Novel Isoforms of Heat Shock Transcription Factor 1, HSF1γα and HSF1γβ, Regulate Chaperone Protein Gene Transcription

Background: HSF1 is the major eukaryotic transcription factor that regulates expression of HSP genes.

Results: We identify two novel HSF1 isoforms and show that HSF1 isoforms differentially regulate chaperone gene transcription.

Conclusion: HSF1 isoforms work synergistically, and the ratio of HSF1 isoforms determines chaperone gene transcription levels.

Significance: Our findings unravel an additional layer of chaperone gene regulation through modulation of HSP expression by HSF1 isoform ratios.

The heat shock response, resulting in the production of heat shock proteins or molecular chaperones, is triggered by elevated temperature and a variety of other stressors. Its master regulator is heat shock transcription factor 1 (HSF1). Heat shock factors generally exist in multiple isoforms. The two known isoforms of HSF1 differ in the inclusion (HSF1α) or exclusion (HSF1β) of exon 11. Although there are some data concerning the differential expression patterns and transcriptional activities of HSF2 isoforms during development, little is known about the distinct properties of the HSF1 isoforms. Here we present evidence for two novel HSF1 isoforms termed HSF1γα and HSF1γβ, and we show that the HSF1 isoform ratio differentially regulates heat shock protein gene transcription. Hsf1γ isoforms are expressed in various mouse tissues and are translated into protein. Furthermore, after heat shock, HSF1γ isoforms are exported from the nucleus more rapidly or degraded more quickly than HSF1α or HSF1β. We also show that each individual HSF1 isoform is sufficient to induce the heat shock response and that expression of combinations of HSF1 isoforms, in particular HSF1α and HSF1β, results in a synergistic enhancement of the transcriptional response. In addition, HSF1γ isoforms potentially suppress the synergistic effect of HSF1α and HSF1β co-expression. Collectively, our observations suggest that the expression of HSF1 isoforms in a specific ratio provides an additional layer in the regulation of heat shock protein gene transcription.

Organisms often face temperature changes in environmental conditions. Molecular chaperones, also termed heat shock proteins, are induced as a cellular response to this proteotoxic stress. Eukaryotes possess a family of transcription factors that regulate this stress response program: the heat shock transcription factors. To date seven members of this family have been identified: HSF1,3 HSF2, HSF3, HSF4, HSF5, HSFY, and HSFX (1). There is very little information about HSF3, HSF5, HSFX, and HSFY as these are mostly yet to be characterized (2). In contrast to HSF1, HSF2 is not activated by a heat shock (3, 4). However, it has been shown to co-localize (5) and interact with HSF1 (5–7) and to mediate transcription of heat shock element-containing genes (6, 8). HSF2 can be induced by proteasome inhibition (9, 10), and it was shown to be required for development and cellular differentiation (4, 11–14). HSF4 is also not activated by heat shock and probably does not act as a classical inducible transcription factor (15), yet it functionally interacts with HSF1 during lens development (16).

HSF1, HSF2, and HSF4 are all known to be expressed as two isoforms with tissue specific patterns of expression (17, 18). Unsurprisingly, this complex expression profile of heat shock factor isoforms has been shown to influence the level of transcription of chaperone genes. The two HSF4 isoforms (HSF4a and HSF4b) have opposing effects on the basal levels of chaperone genes (17). HSF4a attenuates the constitutive expression of chaperones, as well as induction after heat shock, probably by competing with HSF1 for binding at heat shock elements (17, 19). On the other hand, HSF4b can activate transcription of heat shock response genes and at least partially substitute for loss of HSF1 (17). The two isoforms of HSF2 (HSF2α and HSF2β) act mostly as transcriptional regulators of the HSF1-dependent activation of heat shock protein (HSP) genes (7, 20). Recently it was shown that, in response to proteasome inhibition, the HSF2β isoform, in particular, negatively regulates the HSF1-dependent induction of HSP genes (8).

The two known HSF1 isoforms were shown to be generated from the same gene by alternative splicing (21). Inclusion of
exon 11 produces the longer $\alpha$ isoform, whereas exclusion leads to the shorter $\beta$ isoform. Alternative splicing has also been proposed as the mechanism by which multiple HSF isoforms are generated in other species (22–24). In mammals, HSF1 mRNA does seem to be constitutively expressed and transcription is not induced by stress (25). In contrast, fish have two isoforms for the HSF1 homologue, and interestingly, their expression is inducible by various stresses (26–29).

Despite their identification over 20 years ago (2), very little is known about the functional differences of HSF1 isoforms, in particular under heat stress conditions. In this study, we functionally characterize the known HSF1 isoforms and their transcriptional potential in the mouse. We also describe two additional HSF1 isoforms and analyze the time course of their activation by post-translational modifications and nuclear-cytoplasmic transport kinetics. We were able to show that the two novel isoforms are ubiquitously expressed and can be translated into proteins. We also demonstrate that the individual HSF1 isoforms are post-translationally modified to a similar extent, but are exported from the nucleus, or degraded after heat shock, with differential kinetics. Finally, we show that the HSF1 isoform ratio determines the level of heat shock protein gene expression.

**EXPERIMENTAL PROCEDURES**

**Mouse Maintenance, Breeding, and Genotyping—HSF1 knock-out mice (C129-Hsf1tm1Ijb/J) (30) were purchased from The Jackson Laboratory (strain number 010543). They were bred to and maintained on a (CBA × C57BL/6) F1 background (B6CBAF1/OlaHsd, Harlan Olac, Bicester, UK). All experimental procedures were approved by the King’s College London Ethical Review Committee and performed in accordance with United Kingdom Home Office regulations. All animals had unlimited access to food and water and were subject to a 12-h light/dark cycle. Housing conditions and environmental enrichment were as described previously (31). Genomic DNA was isolated from an ear punch. PCR was performed with primers Hsf1KoF (5'-AGACCTGTCCTGTGCTGCTAGC), Hsf1KoR (5'-CAGGTCAACTGCCTACACAGACC), Neo3 (5'-AGGACATAGCGTTGGCTACCCGT), and Neo4 (5'-GCCGCTATTTGTCTTCCCAATCC) for 35 cycles (95°C 25 s, 60°C 20 s, 72°C 45 s) with the GoTaQ system (Promega).

**HSP9090 Treatment—**NVP-HSP990 ((R)-2-amino-7-((R)-4-fluoro-2-((6-methoxypyridin-2-yl)phenyl)-4-methyl-7,8-dihydropyrido[4,3-d][1,4]pyrimidin-5(H6)-one) (32) was obtained from Novartis Pharma AG (Basel, Switzerland). The drug was formulated in 0.2% methyl cellulose in 0.9% saline solution (water with 0.9% NaCl) as vehicle. The HSP9090-vehicle mixture was sonicated in a water bath to create a uniform suspension with very small particle size. The drug was freshly prepared for each round of treatment and administered by oral gavage, with thorough mixing between dosing to ensure a homogenous suspension. Mouse tissue was snap-frozen in liquid nitrogen and stored at −80°C.

**Generation of Primary Cell Lines—**Heterozygous Hsf1 transgenic animals were interbred to obtain homozygous Hsf1 knock-out and wild type littermates. Tissue from two ear punches from the same animal for each cell line was sterilized with 70% ethanol (v/v), chopped into small pieces, and put in a 12.5-ml flask with fibroblast medium (DMEM, high glucose, GlutaMAX with pyruvate, 15% fetal bovine serum, 1× minimum essential medium nonessential amino acids, and penicillin/streptomycin). The tissue pieces were incubated at 37°C until they attached and cells started to migrate. This was defined as passage 0. Cells were subsequently incubated with trypsin solution and transferred into fresh medium. All experiments were performed between passages 3 and 6.

**Heat Shock Treatment—**Flasks or multiwell plates were seeded with Parafilm and submerged in a water bath. Heat shock treatment was 42°C for 45 min unless otherwise stated. Flasks or plates were put back at 37°C to let the cells recover from heat shock. Non-induced cells were maintained at 37°C.

**RT-PCR and Quantification of Hsf1 Isoform Expression—**The transcriptional activities of HSF1 isoforms and their translational HSF1 isoforms and analyze the time course of their activation by post-translational modifications and nuclear-cytoplasmic transport kinetics. We were able to show that the two novel isoforms are ubiquitously expressed and can be translated into proteins. We also demonstrate that the individual HSF1 isoforms are post-translationally modified to a similar extent, but are exported from the nucleus, or degraded after heat shock, with differential kinetics. Finally, we show that the HSF1 isoform ratio determines the level of heat shock protein gene expression.

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**Generation of Hsf1 Isoform Expression Plasmids, Luciferase Assay Plasmids, and Transfections—**HSF1 isoforms were amplified from oligo(dT) reverse-transcribed wild type RNA. Primers for untagged isoforms were mHSF1_kozak_f (5'-GCCGCCCATGGATCTGGTCGTTGCG), and primers for FLAG-tagged isoforms were mHSF1_FLAG_kozak_f (5'-GCCGCCATGGATCTGGTCGTTGCG), both together with mHSF1_rev (5'-CATAGAGACGTGGTGGCCCTTG). PCR products were cloned into pCR8/GW-TOPO vector (Life Technologies). Both untagged and FLAG-tagged versions of Hsf1α and Hsf1γ were obtained. To generate the other isoforms, the α/β-cassette (exon 10 to exon 13) was exchanged by PstI/Sacl digest. All plasmids were verified by sequencing, with National Center for Biotechnology Information (NCBI) accession number NC_000081.6 from base pairs 76477389 to 76500978 being used as a reference. Hsf1α isoforms contain a silent mutation in exon 9 (A1068G from the ATG start codon), Hsf1α isoforms contain a silent mutation in exon 11 (G1323A from the ATG start codon), and Hsf1β and Hsf1γ have no mutations. The gateway entry vectors were subsequently inserted into pT-RExT-DEST30 to give the full expression plasmids. All plasmids are driven by the CMV promoter. The firefly luciferase gene was cloned with primers FFLuc_for (5'-GCCGCTGAGAAAGTGGATCCATGGAAGGACGCCAAAACACTAAAAG) and FFLuc_rev (5'-CGCGAGCTCTTCAATTTGAGACTTCCG) into pcDNA3 to give plasmid pcFLuc. The Hspa1a/b promoter was cloned into

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pcFFLuc with primers hsp70_for (5’-GGGCTCGAGCCCCA-GAAACCTCTGGAGAGT) and hsp70_rev (5’-GGGGTACCCCGGCTCGCTTCGTCGCTTG). All plasmids were transfected with jetPrime (Polyplus-transfection SA) according to the manufacturer’s instructions. The amount of DNA for each transfection, including the isoform combinations, was kept constant (2 μg per 6-well plate, 1 μg per 12-well plate). Until otherwise noted, all experiments were performed 48 h after transfection.

Antibodies and Western Blotting—Anti-HSF1 antibody was raised against peptide LARAPQMSGVARLFCPPSS in rabbit (Davids Biotechnologie GmbH) and used 1:150 in TBS-T (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% (w/v) Tween 20) for 5 h at room temperature. HSF1 preimmune serum was used at 1:5000 in TBS-T for 5 h at room temperature. Other antibodies and dilutions were GAPDH (ab9485, Abcam) 1:1000, HSF1 (ab81279, Abcam) 1:500, HSF2 (sc-13056, Santa Cruz Biotechnology) 1:75, HSF3 (SAB2105376, Sigma) 1:100, HSF4 (sc-ab81279, Abcam) 1:500, HSF2 (sc-13056, Santa Cruz Biotechnology) 1:200, β-actin (ACTB) (sc-47778, Santa Cruz Biotechnology) 1:10000, Hsp70 (SPA-810, StressGen) 1:1000, Hsp40 (SPA-400, StressGen) 1:1000, and Hsp25 (SPA-801, StressGen) 1:1000. Blocking buffer was 5% (w/v) skimmed milk powder in TBS-T. Secondary antibodies were purchased from LI-COR, and Western blots were visualized on an Odyssey Sa (LI-COR) and analyzed with the Image Studio Lite version 3.1 software (LI-COR). Membranes were stripped from bound antibody by incubation at 55°C for 30 min with three changes of buffer during this time (62.5 mM Tris-Cl, pH 6.6, 100 mM β-mercaptoethanol, 2% SDS). After stripping, membranes were extensively washed in TBS-T and reblocked.

Immunoprecipitation—Mouse tissue was homogenized in ice-cold lysis buffer (PBS (Life Technologies), 1% (w/v) Triton X-100, 1 mM dithiothreitol, 5 mM EDTA, Complete protease inhibitors (Roche Applied Science)), used immediately, and never frozen. Sample concentration was determined by measuring absorbance at 280 nm or using a BCA assay (Thermo Scientific). About 5 μg of total protein was incubated with either 3 μg of anti-HSF1 (Abcam) antibody or 3 μg of rabbit IgG (Santa Cruz Biotech) containing exon 9a and exon 11 and consequently does not cause a frameshift when included. The amino acid sequence encoded by exon 9a is shown in Fig. 1A. Exon 9a contains 84 bp that are homologous to other mammalian homologues (Fig. 1D). The identity of the isoform bands was determined by TaqMan RT quantitative PCR, and the evaluation of the data was performed as described previously (33). The quantitative PCR assays for GAPDH and β-actin were purchased from Primer Design. Primers and probes (5’-FAM, 3’-TAMRA) for Hsp70 and β-actin were purchased from Eurofins MWG. Sequences are as follows: Hsp70_Isoform1, 5’-TTGGGGCTTTGTCGCGCTG; Hsp70_Isoform2, 5’-CCCCATGCGATTTTGCTGCT; Dnajb1, 5’-GCCGCTGCCAAGAAAGGAG; Dnajb1-1, 5’-CTTTCGTTGCAGAAACCCCTTTTGAA; Hsp70_Isoform3, 5’-CCCCATGCGATTTTGCTGCT; Hsp70_Isoform4, 5’-GCCGCTGCCAAGAAAGGAG; Dnajb1-2, 5’-GCCGCTGCCAAGAAAGGAG; Dnajb1-3, 5’-GCCGCTGCCAAGAAAGGAG; Dnajb1-4, 5’-GCCGCTGCCAAGAAAGGAG. The identity of the isoform bands was calculated using SPSS (IBM). Analysis of variance with Tukey’s post hoc test was used to calculate significance. p Values less than 0.05 were considered as statistically significant.

RESULTS

Two Novel HSF1 Isoforms Are Expressed in Various Mouse Tissues—We first discovered the additional exon 9a, which gives rise to the two novel HSF1 isoforms, during the cloning of a full-length Hsf1a transcript. This novel exon was present in about 10% of the clones that were analyzed. Sequencing allowed us to map this exon to a genomic sequence of Hsf1a containing exon 9a and exon 11 and thymidine kinase promoter-driven Renilla (Renilla reniformis) luciferase. The amount of DNA for each transfection, including the isoform combinations, was kept constant. The Dual-Luciferase reporter assay system (Promega) was used according to the manufacturer’s instructions. Luminescence signals were read on an Orion II microplate luminometer (Berthold Detection Systems).

Statistics—Data were screened for outliers using Grubb’s test (GraphPad). Statistical significance was calculated using SPSS (IBM). Analysis of variance with Tukey’s post hoc test was used to calculate significance. p Values less than 0.05 were considered as statistically significant.
confirmed by sequencing. In addition to the four major Hsf1 isoforms (H9251, H9252, H9253/H9251, H9253/H9252), we could detect multiple additional bands, all specific for Hsf1 sequences as confirmed by sequencing. The three asterisks in Fig. 2 mark isoforms, with retained partial intronic sequences that were not differentially expressed across tissues. In contrast, the two immature isoforms labeled as unspliced 1 (Hsf1/H9251 with retained intron 11) and unspliced 2 (Hsf1/H9251 with retained introns 10 and 11) did show differences in expression levels between tissues (Fig. 2B). The expression levels of the four mature Hsf1 isoforms and the two immature isoforms were quantified in Fig. 2C. The intensities of all bands in each tissue were normalized to the GAPDH band intensity, and the mean expression of the normalized intensities between experiments was calculated. Either the sum of all Hsf1 isoform band intensities for each tissue was set to 100% (Fig. 2C), or only the sum for the four major Hsf1 isoforms was set to 100% (Fig. 2D and E). Some of the peripheral tissues showed a very high level of the immature Hsf1 isoforms (e.g. spleen in Fig. 2C, unspliced 1 and 2). Hsf1 isoform ratios in brain regions were much more homogenous. Overall, the γ isoforms constituted about 10% of all Hsf1 isoforms in the peripheral tissues and about 15% in the brain regions. The proportion of the Hsf1/H9253/H9251 isoform was quite variable in peripheral tissues, with testes showing exceptionally high levels of about 22% (Fig. 2D). The Hsf1/H9253/H9252 isoform was expressed at almost 3-fold higher levels in brain regions than in peripheral tissues (Fig. 2D, 7.0% brain versus 2.4% periphery). The periphery also showed a slightly lower percentage of α isoforms and a slightly higher level of β isoforms. These Hsf1 isoform ratios remained highly comparable in tissues from mice that had been treated with HSP990 to induce the heat shock response (compare Fig. 2, D and E). The only significant difference (analysis of variance, \( p < 0.05 \)) was a
lower percentage (about 2-fold) of the Hsf1γβ isoform in the hippocampus, striatum, and brain stem after HSP990 dosing (compare Fig. 2, D and E).

The Hsf1γ Isoforms Are Translated into Protein in Vivo—To determine whether the two novel Hsf1 isoforms were translated into protein in vivo, we generated an antibody against the exon
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9a peptide. Validation of the anti-HSF1γ antibody in an Hsf1 knock-out cell line showed that it indeed recognized the two HSF1γ isoforms, but not HSF1α nor HSF1β (Fig. 3A, middle panel). A commercially available antibody against HSF1 (Ab81279, Abcam), which was raised against full-length HSF1 (epitope: amino acids 288–439), recognized all four isoforms (Fig. 3A, right panel). We detected the highest level of total HSF1γ expression with our RT-PCR assay in testis. Therefore, we immunoprecipitated HSF1 from the testes of 8-week-old wild type and Hsf1 knock-out mice and probed with the anti-HSF1γ antibody (Fig. 3B). We could detect a signal in the IP of HSF1 in wild type mice, but not in the knock-out mice. The very small difference in size of only 2.3 kDa between HSF1γα and HSF1γβ made it impossible to separate the two isoforms on a gel. This paradigmatic result demonstrates that HSF1γ isoforms are translated into protein in vivo.

Heat Shock Treatment Induces the Hypershift of HSF1 Isoforms, Indicating Activation through Post-translational Modifications—We next developed a cell model system to characterize the individual HSF1 isoforms in more detail. We established two fibroblast lines each from wild type and from Hsf1 knock-out mice. Using the same RT-PCR assay as before, we analyzed the isoform ratios in the wild type fibroblast lines (Fig. 4A). Only miniscule amounts of the Hsf1γ isoforms could be detected. The ratio of Hsf1α to Hsf1β was about 2 to 1 and did not significantly change after heat shock (Fig. 4A). Furthermore, we analyzed the expression of HSF2, HSF3, and HSF4 in the fibroblast lines and found that there was no apparent change in the basal expression of any HSFs between the wild type and Hsf1 knock-out lines, nor after heat shock (Fig. 4B). On the other hand, the induction of heat shock proteins, as reported previously (30), depends on the presence of HSF1 (Fig. 4B). Interestingly, HSP70 (Hsp70a/b) and HSP40 (DnaJb1) levels under basal conditions were not affected by the Hsf1 knock-out (Fig. 4B, basal lanes). Because the duplicate cell lines for both the wild type and the Hsf1 knock-out cell lines were identical regarding isoform ratio and induction of heat shock proteins, we only focused on one line for each genotype in the following experiments.

Individual isoforms expressed in the Hsf1 knock-out cell line resulted in readily detectable signals at the same apparent molecular weight as in the wild type cell line (Fig. 4C, basal). This implied a much higher expression level of the individual HSF1 isoforms when compared with their endogenous coun-

FIGURE 2. Hsf1 isoform ratios in mouse tissue. A, schematic of the RT-PCR assay to quantify Hsf1 isoform ratio. Primers (yellow arrows) bind in exons 9 and 12 and give distinct product lengths for each isoform. B, representative image of an RT-PCR assay showing Hsf1 isoform ratios in different mouse tissues. Tissues were taken from 8-week-old (CBA × C57BL/6) F1 wild type mice. All visible bands are specific for Hsf1 sequences and were confirmed by sequencing. Asterisks mark Hsf1 isoforms that contain retained introns, but because they show very similar levels in all tissues, were not quantified. Unspliced 1 (Hsf1α with retained intron 11, 292 bp) and unspliced 2 (Hsf1α with retained introns 10 and 11, 369 bp) represent the major immature isoforms and are quantified in C, together with Hsf1α (α), Hsf1β (β), Hsf1γα (γα), and Hsf1γβ (γβ). GAPDH was used as loading control. Marker (M) is low molecular weight marker (New England Biolabs) quad. fem. = quadriceps femoris. C, quantification of Hsf1 isoform ratios in different mouse tissues. The intensity of the GAPDH band was used to average the band intensities from different mice. The sum of the normalized band intensities of all Hsf1 isoforms for each tissue was set to 100%. The data shown are the mean intensity (n = 6; n = 3 for ovary and testes) ± S.E. D, same as in C, but the unspliced isoforms were not considered and the sum of the band intensities for the four main isoforms was set to 100%. E, same as in D, but mice were treated with 12 mg/kg HSP990 h before tissues were taken. Data are mean (n = 2) ± S.E.
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FIGURE 4. HSF1 isoforms are post-translationally modified. A, Hsf1 isoform ratio in wild type primary fibroblast cell lines. The experiment was performed as described in the legend for Fig. 2. Data are mean (n = 4) ± S.E. HS = heat shock. B, characterization of the established primary cell lines. Wild type (Hsf1+/+) and knock-out (Hsf1−/−) fibroblast lines were analyzed for protein expression levels of HSF2, HSF3, HSF4, HSP70 (Hspa1a/b), HSP40 (Dnajb1), and HSP25 (Hspb1). GAPDH and β-actin (ACTB) were used as loading controls. rec = recovery. C, time course of HSF1 isoform activation. Wild type and knock-out fibroblast lines were transfected with vector (−) and the knock-out line in addition to the four HSF1 isoforms (α, β, γα, γβ). Cells were kept at 37 °C (basal) or heat-shocked and recovered for the indicated time period (0–4 h rec) at 37 °C before Western blot analysis. HSF1 isoforms were detected with anti-HSF1 (Abcam), β-actin (ACTB) was used as a loading control. • indicates ~70 kDa.

terparts, most likely because their expression was under the control of the very strong CMV promoter. However, all isoforms were expressed to a similar level when compared with each other (Fig. 3A, right panel, and Fig. 4C). HSF1 activation by heat stress induces a shift of the HSF1 bands toward higher molecular weight (hypershift) due to extensive post-translational modifications (34). A time course of activation for each of the individual HSF1 isoforms showed that all isoforms, as well as the wild type signal, were hypershifted immediately after heat shock (Fig. 4C, 0 h rec lane). This hypershift was still visible even 4 h after heat shock, although at this time point, the very high molecular weight signals had diminished, indicating a deactivation of HSF1 (Fig. 4C). Taken together, all HSF1 isoforms were extensively post-translationally modified after heat shock and were activated with similar kinetics when compared with each other and with the wild type situation.

HSF1 Isoforms Are Imported into the Nucleus after Heat Shock Treatment—We used immunofluorescence to study the intracellular localization of HSF1 isoforms in non-induced conditions or after heat shock. Our attempt to detect endogenous HSF1 with different antibodies (Abcam (ab81279, ab52757, ab2923); Santa Cruz Biotechnology (sc-17757, sc-9144, sc-30443); StressGen (ADI-SPA-901); NeoMarkers (Ab-3); ab2923); Santa Cruz Biotechnology (sc-17757, sc-9144, sc-30443); StressGen (ADI-SPA-901); NeoMarkers (Ab-3 10H8)), in a variety of conditions, was unsuccessful as in each case, we observed a positive signal in the Hsf1 knockout fibroblasts (data not shown). Hence, we used the N-terminal FLAG epitope-tagged HSF1 isoforms to analyze their intracellular distribution. When we analyzed the transfected fibroblast lines, we observed small DAPI-positive foci appearing (Fig. 5A, right panel, white arrows). However, this did not occur when DNA was omitted, i.e. when only the components of the transfection system were used (Fig. 5A, jetPRIME and buffer panels). We concluded that the polyethyleneimine-DNA complexes were very stable and could be stained with DAPI. In the non-induced situation, the HSF1α isoform showed a relatively high level of signal in the nucleus, whereas the other isoforms showed almost exclusive cytoplasmic staining (Fig. 5B, basal panel). Consistent with the observed hypershift due to extensive post-translational modifications (Fig. 4C), the HSF1 isoforms could only be detected in the nucleus immediately after heat shock (Fig. 5B, 0 h rec panel), and a shorter heat shock of only 15 min at 42 °C was also sufficient to induce nuclear translocation of the HSF1 isoforms (Fig. 5B, 15 min HS panel). At 2 h after heat shock, we started to detect a cytoplasmic signal for the HSF1γ isoforms, which became more pronounced at 3 h after heat shock (Fig. 5B, HSF1γα and HSF1γβ, 2 h rec and 3 h rec panels). At both time points, HSF1α and HSF1β were still predominantly localized to the nucleus (Fig. 5B, HSF1α and HSF1β, 2 h rec and 3 h rec panels). The HSF1γβ isoforms could not be detected in the nucleus from 3 h after heat shock onwards (Fig. 5B, Hsf1γβ). At 4 h after heat shock, a cytoplasmic signal was also detectable for HSF1α and HSF1β (Fig. 5B, 4 h rec panel). However, HSFα, HSFβ, and HSF1γα still had a rather large fraction of signal visible in the nucleus (Fig. 5B, 4 h rec panel). At 6 h after heat shock, the intracellular distribution of the isoforms was comparable with the non-induced conditions (Fig. 5B, compare basal and 6 h rec panels).

HSF1 Isoform Ratio Regulates the Level of Transcription of Heat Shock Protein Genes—Under basal conditions, expression of Hspa1a/b (Hsp70) and Dnajb1 (Hsp40) was not significantly changed between the Hsf1 knock out line and wild type cells, and the comparative expression levels did not change on expression of the individual isoforms (Fig. 6, A and C). Only Hspb1 (Hsp25) showed a significantly lower amount of transcripts in the knock-out line when compared with wild type (Fig. 6E; see also Fig. 4B). This reduced level could not be rescued by the expression of individual isoforms (Fig. 6E). However, expression of combinations of Hsf1 isoforms under non-induced conditions resulted in higher levels of transcripts for all three HSP genes, when compared with the expression of individual isoforms (Fig. 6, A, C, and E). The amount of DNA used for transfections was kept constant for all conditions, and the
relative isoform proportions were (given in %): \( \alpha = 68.7\) and \( \beta = 31.3; \) \( \alpha, \) \( \beta, \) and \( \gamma = 64.2, 29.2, \) and \( 6.6; \) \( \alpha, \beta, \) and \( \gamma = 67.3, 30.6, \) and \( 2.1; \) \( \alpha, \) \( \beta, \) \( \gamma, \) and \( \gamma = 62.7, 28.6, 6.6, \) and \( 2.1. \) These proportions were derived from the average levels in mouse peripheral tissues (with testis excluded) as determined in Fig. 2: \( 6.6\% \) for the \( Hsf1^\alpha \) isoform and \( 2.1\% \) for the \( Hsf1^\beta \) isoform. 

Similar to the induction of heat shock proteins in the wild type fibroblast line (Fig. 4B), HSP genes were highly induced 2 h after heat shock (Fig. 6, \( Hsf1^\alpha /H11001/H11001 \)). Consistent with previous publications (8, 30), \( Hsf1 \) was in all cases required for induction of HSP genes (Fig. 6, compare \( Hsf1^+/+ \) vector with \( Hsf1^{-/-} \) vector). However, each individual isoform was sufficient to significantly induce transcription at the \( Hspa1a/b \) locus after heat shock (Fig. 6B). \( Hsf1^\alpha, Hsf1^\beta, \) and \( Hsf1^\gamma \) were sufficient to significantly induce \( DnaJb1 \) (Fig. 6D), and for the \( Hspb1 \) gene, \( Hsf1^\alpha \) and \( Hsf1^\gamma \) were sufficient to significantly induce transcription (Fig. 6F). As for non-induced conditions, expression of \( Hsf1 \) isoform combinations resulted in higher levels of transcripts for the respective HSP gene after heat shock when compared with expression of the individual isoforms (Fig. 6, B, D, and F). Although this was not statistically significant, we observed a clear trend for this synergistic enhancement of transcription induction for all three HSP genes (Fig. 6, B, D, and F).

Generally, we noticed a higher variability in transcript levels when \( Hsf1 \) combinations were used, which was more pronounced when the cells were heat-shocked (Fig. 6). This could be the result of slightly different isoform ratios due to different transfection efficiencies between experiments. However, primary fibroblast lines usually show lower transfection efficiencies when compared with commonly used immortalized cell lines, which could also be a contributing factor. To at least partly solve this issue by only analyzing the transected population of the cells, we used an \( Hsp70 \) promoter luciferase construct to assay the transcriptional activities of the \( Hsf1 \) iso-

FIGURE 5. ***HSF1 isoforms show different nuclear export or degradation kinetics after heat shock.*** A, DAPI stain (blue) of \( Hsf1 \) knock-out cells with different transfection conditions. Cells were completely untreated (not treated panel), transfected with only the jetPRIME reagent (jetPRIME panel) or only with the buffer (buffer panel), or transfected with both together with plasmid DNA (jetPRIME, buffer and plasmid DNA panel). The white arrows indicate the appearance of DAPI-positive small puncta when DNA is transfected. B, immunofluorescence of \( HSF1 \) isoforms after heat shock (HS). Cells were either kept at \( 37^\circ C \) (basal panel) or heat-shocked for 45 min at \( 42^\circ C \) and recovered for the indicated time period (0–6 h rec) at \( 37^\circ C \). The second column shows cells that were only heat-shocked for 15 min (15 min HS panel) at \( 42^\circ C \) and immediately fixed. Images are shown as merged color with DAPI in blue and anti-FLAG (FLAG-tagged \( HSF1 \) isoforms) in green. White bars correspond to 50 \( \mu \)m. rec = recovery.
forms. As for the endogenous Hsp70 locus, we did not observe any significant differences under non-induced conditions for any of the transfection combinations used (Fig. 6G). Furthermore, as for the endogenous Hspa1a/b gene, expression of the individual Hsf1 isoforms was sufficient to significantly induce transcription after heat shock (Fig. 6H). In this assay, the individual isoforms induced transcription of the luciferase reporter after heat shock to a similar extent as the wild type cell line (Fig. 6H, compare Hsf1^{+/+} vector with the individual Hsf1 isoforms). There was no significant difference in luciferase signal between the individual isoforms, although the α isoform showed a trend toward higher activity (Fig. 6H, individual isoforms). Expression of a combination of the Hsf1α and Hsf1β isoforms in a ratio of about 2:1 (see above) resulted in a highly significant syner-

**FIGURE 6. The transcriptional activities of HSF1 isoforms.** A–F, quantitative PCR analysis of HSP transcript levels in wild type (Hsf1^{+/+}) and Hsf1 knock-out (Hsf1^{-/-}) fibroblast lines. Cell lines were transfected with plasmids as indicated below the graphs. Quantitative PCR signals for Hspa1a/b (A and B), Dnajb1 (C and D), and Hspb1 (E and F) were normalized to the geometric mean of GAPDH and β-actin. Graphs show either the HSP transcript levels of cells that were kept constantly at 37 °C (basal) (A, C, and E) or the basal conditions as before together with the transcript levels 2 h after heat shock (HS) (45 min of heat shock, 2 h of recovery at 37 °C) (B, D, and F). The mean expression values are shown normalized to wild type levels (set to 1). Data are mean (n = 4) ± S.E. Statistics for basal versus heat shock conditions: #, p < 0.05, ##, p < 0.01, ###, p < 0.001. Statistics for comparison of isoforms for each condition: *, p < 0.05. AU = arbitrary units. G–H, Hsp70 promoter firefly luciferase assay. Cell lines were transfected with plasmids as indicated below the graphs. In addition, thymidine kinase promoter-driven Renilla luciferase was added in all conditions to normalize the obtained Hsp70 promoter firefly luciferase signals. Graphs show either the signals for cells that were kept constantly at 37 °C (G, basal) or the basal conditions as before together with the transcript levels 2 h after heat shock (45 min of heat shock, 2 h of recovery at 37 °C) (H). The mean signal values are shown normalized to the wild type signal (set to 1). Data are mean (n = 3) ± S.E. Statistics for basal versus heat shock conditions: #, p < 0.05, ##, p < 0.01, ###, p < 0.001. Statistics for comparison of isoforms for each condition: *, p < 0.05, ***, p < 0.001. n.s. = not significant.
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The mammalian heat shock factor 1 was first cloned 23 years ago (35). It soon became evident that there are two major isoforms created by alternative splicing (21). However, surprisingly little information is available about the molecular functions of HSF1 isoforms, in particular their role in regulation of the heat shock response. We therefore analyzed the molecular functions of HSF1 isoforms and in particular their transcriptional activities during heat shock. Furthermore, we show that two novel HSF1 isoforms exist and are expressed in a wide range of mouse tissues.

We found that the signals for the Hsf1 isoforms in different tissues were not fully comparable on an absolute level because the GAPDH signals might differ between tissues. However, Fig. 2C clearly shows considerable differences in the ratios of the four major isoform transcripts as well as some immature isoforms between tissues. Interestingly, tissues that induce the heat shock response to a much lower extent, such as e.g. the brain regions when compared with skeletal muscle (36), have a much lower Hsf1α/Hsf1β ratio (Fig. 2). They also show more immature Hsf1 transcripts (Fig. 2C). The high levels of immature Hsf1 transcripts in some tissues (Fig. 2, e.g. spleen) open the possibility that HSF1 protein levels are not regulated by changes in transcription rates, but rather through modulation of splicing. This observation is consistent with previous reports that HSF1 expression is stable and not influenced by stress (25).

The ratio of HSF1 isoforms had previously only been studied in heart, brain, and testis (21). Although the ratios for heart and brain are in good agreement with our data, the ratio for testis is different (see Ref. 21 and Fig. 2). In contrast to Goodson and Sarge (21), who observed an Hsf1α/Hsf1β isoform ratio of about 1 to 2 in testis, we observed an Hsf1α/Hsf1β isoform ratio of about 4 to 1 in testis (Fig. 2). Unfortunately, it is not easy to compare the data as the sample numbers and error bars have been omitted from the other study (21), although if a difference does exist, it could possibly be due to the different mouse strains used: CBA/J (21) versus (CBA/Ca × C57BL/6) F1. In this study, we analyzed the Hsf1 isoform ratios in 8-week-old mice. It would be very interesting to determine whether these ratios change during aging, which could explain some of the changes in the proteostasis capacity of aged tissue (36–42).

Using an antibody against the newly identified exon 9a, which encodes for the 28-amino acid stretch of the two γ isoforms, we showed that the Hsf1γ isoforms are indeed translated into protein (Fig. 3). Exon 9a is located between the regulatory domain and the heptad repeat C (Fig. 1A). This additional sequence could therefore easily influence the regulation of HSF1 activity, as well as its DNA binding status or protein stability. The activation of HSF1 isoforms after heat shock, which is composed of nuclear import concomitant with post-translational modifications (Figs. 4C and 5B), seems to be similar between the isoforms. We could detect extensive and comparable post-translational modifications for each isoform as indicated by a hypershift on Western blot (Fig. 4C). However, we cannot exclude the possibility that individual isoforms are modified with distinct patterns of post-translational modifications. A decrease in the hypershift begins to occur between 2 and 4 h after heat shock, indicating an attenuation of HSF1 activation (Fig. 4C, 2 h and 4 h rec panels). At the same time points, the HSF1 isoforms start to appear in the cytoplasm (Fig. 5B). Interestingly, for the two HSF1γ isoforms, a cytoplasmic signal is evident by 2 h after heat shock when compared with 4 h for the α and β isoforms (Fig. 5B). At 4 h after heat shock, all HSF1 isoforms exhibit a strong cytoplasmic signal, yet they are still to some extent post-translationally modified (compare Figs. 4C and 5B, 4 h rec panels). These modifications likely represent inhibitory signals for the transcriptionally active HSF1, e.g. phosphorylation on Ser-303 followed by sumoylation on Lys-298 (34). At 6 h after heat shock, all isoforms have returned to the same steady state localization indicative of the non-induced condition (compare Fig. 5B, basal and 6 h rec panels).

The steady state localization of HSF1 is however somewhat controversial. Very often, predominant nuclear localization of HSF1 has been reported (see for example Ref. 43 and references therein). However, we found that various antibodies against endogenous HSF1 detect a nonspecific staining in Hsf1 knock-out cells that it predominantly localized to the nucleus, albeit of lower intensity when compared with the wild type cell line (data not shown). Many studies also rely on GFP-fused constructs to detect intracellular localization, with the problem of GFP having an intrinsic propensity to localize to the nucleus (44). Furthermore, many of the cell lines that have been used previously are derived from oncogenic tissue, or are immortalized. These conditions themselves have the potential to induce a relocalization of HSF1 to the nucleus, as for example cancer cells are highly dependent on HSF1 to induce a non-heat shock response transcriptional network (45). Formally, we cannot exclude the possibility that the presence of a FLAG tag and/or the fact that our constructs are overexpressed could also change the localization of the isoforms. However, in the non-induced conditions, only FLAG-tagged HSF1α showed a relatively high fraction of nuclear signal (Fig. 5B, α basal panel). Taken together, these data are in good agreement with the proposed constitutive nuclear import of HSF1 isoforms (43), balanced by nuclear export and/or degradation (46). The later mechanism has recently been described to quickly attenuate the heat shock response by degrading activated HSF1 (46).

Interestingly, mouse testes showed a very high level of the Hsf1γ isoform, with about 22% of total Hsf1 levels (Fig. 2D). The activation temperature of HSF1 has been shown to be dependent on the average environmental temperature in which the organism lives (47–49), but it is also tissue-specific (47, 50, 51). For example, the threshold temperature to
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activate HSF1 in male gonad cells is lower than the temperature for the rest of the body (47, 50). Thus, it would be interesting to see whether HSF1γαα could be activated at lower temperatures or whether its altered attenuation kinetics (Fig. 5B) might contribute to an adaption mechanism to fast changing environmental temperatures.

Knock-out of Hsf1 does not lead to significantly changed levels of Hspa1a/b (HSP70) or Dnaj1 (HSP40). Hspb1 (HSP25), however, is down-regulated at both transcript and protein levels (see Ref. 30 and Figs. 4B and 6E). The expression of many HSP genes depends on the concerted action of multiple transcription factors, e.g. members of the V-maf musculoaponeurotic fibrosarcoma oncogene homolog (MAF) family, PAX6, NF-Y, NF-xB, or cAMP-response element-binding protein (CREB) (52, 53). This might explain why the relatively high overexpression of the HSF1 isoforms when compared with the wild type HSF1 amount (see Fig. 4C) does not result in elevated levels of HSP transcripts under basal conditions (Fig. 6, basal).

When we analyzed the endogenous levels of Hspa1a/b in non-induced cells, we observed a statistically significant higher expression when combinations of isoforms, in particular Hsf1α and Hsf1β, were expressed (Fig. 6A). We also observed a similar pattern of Dnaj1 expression, although this did not reach statistical significance (Fig. 6C). Furthermore, expression of the individual isoforms was not sufficient to rescue the reduced level of Hspb1 in non-induced conditions (Fig. 6E). However, expression of HSF1 isoform combinations restored the basal expression levels of Hspb1 to be similar to wild type levels (Fig. 6E). In general, the HSF1α isoform seems to have the highest transcription activation potential of the four isoforms, although we did not observe statistically significant differences between the individual isoforms (Fig. 6).

We noticed a rather high variability in endogenous HSP gene levels following transfection with the HSF1 isoform combinations, which was most probably due to unequal ratios in the percentage of transfected fibroblasts. To partly alleviate this problem by analyzing only the transfected subpopulation of the cells, we used an Hsp70 promoter luciferase assay. Here we did not observe, in contrast to the analysis of endogenous Hspa1a/b (Fig. 6A), a synergistic effect for HSF1 isoform combinations under basal conditions (Fig. 6G). However, 2 h after heat shock, we saw a dramatic, highly statistically significant synergistic enhancement when expressing HSF1α and HSF1β together when compared with the expression of only the individual isoforms (Fig. 6H). It is intriguing that the average ratio of Hsf1α to Hsf1β is ~2 to 1 (Figs. 2 and 4A). Therefore, stochastically each HSF1β isoform could heterotrimerize with two HSF1α isoforms. This 2 to 1 ratio could potentially create a different structure in the trimeric transcription activation domain and thereby influence HSF activation, for example by inhibiting binding of HSP70 and HSP40 (54) or by forming a platform for interaction with other transcriptional regulators (55, 56).

During stress and development, HSF1 can form heterodimers with HSF2 and possibly other HSFs (57). Furthermore, a recent study that analyzed HSF1 and HSF2 isoforms, mainly under normal conditions or proteasome inhibition, suggested that the HSF2β/HSF2α ratio influences HSF1 activity, in particular HSF1β (8). Thus, HSF heterotrimer formation or changes in the expression pattern of HSF2 isoforms are most probably additional determinants of the level of the heat shock response.

In our luciferase assay, the addition of the HSF1γ isoforms seemed to have an inhibitory effect on transcriptional activation (Fig. 6H) by almost completely abolishing the synergistic effects of Hsf1α and Hsf1β co-expression. Such antagonistic functions of isoforms generated from the same transcript have been previously described for other transcription factors, e.g. acute myeloid leukemia 1 (AML1) (58) and cAMP-responsive-element modulator (CREM) (59). Hence, if this inhibitory effect of the γ isoforms can be confirmed in further experiments (other cell lines, different HSP genes, etc.), the varying expression of HSF1γ isoforms in different tissues (Fig. 2) could be a major determinant of the HSP mosaic of each tissue type.

Taken together, our data provide proof for the existence of two novel HSF1 isoforms, which are expressed across a wide range of mouse tissues. We have also shown for the first time that the ratio of HSF1 isoforms determines the rate of heat shock protein gene transcription, under both non-stressed and heat shock conditions. Furthermore, expression of isoforms in a distinct ratio leads to a synergistic enhancement of transcription of HSP genes. In contrast, the physiological function of the HSF1γ isoforms might be to attenuate the level of transcriptional activation by the two major isoforms, HSF1α and HSF1β. We propose that HSF1 isoform ratios are a major factor in the regulation of the expression of heat shock protein genes and that modulation of this ratio could influence the stress response capacity of various tissues.

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