 co-localize in the nuclear stress bodies, and a direct interaction between HSF1 and HSF2 during both control and heat shock treatment has been demonstrated (36, 37). Reporter assays have suggested that HSF2 is able to potentiate HSF1-mediated transactivation (37). However, evidence for a functional relationship between these two factors on endogenous promoters is still missing.

Our aim was to elucidate the specific contributions of HSF1 and HSF2 on hsp regulation during heat shock and hemin-induced erythroid differentiation of K562 cells, two conditions suggested to activate mainly HSF1 and HSF2, respectively. Using chromatin immunoprecipitation (ChiP), we show that both HSF1 and HSF2 occupy the hsp70 promoter in response to both heat shock and hemin. Complete knock-out or RNA interference (RNAi)-mediated knockdown of HSF1 markedly affected HSE binding activities, suggesting that HSF1 is able to either directly or indirectly modulate the DNA binding activity of HSF2. Furthermore, we show that an intact HSF1 DNA-binding domain is required to reach maximal levels of HSF2 DNA binding during both hemin-mediated differentiation and heat shock. On the transcriptional level, we demonstrate using real-time RT-PCR analyses on Hsf2−/− mouse embryonic fibroblasts (MEFs) that HSF2 is able to modulate expression of hsp70, as well as hsp25, hsp40, and hsp110, not only in response to heat shock but also during proteasome inhibition. This study provides novel evidence for a close relationship between the two well conserved transcription factors HSF1 and HSF2 in regulation of hsp expression during stress.

EXPERIMENTAL PROCEDURES

Cell Culture and Experimental Treatments—Human K562 erythroleukemia cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, l-glutamine, penicillin, and streptomycin. MEFs from Hsf1-deficient and wild-type mice (20) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 10 mM non-essential amino acids, 0.96 μl of β-mercaptoethanol/100 ml, l-glutamine, penicillin, and streptomycin. MEFs from Hsf2-deficient and wild-type mice (31) were immortalized using the plasmid 830Tt and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1.2 mM sodium pyruvate, l-glutamine, penicillin, and streptomycin. Hemin (Fluka) was used at a final concentration of 40 μM. Heat shock treatments were performed in a 42 °C (K562) or 43 °C (MEFs) water bath. The proteasome inhibitor MG132 (Peptide Institute Inc.) was used at a concentration of 10 μM for 5 h.

Chromatin Immunoprecipitation—The ChiP protocol was modified from Takahashi et al. (38). K562 and MEF cells were cross-linked with a final concentration of 1% formaldehyde followed by quenching with a final concentration of 125 mM glycine. Chromatin samples were fragmented by sonication with Bioruptor (Diagenode) to an approximate size of 500 bp. Immunoprecipitation was performed after preclearing with 50% slurry of protein G-coated Sepharose beads containing bovine serum albumin (100 μg/ml, Amersham Biosciences) at 4 °C overnight. The following antibodies were used: HSF1 (SPA-901, Stressgen); HSF2 (Fig. 1; clone 3E2, NeoMarker, Fig. 3; Ref. 24 and rabbit polyclonal antibodies specific to mouse HSF2 produced in the Sistonen laboratory (SFI57 and SFI58); acetylated histone H4 antibody (positive control, Upstate Biotechnology, 06-866); and normal rabbit serum (Jackson Immunoresearch Laboratories). Washing of immunocomplexes was performed three times in wash buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0), twice in wash buffer 2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0), and three times in wash buffer 3 (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 10% glycerol). Cross-links were reversed by incubating the samples overnight at 65 °C. DNA was purified with phenol:chloroform, and PCR analysis was performed on 10% of each ChiP sample using puRe Taq Ready-to-go PCR beads (Amersham Biosciences). The following primers were used (‘h’ for human, “m” for mouse): hHsp70.1 US, 5’-CCA TGG AGA CCA ACA CCC T-3’; hHsp70.1 DS, 5’-CCC TGG GCT TTT ATA AGT CG-3’; hβ-actin US, 5’-AAC TCT CCC TCC TCC TCT TC-3’, and hβ-actin DS, 5’-GAG CCA TAA AAG GCA ACT TTC GG-3’; mHsp70.1 US, 5’-CAC CAG CAC GTT CCC CA-3’, and mHsp70.1 DS, 5’-CGC CCT GCC CCT TTA AG-3’; mPK C US, 5’-GAG TGA CAC CTC ACA GCT GTG G-3’, and mPK DS, 5’-GCC AGG CCT TTG GAT CAT AGC C-3’ (39). All primers were used at a final concentration of 0.3 μM except those for mPKC that were used at 0.2 μM. The EtBr-agarose gel was scanned with FluorImager 958 (GE Healthcare), and the quantification was performed from three ChiP experiments. The signal for the nonspecific antibody control (αNS) was subtracted from all samples before quantification. The values represent -fold inductions as compared with the control sample, which was arbitrarily set to 1. The input lanes represent 1% of the material used in the immunoprecipitation assay.

Western Blot Analysis—Soluble cell extracts were prepared as described previously (40) and subjected to SDS-PAGE followed by transfer to nitrocellulose membrane (Protran nitrocellulose, Schleicher & Schuell). Proteins were detected with the following antibodies: HSF1 (41), HSF2 (24), Hsc70 (SPA-815, Stressgen), Hsp70 (SPA-810, Stressgen). Secondary antibodies were horseradish peroxidase-conjugated and purchased from Promega or Amersham Biosciences. The blots were developed with an enhanced chemiluminescence method (ECL kit, Amersham Biosciences).

RNAi by Small Hairpin RNA (shRNA)—The pSUPER vector (Oligoengine) was used for generating specific hairpin-loop RNA that is processed to functional shRNA in transfected cells (42). The pSUPER vector was ligated at BglII and HindIII restriction sites with a double-stranded 64-nucleotide oligonucleotide containing the unique 19-nucleotide sequence from the target transcript both in sense and antisense orientation, separated by a 9-nucleotide spacer sequence (TTCAAGAGA). The double-stranded oligonucleotides were specific for HSF1 (GCT CAT TCA GTT CCT GAT C) or HSF2 (CAG GGC AGT ACA ACA GCA T), and these sequences did not have any significant homology to the other HSFs or any other known genes using BLAST (43). The scrambled sequence (GCG CGC TTT...
GTATTTCCGGGTGAAG-3’ (1 μM); mHsp40 probe, 5’-FAM GGAGGAAG Q-3’ (0.15 μM); US, 5’-ACCGCTATGGAGAGGAAGG-3’ (0.4 μM); DS, 5’-GAGGTACCATGACACCACTCCCA-3’ (0.4 μM); mHsp110 probe, 5’-FAM CAGAGCCT Q-3’ (0.2 μM); US, 5’-ACCGCTATGGCGACAGAG-3’ (0.4 μM); DS, 5’-CCGGAGAAAAGATGTGACAC-3’ (0.4 μM); mGAPDH probe, 5’-FAM CAGAGAAGCTGGATGCGCCTC TAMRA-3’ (0.2 μM); US, 5’-TGACACCACTCTGTTAGT-3’ (0.3 μM); DS, 5’-GATGCAGGGATGATGTTCC-3’ (0.3 μM). The hHsp70.1, mHsp70.1, mHsp25.1, and mGAPDH probes were purchased from CyberGene, the hGAPDH probe from MedProbe, and the mHsp40 and mHsp110 probes from Roche Applied Science. Q stands for dark quencher dye (Roche Applied Science). Absolute QPCR ROX Mix (Advanced Biotechnologies Ltd.) was used to prepare the reaction mixes, and the PCR was performed with ABI Prism 7700 (Applied Biosystems). Relative quantities of the hsp mRNAs were normalized against GAPDH, and the fold inductions were determined. For rescue experiments equal amounts of mHSF2-α and mHSF2-β were reintroduced into Hsf2−/− MEFs with Lipofectamine 2000 (Invitrogen). Mock cells were transfected with an empty vector. On the following day cells were subjected to heat shock for 1 h, and hsp mRNAs were analyzed as described above.

RESULTS

Both HSF1 and HSF2 Are Recruited to the hsp70 Promoter—The binding of HSF1 and HSF2 to the proximal human hsp70.1 promoter (hereafter hsp70), as illustrated schematically in Fig. 1A, was analyzed in K562 erythroleukemia cells treated with either heat shock or hemin by ChIP. During hemin treatment we observed binding of HSF1 and HSF2 to the hsp70 promoter (Fig. 1B), which is in line with previous studies (23, 25, 36, 45–47). To our surprise, HSF2 was found on the hsp70 promoter also upon heat shock (Fig. 1B), which is in contrast to previous studies (23, 25, 36, 45–47). This finding was consistent in repeated experiments and is quantified in Fig. 1C.

DNA Binding Activity of HSF2 Is Affected by HSF1—To study the interplay of HSF1 and HSF2 on DNA, not only during the heat shock response but also during hemin-mediated erythroid differentiation of human K562 cells, we employed RNAi down-regulation with the pSUPER vector-based method (42). We chose 19-nucleotide targeting sequences that down-regulate either HSF1 or HSF2 in addition to a scrambled control to monitor that the short hairpin did not affect the results. Transient transfections with the specific constructs demonstrated strong and specific down-regulation of HSF1 (Fig. 2A, lane 2) or HSF2 (lane 3), and the scrambled control did not affect the levels of either factor (lane 1).

HSE binding activity was examined by EMSA, and as expected, both heat shock and hemin treatment induced a prominent HSE binding activity in cells transfected with the shRNA control vector (Fig. 2B, lanes 2 and 3). Upon heat shock, the HSE binding activity was markedly diminished when HSF1 was down-regulated (Fig. 2B, lane 5), whereas HSF2 down-regulation did not markedly affect the heat-induced HSF-HSE complex (lane 8). This result corresponds to previous supershift EMSA results, suggesting that HSF1 is the dominant factor upon heat shock (23–25).
HSF2 Requires HSF1 DNA Binding Activity to Be Able to Participate in HSF-HSE Complex Formation—Our results from K562 cells indicated that both HSF1 and HSF2 are able to bind HSEs during heat shock. However, their stoichiometry on DNA cannot be determined by the EMSA assay (48). To resolve the individual contributions of HSF1 and HSF2 to the HSE-binding complex and whether HSF1 indeed regulates the participation of HSF2, we performed in vitro DNA binding studies using biotin-mediated oligonucleotide pulldown assays with MEFs derived from mice deficient in HSF1 (Fig. 3A, left panel) (20). The oligonucleotides consisted either of an HSE sequence or a scrambled control sequence. Hsf1+/−/ MEFs displayed binding of both HSF1 and HSF2 to the HSE during heat shock (Fig. 3B). The stress-induced DNA binding ability of HSF2 was severely hampered in the absence of HSF1 (Fig. 3B, left panel), which is in line with the EMSA assay on heat-shocked K562 cells with shRNA-mediated down-regulation of HSF1 (Fig. 2). To investigate whether interaction with HSF1 would be sufficient to rescue HSF2 DNA binding activity, we used the same experimental setup as described for Fig. 2D and transfected back either wild-type HSF1 or the R71A mutant that is able to trimerize but not bind HSEs. In line with the results shown in Fig. 2D, only the wild-type HSF1 was able to rescue the HSE binding activity of HSF2 (Fig. 3B, right panel), suggesting that the DNA binding activity of HSF1 is required to enable HSF2 to participate in the HSE-binding complex.

Heat-inducible Binding of HSF2 to the Endogenous hsp70 Promoter Is Lost in the Absence of HSF1—To analyze the dependence of HSF-HSE complex formation on HSF1 in vivo, we performed ChIP analyses in MEFs lacking either HSF1 or HSF2 (Fig. 3A) (20, 31). Both HSF1 and HSF2 occupied the murine hsp70 promoter in heat-shocked Hsf1+/−/ and Hsf2+/−/ MEFs (Fig. 3, C and D), which is in line with our results from human K562 cells (Fig. 1B) and the biotin-mediated oligonucleotide pulldown assays on MEFs (Fig. 3B). In cells lacking HSF1 (Hsf1−/−), HSF2 could not be detected on the proximal hsp70 promoter (Fig. 3C), demonstrating that also in vivo the DNA binding activity of HSF2 upon heat shock seems to depend on HSF1. The finding was not the result of an antibody-specific effect, because the same results were obtained using several different HSF2 antibodies (data not shown). Strong binding of HSF1 was observed in both Hsf2+/−/ and Hsf2−/− MEFs upon heat shock (Fig. 3D).

HSF1 Is the Major Transcriptional Activator of hsp70, but HSF2 Modulates Its Hemin-inducible Expression—Because binding of HSF1 and HSF2 to the hsp70 promoter was...
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FIGURE 2. A, Western blot analysis of K562 cells transiently transfected with control, HSF1, or HSF2 shRNA constructs with HSF1, HSF2, and Hsc70 antibodies. B, K562 cells were transiently transfected with control, HSF1, or HSF2 shRNA and were left untreated (C), heat-shocked (HS, 42 °C, 1 h), or hemin-treated (He, 16 h, 40 μM). Whole cell extracts were analyzed by EMSA using a radiolabeled probe corresponding to the proximal HSE from the hsp70 promoter. HSF-HSE represents the specific DNA-binding complex, CHBA stands for constitutive HSE binding activity reported previously (40, 55), and NS indicates nonspecific DNA-protein complexes. C, K562 cells were transiently transfected with control, HSF1 shRNA (HSF1) in addition to RNAi-resistant silently mutated wild-type HSF1 (+HSF1*) or RNAi-resistant HSF1 mutated on arginine 71 to alanine (+R71A), which is unable to bind DNA. Western blot analysis was performed with antibodies against HSF1, HSF2, and Hsc70. D, EMSA analysis of samples prepared as in C, using the same probe as in B.

detected in K562 cells exposed to hemin treatment, in both ChIP and EMSA analyses, we analyzed their specific contributions to hsp70 expression. The expression of human hsp70 was analyzed by real-time RT-PCR in hemin-treated K562 cells with either scrambled control or transiently down-regulated HSF1 or HSF2. Our results show that, in addition to the dramatically decreased amounts of hsp70 mRNA when HSF1 was down-regulated, HSF2 down-regulation also diminished hemin-induced hsp70 mRNA (Fig. 4A). Similar results were obtained on the protein level with down-regulation of HSF1 displaying a more prominent reduction in Hsp70 protein than knockdown of HSF2 (Fig. 4B, lanes 5 and 6 versus 8 and 9).

Both HSF1 and HSF2 Contribute to the Transcriptional Regulation of hsp Genes upon Stress—The importance of HSF1 for hsp expression during the heat shock response is evident from HSF2 (Fig. 5B). The hsp genes display distinct activation kinetics (1), and because hsp40 and hsp110 do not display activation by a 1-h heat shock, the results for these two genes are shown only for the recovery phase after heat shock and for proteasome inhibition by MG132 (Fig. 5A). The increased levels of hsp40 and hsp110 mRNAs in Hsf2−/− MEFs in both the recovery phase after heat shock and during proteasome inhibition, suggest that HSF2 is able to repress gene expression driven by these two promoters (Fig. 5B).

To further investigate the role of HSF2 in stress-inducible hsp expression, we included hsp25, hsp40, and hsp110 in the real-time RT-PCR analyses of cells exposed to the same treatments as shown in Fig. 5A. In the absence of HSF2, the induction of hsp25 mRNA was only slightly reduced during a 1-h heat shock, whereas this effect was not apparent in the recovery phase (Fig. 5B). During MG132 treatment the absence of HSF2 rather increased the levels of hsp25 mRNA compared with the wild-type MEFs, indicating an inhibitory role for HSF2 earlier studies (20, 21, 49), whereas the potential impact of HSF2 on stress-inducible hsp gene expression is unclear. Therefore, we first performed real-time RT-PCR analysis of hsp70 expression in Hsf2+/+ and Hsf2−/− MEFs subjected to various forms of stress. The cells were exposed to a 1-h heat shock or a 0.5-h heat shock followed by a 3-h recovery or were treated for 5 h with the proteasome inhibitor MG132. In all three treatments, the hsp70 mRNA induction was diminished in Hsf2−/− MEFs as compared with Hsf2+/+ MEFs (Fig. 5A), clearly demonstrating that HSF2 participates in the transcriptional regulation of hsp70 under stressful conditions. Because hemin has been suggested to mediate its effect also through proteasome inhibition in certain cells (50), we treated MEFs with hemin. However, no stress-induced activation was observed (data not shown).

To determine whether the observed effects on hsp genes in Hsf2−/− MEFs were due to the lack of HSF2, we reintroduced HSF2 to these cells by transient transfections. In Fig. 6, a selection of the stress conditions used in Fig. 5 are shown, and the results demonstrate that the normal stress-inducible expression patterns of the different hsp genes can efficiently be restored by exogenous HSF2 (Fig. 6). These results confirm that
HSF2 indeed contributes to the transcriptional regulation of multiple *hsp* genes under stressful conditions.

**DISCUSSION**

There is unambiguous genetic evidence provided by the HSF1 knock-out mouse models that HSF1 is essential for the heat shock response (20–22, 49). In contrast, the role of HSF2 in the heat shock response has remained unresolved (51, 52). We report here that upon exposure to heat stress, HSF2 binds to chromatin on the *hsp70* promoter and modulates the expression. In addition to heat shock, both HSF1 and HSF2 are recruited to the *hsp70* promoter to transactivate the gene during hemin-mediated erythroid differentiation of K562 cells. Furthermore, HSF2 modulates HSF1-mediated regulation of other *hsp* genes, such as *hsp110, hsp40* and *hsp25*, during heat shock and proteasome inhibition, clearly demonstrating a functional role for HSF2 in the stress response. Moreover, the stress-induced DNA binding ability of HSF2 seems to be modulated by HSF1, proposing a close functional relationship for these two well conserved transcription factors.

The results from the two loss-of-function model systems used in our study suggest that the amount of HSF-HSE complex formation is determined by HSF1 (Figs. 2 and 3). The depletion of HSF1 severely decreased the amount of HSE binding activity in EMSA, biotin-mediated oligonucleotide pulldown, and ChIP analyses. The formation of the HSE-binding complex requires an intact HSF1 DNA binding activity, because the HSF1 R71A mutant could not rescue the stress-induced HSF-HSE complex in cells with normal levels of HSF2. The exact composition of the HSF-HSE complex cannot be determined by EMSA assays (48), but our ChIP analyses from K562 cells suggest that similar levels of HSF1 occupy the *hsp70* promoter during hemin and heat treatments. Similarly, no marked difference in the levels of HSF2 binding could be detected under these conditions (Fig. 1, B and C). However, the stoichiometry of individual HSFs in the HSE-binding complex, *i.e.* the specific amounts of HSF1 and HSF2 binding on endogenous promoters, remains to be established.

An elegant control mechanism for the HSF1-HSF2 interplay on different promoters could be through formation of heterocomplexes. Because of the highly homologous trimerization domains, it is plausible that HSF1 and HSF2 could form either homo- or heterotrimers *in vivo*. Recent results published by Loison et al. (53) indicate that heterocomplexes can occupy the *clusterin* promoter. In response to proteotoxic insults, caused either by the proteasome inhibitor MG132 or by incorporation of the amino acid analog azetidin, both HSF1 and HSF2 bind to the *clusterin* promoter. The promoter contains two canonical and one non-canonical nGAAn pentamer, suggesting occupancy by one HSF trimer, which in turn implies binding of a heterocomplex. The probe used in our biotin-mediated oligonucleotide pulldown assay contains a consensus HSE composed of three pentameric sequences, indicating binding of a heterocomplex. The formation of heterotrimers and higher order homo-oligomers or mixtures of hetero-oligomers *in vivo* on DNA is conceivable, as the composition of HSEs on different promoters varies (47, 54). The human *hsp70* promoter used in our study contains a proximal and a distal HSE composed of five and six nGAAn pentamers, respectively, and has been sug-

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![FIGURE 4. A, real-time RT-PCR analysis of hsp70 expression in K562 cells with either scrambled control or transiently down-regulated HSF1 or HSF2 treated with hemin (16 h, 40 μM). Relative quantities of hsp70 mRNA were normalized to GAPDH, and all reactions were made in duplicates using samples derived from at least three biological repeats. Error bars indicate ± S.D. The relative expression was calculated from the scrambled control, which was arbitrarily set to 100. B, samples prepared as described in A were treated with hemin (He, 40 μM) for the indicated times. Western blot analysis was performed with antibodies against HSF1, HSF2, Hsp70, and Hsc70.

A, Western blot analysis of HSF1, HSF2, and Hsc70 in Hsf1+/+ and Hsf1−/− MEFs (left panel) and Hsf2+/+ and Hsf2−/− MEFs (right panel). B, biotin-mediated *in vitro* oligonucleotide pulldown assays. Extracts from heat-shocked (15 min, 43 °C) wild-type (Hsf1+/+) and Hsf1−/− MEFs (left panel) were incubated with a biotinylated HSE oligonucleotide (b) or a scrambled control (s). After the binding reaction, unbound proteins were removed by washing. The bound fraction was released by the addition of denaturing buffer and analyzed by Western blotting using HSF1 and HSF2 antibodies. Right panel, Hsf1+/+ MEFs were transfected with empty vector, wild type, or a DNA-binding deficient HSF1 mutant (+/R71A) and heat-shocked (15 min, 43 °C). Oligonucleotide pulldowns were performed as described for the left panel. Input samples are shown on the right. C and D, ChIP analyses of the recruitment of HSF1 (αHSF1) and HSF2 (αHSF2) to the murine *hsp70* promoter. Wild-type (Hsf1+/+) and Hsf1−/− MEFs were either left untreated (C, control) or heat-shocked (HS, 43 °C, 1 h) before cross-linking and immunoprecipitation. Nonspecific antibody (αNS) did not pull down the promoter fragments, whereas acetylated histone H4 was found on the *hsp70* promoter (+). The input lanes represent 1% of the material used in the ChIP assay. No significant recruitment of HSF1 or HSF2 was observed on the phosphoenolpyruvate carboxykinase (PCK) promoter, and the weak signal detected on the PCK promoter was subtracted during quantification. Quantifications are shown in the lower panels. The percent input indicated for each sample has the nonspecific antibody control value subtracted to account for general background. Input values were arbitrarily set to 100%.
gested to be occupied by several trimers (55). Thus, HSF1 and HSF2 binding on the hsp70 promoter could be envisioned to occur either on separate HSEs through homotrimerization or on both HSEs through formation of heterocomplexes. Further characterization of target gene promoters will elucidate whether they depend on the activity of a single HSF or whether heterocomplex formation is a common denominator in HSF-mediated transcription.

Distinct heterocomplexes formed on different promoters might provide an efficient way of integrating the functions of mammalian HSFs in response to a plethora of physiological and environmental stimuli. By forming heterocomplexes with different compositions, a gradient of transactivation could be achieved. The transcriptional capacity of HSF1 in reporter assays is stronger than that of HSF2, and functional interaction on the level of transactivation had been studied previously only in reporter assays (37, 56). Our results regarding the role of HSF2 on the heat-induced activation of hsp70 promoter are in line with the reporter assays performed by He et al. (37), who reported that overexpression of the HSF2-α isoform in the presence of HSF1 enhanced heat-induced hsp70B′ promoter activity. Our study expands beyond the previous ones by demonstrating the modulatory role of HSF2 on multiple endogenous hsp promoters under diverse stressful conditions. We demonstrate that HSF2 participates in the heat-induced expression of hsp70 and hsp25, which earlier was considered to require only HSF1 (52). Importantly, HSF2 seems also to be involved in repression of gene expression during stress, because the stress-inducible expression of hsp40 and hsp110 was lower in the presence of HSF2 than in its absence (Figs. 5 and 6).

The modulation of hsp expression by HSF2 differs from the previously reported Northern blotting analyses of hsp expression in heat-shocked Hsf2−/− MEFs that did not show any differences between wild-type and Hsf2−/− cells (52). The different results could arise from the fact that the MEFs used in the two studies originate from mice with different genetic background. Unlike McMillan et al. (52), who used primary MEFs, our study was conducted with immortalized MEFs. The additive effects of HSF2 on hsp70 might be a characteristic of transformed cells, because potentiating effects of HSF2 on HSF1-mediated transcription have been observed in immortalized MEFs, K562 (this study), and mouse erythroleukemia cells (37). Hsp70 and other Hsps are known to act as potent survival proteins protecting cells against numerous death-inducing stimuli (57), and perhaps HSF2, in addition to HSF1, should be considered as an important regulator in diseases such as cancer and neurodegeneration, where dysregulation of molecular chaperones is evident (58, 59).

The hemin-mediated erythroid differentiation of K562 cells and the heat shock response represent two different modes of HSF activation, hemin-mediated differentiation being a slow process that requires several hours, whereas heat shock induces
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To date, the presumption of HSF1 being the sole stress-responsive factor has served as a base for most studies on the heat shock response, whereby the putative effects of HSF2 might have been overseen. Our study provides novel evidence that HSFs are able to function together both in the hemin-induced erythroid differentiation of K562 cells and during heat stress. Notably, HSF1 has been proposed as a target for different therapeutic strategies (61). Given the results obtained in this study, HSF1 and HSF2 exhibit a functional interdependency, which is most important to be taken into consideration when planning further HSF-targeted therapies.

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