HSF2 autoregulates its own transcription

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Abstract. Heat shock factor 2 (HSF2) is one of the most important regulators affecting stress mechanisms, and is frequently amplified in the ubiquitin proteasome pathway. Despite its significance, the mechanisms which regulate HSF2 expression remain unclear. In the present study, we describe the existence of a negative autoregulatory mechanism of HSF2. Transfection assays demonstrated that HSF2 decreased endogenous HSF2 mRNA expression in human K562 erythroleukemia cells. Luciferase reporter assays revealed that HSF2 inhibited the activity of its own promoter in a dose-dependent manner and that the downstream region (-1.5 kb) relative to the transcription start site was responsible for this inhibitory effect. Furthermore, chromatin immunoprecipitation (ChIP) assat indicated that HSF2 is directly recruited onto its own promoter, which contains a putative heat shock element (HSE). Collectively, the findings of our studys suggest that HSF2 contributes to its own expression by forming a negative autoregulatory loop.

Introduction

In response to various stimuli under conditions of physiological stress, heat shock transcription factors (HSFs) regulate the dynamic expression of various heat shock proteins (HSPs), which are responsible for the subsequent downstream effects, including stress-related cytoprotective events, the folding and assembly of nascent polypeptides and the intracellular transport of proteins (1-4).

Heat shock factor 2 (HSF2), which belongs to the HSF family, has been proven to play a key role in regulating the ubiquitin proteasome pathway and differentiation (1,2,5). HSF2 is abundantly expressed and is activated in stem cells and embryonic carcinoma cells and also during embryogenesis and spermatogenesis (1,6,7). The transcription of HSF2 is complicated by the existence of two isoforms, HSF2-α and HSF2-β, which are generated by alternative splicing events. The ratio of HSF2-α and HSF2-β isoforms varies significantly between different adult tissues, such as the brain, heart and testes, suggesting that these two proteins are functionally distinct (1,4). Similar to heat shock factor 1 (HSF1), HSF2 was recently found to be activated during heat shock and its expression is induced upon exposure to proteasome inhibitors; it was also reported that its deficiency increased the sensitivity of vertebrate cells to heat shock (8-11). *Hsf2*$^{-/-}$ mice have a male hypofertile phenotype that is characterized by reduced testis size and brain abnormalities associated with enlarged ventricles (6,12). Although HSF2 typically functions as a transcription factor, it also induces gene bookmarking, such as for the hsp70i gene, as demonstrated in mitotic cells (13). Additionally, HSF2 modulates the expression of heat shock genes by interacting directly with HSF1 or heat shock factor 4 (HSF4) (14-17). However, little is known concerning the exact transcriptional regulation of HSF2 during cellular processes.

To the very best of our knowledge, in the present study, we provide the first direct evidence that HSF2 transcription is inhibited and regulated by an autoregulatory mechanism through regions of its own promoter, thus providing a novel mechanism responsible for the regulation of HSF2 through various cellular signals.

Materials and methods

Cell culture and reagents. Human K562 erythroleukemia cells [from the Americal Type Culture Collection (ATCC), Manassas, VA, USA] were cultured in a 5% CO_2 atmosphere at 37˚C in RPMI-1640 (Sigma-Aldrich, Co. Wicklow, Ireland) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA) and antibiotics (penicillin 10,000 U/ml, streptomycin 10,000 μg/ml; WelGENE Inc, Daegu, Korea). HEK293 embryonic kidney cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% (v/v) FBS. Hemin

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Abbreviations: HSF2, heat shock factor 2; HSF1, heat shock factor 1; HSE, heat shock element; ChIP, chromatin immunoprecipitation; TAT PDT, protein transduction domain of Tat

Key words: heat shock factor 1, heat shock factor 2, autoregulation, heat shock element, heat shock factor 2 promoter

was purchased from Sigma. Wild-type, *Hsf1*$^{-/-}$ and *Hsf2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were maintained in DMEM with 10% FBS. Wild-type MEFs and *Hsf1*$^{-/-}$ MEFs were a gift from Ivor Benjamin (University of Texas Southwestern Medical Center, Dallas, TX, USA). *Hsf2*$^{-/-}$ MEFs were kindly provided by Dr Valérie Lallemand-Mezger (Paris Diderot University, Paris, France).

Plasmid constructs. Human HSF1 and HSF2 coding regions were generated by PCR amplification and subcloned into the pcDNA3 plasmid (Invitrogen™, Carlsbad, CA, USA). The HSF2 promoter (pGL3-HSF2-luc-P1, -2.68 kb) was constructed by PCR, with human genomic DNA as the template. The HSF2 promoter was PCR-amplified using the following primers: forward, 5'-CTAGCT AGCGCCAGTAGCATCTGCGT CATCT-3', and reverse, 5'-AGCTCATTAGCCAAATGCA TGAGCCTC-3'. The deleted HSF2 promoters were PCR-amplified using the following forward primers: pGL3-HSF2-P2, 5'-GGAAAGGGCACATACTTTTGAG CTC-3'; pGL3-HSF2-P3, 5'-CTAGCTAGCACTCTCCCATTTAC TTGCTGTGACTG-3'; and pGL3-HSF2-P4, 5'-CTAGCT AGCCTAGTTCATTGGGTTGTTGTGAGGATTC-3'. The reverse primer was 5'-AGCTCATTAGCCAAATGCATG AGCCTC-3'. pGL3-HSF2-P5 was created by the *Hin*dIII digestion of pGL3-HSF2-P1. Luciferase reporter assays were performed as previously described (16). Briefly, the wild-type, Hsf1$^{-/-}$ and Hsf2$^{-/-}$ MEFs were grown in 12-well plates and then co-transfected with HSF2 promoter-luciferase plasmid DNA and either with pcDNA3-HSF1 or -HSF2. After 48 h of transfection, cell lysates were analyzed for luciferase activity. The luciferase reporter assays were performed using the Dual-Luciferase Reporter assay system (Promega, Madison, WI, USA) following the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was prepared using TRIzol reagent (Invitrogen). RNA (1 μg) was treated with DNase (Promega) and reverse transcribed using the Maxime RT PreMix (iNtRON Biotechnology, Seongnam-si, Korea). The following primers were used for RT-PCR: hHSF2-ORF-forward, 5'-TAGA GAACCCACTGCTTACTGG-3' and hHSF2-ORF-reverse, 5'-GTTGCTCATCCAAGACCAGAA-3'; hHSF2-endo-forward, 5'-CCCCAGGAAGTGGACTTTACATGTA-3' and hHSF2-endo-reverse, 5'-TATGGAGCTGGAACCCTATCA GACA-3'. The GAPDH RT-PCR primers were: forward, 5'-AGCCAAAAGGGTCATCATCTCTGC-3' and reverse, 5'-GCATTGCTGATGATCTTGAGGCTG-3'. GADPH was used as an internal control. The following primers were used for quantitative (real-time) PCR: hHSF2-ORF-forward, 5'-ATTCAGAGTGGAGAGCAGAATG-3' and hHSF2-ORF-reverse, 5'-CTG GACAGCACTAGACATGAGA-3'; hHSF2-endo-forward, 5'-CCGCGTTAACAATGAAGCAG-3' and hHSF2-endo-reverse, 5'-CATTCTGGCTCCAGGTG ATG-3'. After the reaction mixture was loaded into a glass capillary tube, the following cycling conditions were used: initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 55˚C for 30 sec and a final extension at 72˚C for 10 sec. In the final cycle, the melting curve was obtained by initially heating to 95˚C and subsequently cooling to 40˚C for 30 sec. Our method was optimized for the relative quantification module of LightCycler software, version 4.0.

Western blot analysis. The cells were treated with 30 μM hemin for 24 h or transiently transfected with the pcDNA3 plasmids containing HSF1 or HSF2. The cells were then washed with PBS and harvested in lysis buffer. Samples containing equal amounts of protein were loaded into each lane of an SDS-polyacrylamide gel for electrophoresis and subsequently transferred onto polyvinylidene difluoride membranes. The membranes were blocked and then incubated with the following antibodies: antibodies against HSF1 (sc-17757) and HSF2 (sc-13517) obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Chromatin immunoprecipitation (ChIP) assay. The K562 cells were grown to almost 80% confluence and cross-linked with formaldehyde (Sigma) at room temperature for 10 min. The cross-linked chromatin was prepared with a commercial ChIP assay kit (EZ-Magna ChIP; Millipore, Billerica, MA, USA) and immunoprecipitated using 4 μg of normal rabbit anti-IgG (Santa Cruz Biotechnology) or 4 μg of anti-HSF2 antibody (Santa Cruz Biotechnology). The HSF2 binding site was PCR-amplified using the input DNA or DNA isolated from the precipitated chromatin as the template, in combination with primers flanking the putative HSF binding sites in the HSF2 promoter. The primer sequences were as follows: forward, 5'-CTCTCCCATTTACTTGCTGTGACTGAAG-3' and reverse, 5'-GAGCCCTTATATATGCCAAGGGCTT TAC-3'.

Purification of TAT fusion proteins. The TAT-Hsp40 expression vector was constructed as previously described (18). The TAT-HSF2 protein was expressed in *E. coli* BL21(DE3) pLysS cells (Invitrogen) and purified using the urea-denaturing protein purification method, as previously described (18,19). The cells were lysed by sonication in lysis buffer (1 mM imidazole, 100 mM NaCl, 20 mM HEPES, pH 8.0) containing 8 M urea. The cell lysates were centrifuged at 12,000 x g for 30 min at 4˚C, and 1 ml Ni^{2+}-NTA agarose was added to the cleared supernatant. Following 2 h of gentle mixing at 4˚C, the resin was transferred to a column and subsequently washed 3 times with 10 ml washing buffer (20 mM imidazole, 300 mM NaCl, 50 mM phosphate buffer, pH 8.0). The proteins were eluted 4 times with 1 ml elution buffer (500 mM imidazole, 300 mM NaCl, 50 mM phosphate buffer, pH 8.0). The urea denaturant was removed with a Mono Q ion exchange column and desalinated with a PD-10 Sephadex size exclusion column (both from GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The protein concentration was quantified using the Bradford assay and confirmed by SDS-PAGE.

Statistical analysis. All data are expressed as the means ± SD of at least 3 independent experiments. One-way analysis of variance (ANOVA), followed by the Student's t-test, was used for statistical evaluations. Values significantly different from the relative control are indicated with an asterisk, and values significantly different from another group are indicated with a hash symbol. A P-value <0.05 was considered to indicate a statistically significant difference.

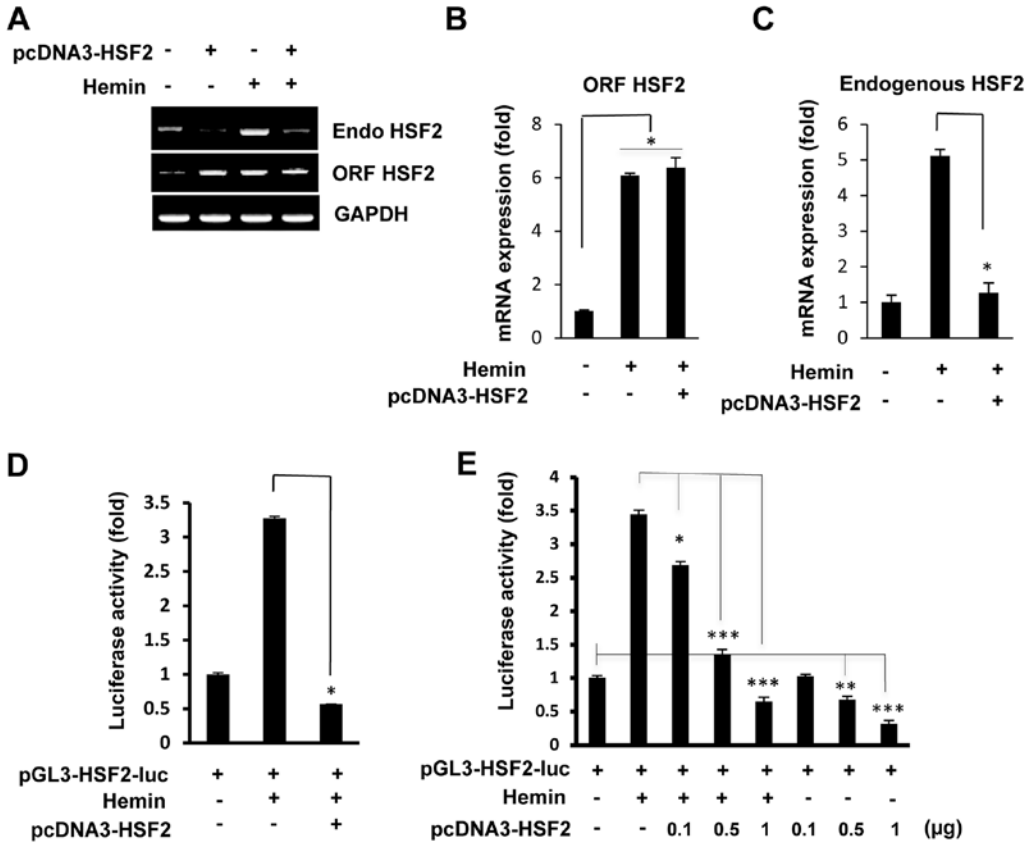

Figure 1. Overexpression of heat shock factor 2 (HSF2) leads to a reduction in the level of endogenous HSF2 mRNA. (A) Expression levels of endogenous HSF2 mRNA. K562 erythroleukemia cells were transiently transfected with pcDNA3-HSF2 and/or treated with 30 μM hemin for 24 h . Forty-eight hours after transfection, total RNA was prepared and subjected to RT-qPCR; GADPH was used as an internal control. (B and C) RT-qPCR. Total RNA was prepared as in (A) and subjected to RT-qPCR to examine the expression levels of exogenous- and endogenous HSF2 mRNA. ORF HSF2; exogenous plus endogenous HSF2 mRNA; *P<0.001. (D and E) HSF2 promoter activity is repressed by the overexpression of HSF2. K562 cells were transfected with pGL3-HSF2-luc (-2.68 kb/+19) and the *Renilla* luciferase reporter plasmid (pRL) together with pcDNA3-HSF2 expression vector. Cells were treated with hemin, or left untreated, as indicated. Forty-eight hours after transfection, the cells were lysed and luciferase activity was measured. The firefly luminescence signal was standardized to the *Renilla* luminescence signal. The results are shown as the fold induction of the luciferase activity compared with the control cells transfected with the pGL3-HSF2-luc plasmid and not treated with hemin. *P<0.001, **P<0.01 and ***P<0.05.

Results

HSF2 transcription is downregulated by HSF2 overexpression. To examine whether HSF2 directly regulates its own expression, the K562 cells were transiently transfected with an HSF2 expression plasmid, and the mRNA expression levels of endogenous HSF2 were measured by RT-qPCR. The primer set used for endogenous HSF2 mRNA was designed to detect the 5'-untranslated region (5'-UTR) of HSF2 mRNA. We also analyzed total HSF2 RNA using open reading frame (ORF)-specific primers. As shown in Fig. 1A, the overexpression of HSF2 markedly inhibited the mRNA expression of endogenous HSF2. Hemin is a well-established inducer of HSF2 in K562 cells, and in accordance with our previous study (16), hemin treatment induced the mRNA expression of endogenous HSF2. However, even after treatment with hemin, the induction of the overexpression of HSF2 in the K562 cells by transfection with the HSF2 expression vector markedly inhibited the levels of endogenous HSF2 mRNA. The total expression levels of HSF2 (ORF HSF2) were similar between the HSF2-overexpressing and hemin-treated cells (Fig. 1A). RT-qPCR confirmed that the increased levels of endogenous HSF2 mRNA induced by hemin were significantly decreased in the cells which overexpressed HSF2 and that the total expression levels of HSF2 (ORF HSF2) were similar between the HSF2-overexpressing and hemin-treated cells (Fig. 1B and C).

To investigate the effects of HSF2 on its own promoter, a plasmid expressing HSF2 was co-transfected with the human HSF2 promoter (2.68 kb/+19)-luciferase construct into the K562 cells. As shown in Fig. 1D, the overexpression of HSF2 markedly reduced the hemin-induced HSF2 promoter activity, indicating the specific repression by HSF2 of its own promoter. This repression was shown to be concentration-dependent by co-transfection with a fixed amount of pGL3-HSF2-luc and increasing amounts of the plasmid pcDNA3-HSF2. Both with and without hemin treatment, transfection with increasing amounts of the expression plasmid, pcDNA3-HSF2, led to a marked reduction in HSF2 promoter activity (Fig. 1E). These results strongly suggest that the promoter of HSF2 (at position 2.68 kb/+19) contains an HSF2-responsive region.

HSF2 binds to heat shock element (HSE) sites in its own promoter. As HSF2 is known to be a transcription factor involved in DNA-binding activity (20), we examined whether

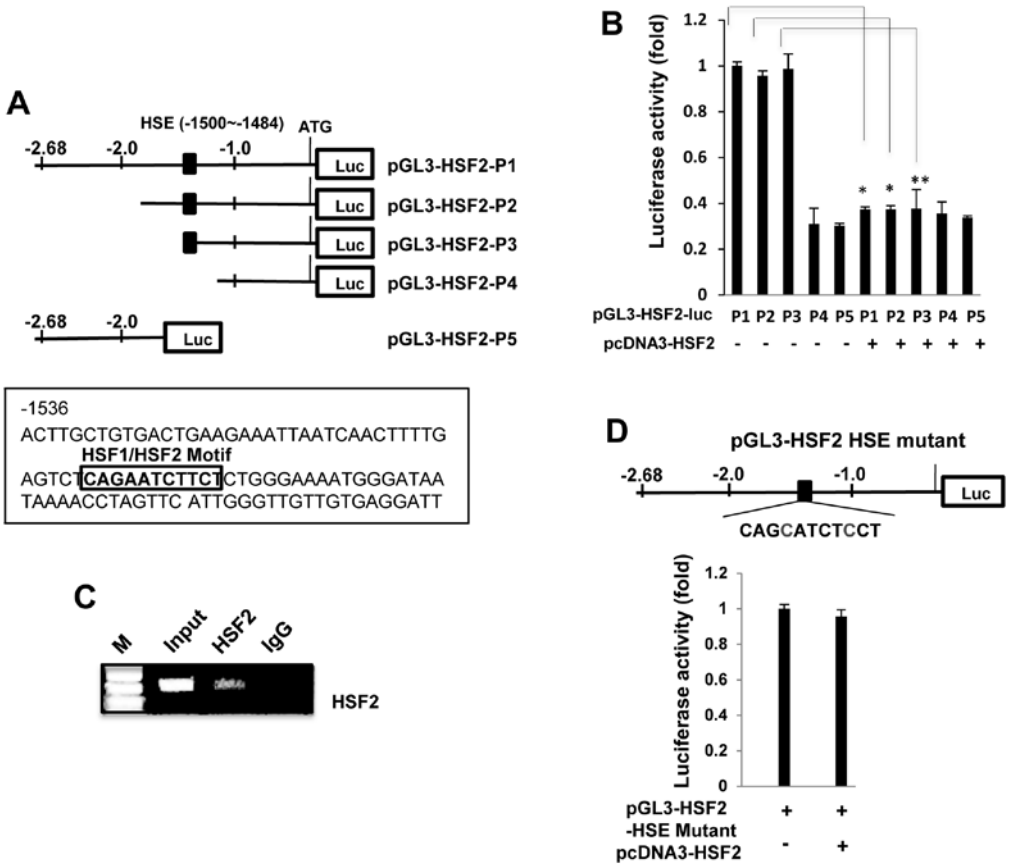

Figure 2. Heat shock factor 2 (HSF2) binds to the heat shock element (HSE) motif in its own promoter. (A) A schematic representation of pGL3-HSF2-luc-P1 containing HSF2 promoter is shown on the left. The putative HSF transcription factor binding sites (HSEs) are marked with a black box. (B) K562 cells were transfected with HSF2 promoter WT (pGL3-HSF2-luc-P1) or deleted HSF2 promoter and the *Renilla* luciferase reporter plasmid (pRL) together with HSF2 expression plasmid. The results presented are the means ± SD of 3 independent experiments. $^{*}p<0.001$ and $^{**}p<0.01$. (C) Chromatin immunoprecipitation analysis. Following transfection with HSF2 for 48 h, chromatin was prepared and immunoprecipitated with specific antibodies against HSF2 or IgG. The input DNA and DNA isolated from the precipitated chromatin were amplified by PCR and separated on a 1.5% agarose gel. (D) pGL3-HSF2 promoter assay with HSE point mutation constructs in K562 cells. Cells were transfected with the wild-type pGL3-HSF2 promoter or point-mutated pGL3-HSF2 (as indicated); the pRL (*Renilla* luciferase) plasmid was co-transfected as an internal control. The cells were harvested at 48 h after transfection. The promoter activity of each preparation was normalized to the *Renilla* value. The relative promoter activity is the average of at least 3 independent experiments.

the HSF2 promoter contains typical HSF-binding sites. A HSF binding site sequence analysis (http://molbiol tools.ca/Transcriptional_factors.htm) of the HSF2 promoter revealed one potential HSE site at -1500/-1484, and therefore we examined whether this HSE motif plays a role in the negative regulation of the HSF2 promoter. Deleted HSF2 promoters were constructed (Fig. 2A) and transfected into the K562 cells in order to analyze promoter activity. As shown in Fig. 2B, deletion of the HSE motif reduced promoter activity by up to 60% relative to the non-deleted promoter (pGL3-HSF2-luc-P1), indicating that this HSE site is a critical region. The overexpression of HSF2 significantly decreased the luciferase activity of the wild-type promoter (pGL3-HSF2-luc-P1), but not the HSE truncated-promoter (pGL3-HSF2-P4 and pGL3-HSF2-P5), indicating that the HSE site contributes to the observed responsiveness to HSF2-mediated repression.

To further confirm these results, we performed a ChIP assay using cross-linked genomic DNA prepared from HSF2-transfected K562 cells. As clearly shown in Fig. 2C, the PCR product containing the putative HSE region was specifically and markedly amplified, indicating that exogenous HSF2 directly binds to the HSE site; IgG was employed as a negative control for this experiment. Additionally, an HSF2 promoter (pGL3-HSF2-HSE mutant-P1) plasmid containing a mutation at the HSE site was constructed (Fig. 2D) and transfected into the K562 cells in order that we could analyze promoter activity. Notably, the pGL3-HSF2 HSE mutant did not significantly reduce the promoter activity induced by HSF2 overexpression, indicating that the HSE site contributes to the observed responsiveness to HSF2-mediated repression.

HSF1 is partially involved in the regulation of HSF2 promoter activity. HSF1 is a transcription factor that contains a DNA-binding domain and exhibits DNA-binding activity at the same DNA sequences (HSE) (20). To further analyze the transcriptional regulation of the HSF2 promoter by HSF1, the cells were co-transfected with the pcDNA3-HSF1 and HSF2 promoter. The protein levels of HSF1 or HSF2 increased in the HSF1- or HSF2-transfected cells compared to the empty vector (pcDNA2)-transfected cells (Fig. 3A). As shown in Fig. 3B-D, as observed with HSF2, HSF1 overexpression also led to reduced the levels of hemin-induced endogenous HSF2 mRNA and HSF2 promoter activity. In addition, increasing the amounts of the expression plasmid, pcDNA3-HSF1, inhibited HSF2 promoter

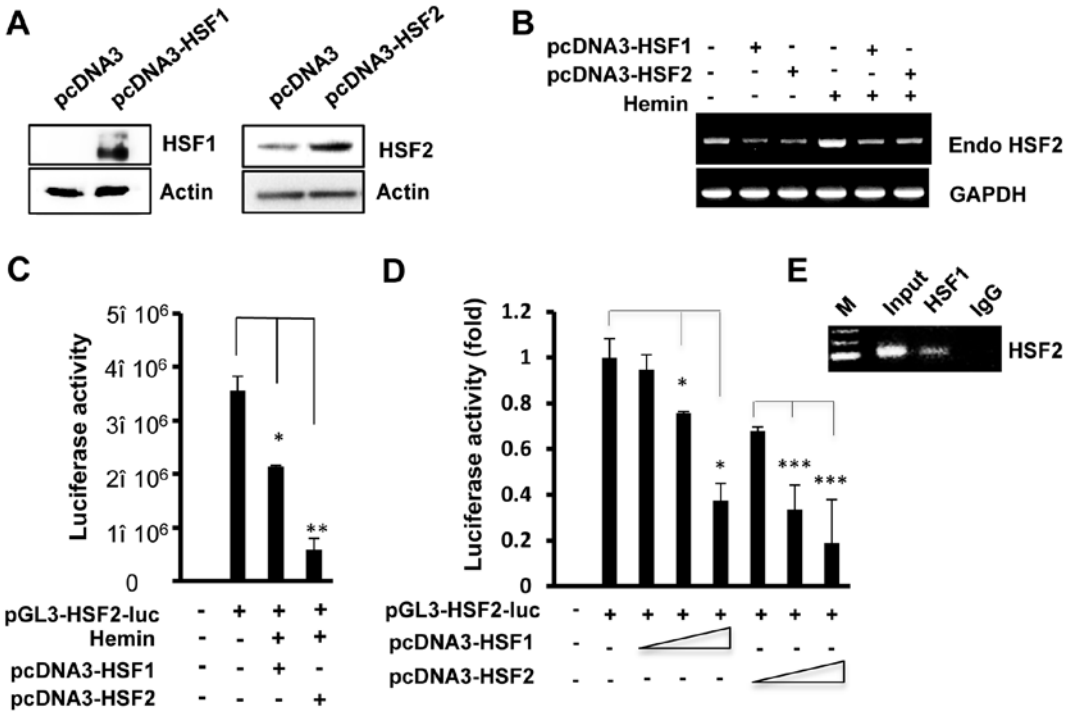

Figure 3. Heat shock factor 2 (HSF2) is partly inhibited by the overexpression of heat shock factor 1 (HSF1). (A) Expression levels of heat shock factor 1 (HSF1) or HSF2 following transient transfection of K562 cells with the pcDNA3-HSF1 or -HSF2 plasmid. Total lysates, prepared as described in the Materials and methods were subjected to western blot analysis using antibodies against HSF1 and HSF2. (B) Effects of HSF1 on endogenous HSF2 (Endo HSF2) expression. Total RNA was prepared as described in the legend of Fig. 1 and subjected to RT-qPCR to examine the expression levels of endogenous HSF2 mRNA. (C andD) Effects of HSF1 on the HSF2 promoter assay. K562 cells were transfected with pGL3-HSF2-luc-P1 with the *Renilla* luciferase plasmid (pRL), pcDNA3-HSF1 or -HSF2 expression plasmids, and treated with 30 μM hemin for 24 h. At 48 h after transfection, the luciferase activity was normalized to the *Renilla* luminescence activity in each sample. *P<0.001, **P<0.01 and ***P<0.05. (E) ChIP assay detected *in vivo* binding of HSF1 to the HSF2 promoter. K562 cells were transiently transfected with the pcDNA3-HSF1 plasmid. Forty-eight hours after transfection, whole-cell lysates were prepared and subjected to ChIP assay. Chromatin was prepared and immunoprecipitated with specific antibodies against HSF1 or IgG. The input DNA and DNA isolated from the precipitated chromatin were amplified by PCR and separated on a 1.5% agarose gel. IgG was used as a negative control.

activity in a concentration-dependent manner (Fig. 3D). However, HSF2 resulted in a stronger inhibition of its own promoter compared to when HSF1 was overexpressed.

To examine whether the sequence containing the HSE site in the HSF2 promoter is recognized by HSF1, we performed a ChIP assay. HSF1 antibody was used to immunoprecipitate the chromatin from HSF1-transfected cells, and the associated DNA fragments were amplified using primers flanking the HSE region in the HSF2 promoter. As shown in Fig. 3E, the PCR product containing the putative HSE region was specifically amplified, indicating that exogenous HSF1 also binds to the HSE region. In addition, similar results were observed with the HEK293 cells (data not shown). Thus, we speculate that the binding of HSF1 and/or HSF2 on the HSE motif mediates the repressive effect of the HSF2 promoter.

HSF1/HSF2 is recruited to the HSF2 promoter to regulate promoter activity. In a previous study, it was shown that HSF1 and HSF2 can form heterotrimers and bind to DNA following proteasome inhibitor treatment (14,15). We thus examined whether HSF2 transcriptional activity is synergistically inhibited by complexes of HSF1 and HSF2 proteins. We co-transfected the expression plasmids HSF1 and/or HSF2 with the HSF2 reporter construct into the MEFs. HSF2 promoter activity in the MEF wild-type cells was significantly inhibited by up to 40 and 80% when HSF1 and HSF2 were overexpressed, respectively; however, following co-transfection with HSF1 and HSF2, promoter activity was decreased to an event greater extent than following transfection with HSF1 or HSF2 alone (Fig. 4A). Similar results were also observed with the *Hsf1*$^{-/-}$ or *Hsf2*$^{-/-}$ MEFs transfected with the HSF2 promoter luciferase construct (data not shown). These results clearly suggest that HSF2 regulates its own promoter activity through interplay with HSF1. As expected, HSF2 promoter activity was markedly inhibited in the *Hsf1*$^{-/-}$ or *Hsf2*$^{-/-}$ MEFs transfected with HSF1 or HSF2 (Fig. 4B and C).

To determine whether HSF2 transcriptional activity is functionally inhibited by the HSF2 protein, we assessed the effects of a purified TAT-tagged HSF2 protein on HSF2 promoter activity. Previous studies have demonstrated the potential ability of the HIV-1 TAT protein transduction domain to modulate the biology of living organisms through the direct cellular delivery of proteins and peptides (18,19). In the present study, the purified TAT-HSF2 fusion protein was directly added to wild-type MEFs for 24 h, and the level of transduced HSF2 was determined by a western blot analysis. As shown in Fig. 4D, TAT-HSF2 was delivered successfully into the MEFs in a dose-dependent manner. Consistent with the results shown in Fig. 1, treatment with hemin alone induced HSF2 promoter activity. However, the TAT-HSF2-transduced cells exhibited lower levels of promoter activity following treatment with hemin (Fig. 4E).

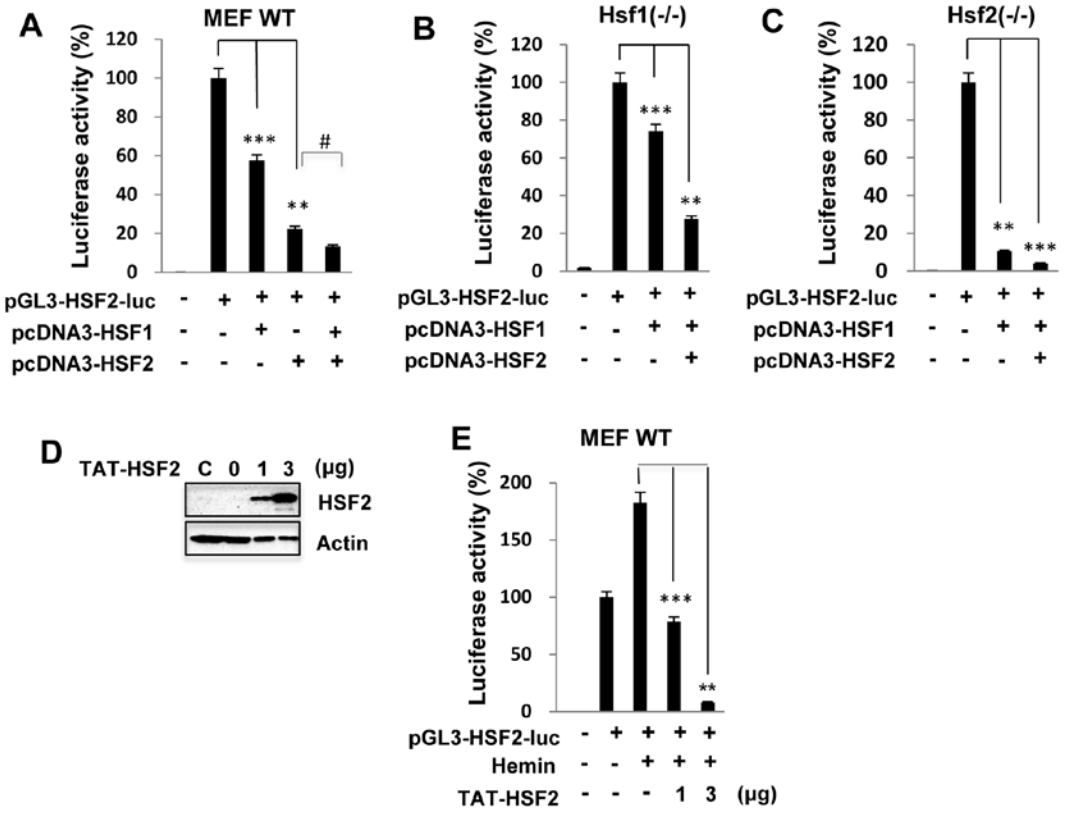

Figure 4. Relative luciferase activity of the heat shock factor 2 (HSF2) promoter with heat shock factor 1 (HSF1) and HSF2 in mouse embryonic fibroblasts (MEFs). (A-C) Wild-type MEFs, and *Hsf1(-/-)* MEFs and *Hsf2(-/-)* MEFs were co-transfected with pGL3-HSF2-luc and pRL plasmid or pcDNA3-HSF1 and -HSF2 expression plasmids, as indicated. Forty-eight hours after transfection, the cells were lysed and their luciferase activities were measured. (D) Transduction of purified TAT-HSF2 protein inhibits HSF2 promoter activity in MEFs. MEF wild-type cells were transduced with 1 or 3 μg of TAT-HSF2 protein for 24 h, and the transduced TAT-HSF2 was detected by western blot analysis. (E) MEFs were pre-treated with TAT-HSF2 for 6 h and exposed to 30 μM hemin for 24 h. Luciferase activity of the HSF2 promoter was measured. The data are the means ± SD from 3 independent experiments. $^{\#}$P<0.001, **P<0.01 and ***P<0.05.

Discussion

HSF2 is a transcription factor that displays tightly regulated gene expression. Its expression can be stimulated by physiological signals triggered by differentiation or development (1,21,22) and also by environmental stress conditions, such as heat shock or proteasome inhibition (10,11). Although the HSF2 gene promoter contains many putative responsive elements (23), the precise transcription factors involved in the regulation of HSF2 transcription with various stimuli remain unknown.

The majority of studies on HSF2 have focused on protein misfolding diseases, delaying aging, and the development of the embryo and sperm. It has been proposed that HSF2 is an upstream regulator of oncogenic mechanisms relevant for tumor progression and invasion, which are attractive therapeutic targets. Understanding the mechanisms underlying the variable expression of HSF2 is crucial to understanding the possible role of HSF2 under both physiological and pathophysiological conditions. Previously, a molecular characterization of the human HSF2 promoter was published by Lee *et al* (24), who observed that the several transcription factors play a critical role in determining the levels of Hsf2 expression. However, no information is available concerning the role of HSF2 in the activity of its own promoter.

In the present study, we demonstrated that HSF2 transcription is regulated by its overexpression in a negative manner. Promoter activity analysis revealed that HSF2 regulates its own promoter, thus providing evidence for the hypothesis that an autoregulatory mechanism exists at the transcriptional level, and ChIP assays confirmed that the promoter binding of HSF2 is mediated by a putative HSE motif. Our results also suggest that HSF2 transcription is partially repressed by HSF1.

In our previous study, we demonstrated that HSF4a was able to inhibit hemin-induced HSF2 mRNA and protein expression (16). Based on the results of this study, and other previous studies, we suggest that HSF2 expression is regulated by the HSF family and by the transcriptional and/or functional association between HSFs. It is also possible that overexpressed HSFs regulate HSF2 expression by preventing the induction of HSF2 or through the expression of other factors controlled by HSF-mediated signaling. An alternative explanation involves the presence of post-translational modifications, such as phosphorylation and/or sumoylation, which may stabilize the binding of HSF2 to the promoter (25).

It has been reported that the heterotrimerization HSF1-HSF2 provides a transcriptional switch in response to stress and developmental stimuli (14), and previous studies have shown that HSF2 is associated with HSF1 and activates the *hsp70* promoter *in vitro* and *in vivo* (15,17). Furthermore, it is possible that with various stimuli, HSF1 and HSF2 interact and form heterocomplexes that can be recruited to specific promoters (16). For example, in endothelial cells, the arsenite-

inducible RNA-associated protein (AIRAP) transcriptional level is regulated by HSF1-HSF2 heterotrimeric complexes following treatment with the anticancer drug, bortezomib, suggesting that these two factors have a close functional association and also that HSF2 alone can negatively regulate bortezomib-induced AIRAP expression (26). Treatment with the proteasome inhibitor MG132 or the amino acid analog L-azetidine-2-carboxylic acid (AZC) has been shown to induce the formation of a HSF1/HSF2 heterocomplex that binds to the clusterin element and increases both clusterin protein and mRNA levels in the human glial cell line, U-251 MG (27).

In the present study, we also demonstrated that both HSF1 and HSF2 are recruited to the HSF2 promoter under overexpression conditions. The activity of the HSF2 promoter was decreased to a greater extent by the overexpression of both HSF1 and HSF2 than by HSF1 or HSF2 alone, which suggests that HSF1 and HSF2 interacts directly and/or form heterocomplexes to bind to the HSF2 promoter. Notably, HSF1 is able to inhibit HSF2 promoter activity in *Hsf2*$^{-/-}$ MEF cells, indicating that HSF1 plays an important role in HSF2 transcription. Although the potential impact of HSF1 on stress-regulated HSF2 transcriptional expression is not yet well defined, at the transcriptional level, we do know that an HSF can positively and/or negatively modulate the expression of other HSF genes as well as that of its own gene.

In conclusion, in the present study, we provide molecular evidence for an autoregulatory mechanism that allows HSF2 to control its own expression. We believe that these findings provide new insight into the pathogenetic mechanisms of human HSF2-related diseases.

Acknowledgements

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