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A Novel Mouse HSF3 Has the Potential to Activate Nonclassical Heat-Shock Genes during Heat Shock

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The heat-shock response is characterized by the expression of a set of classical heat-shock genes, and is regulated by heat-shock transcription factor 1 (HSF1) in mammals. However, comprehensive analyses of gene expression have revealed very large numbers of inducible genes in cells exposed to heat shock. It is believed that HSF1 is required for the heat-inducible expression of these genes although HSF2 and HSF4 modulate some of the gene expression. Here, we identified a novel mouse HSF3 (mHSF3) translocated into the nucleus during heat shock. However, mHSF3 did not activate classical heat-shock genes such as *Hsp70*. Remarkably, overexpression of mHSF3 restored the expression of nonclassical heat-shock genes such as *PDZK3* and *PROM2* in HSF1-null mouse embryonic fibroblasts (MEFs). Although down-regulation of mHSF3 expression had no effect on gene expression or cell survival in wild-type MEF cells, it abolished the moderate expression of PDZK3 mRNA and reduced cell survival in HSF1-null MEF cells during heat shock. We propose that mHSF3 represents a unique HSF that has the potential to activate only nonclassical heat-shock genes to protect cells from detrimental stresses.

INTRODUCTION

When exposed to high temperatures, cells produce a set of heat-shock proteins (Hsps) that facilitate the folding of proteins and maintain protein homeostasis. This phenomenon is known as the heat-shock response and is an adaptive response to proteotoxic stress (Parsell and Lindquist, 1993; Morimoto, 2008). In mammals, the expression of classical Hsps is regulated by heat-shock transcription factor 1 (HSF1; Wu, 1995; Morimoto, 1998). HSF1 remains as a monomer in both the cytoplasm and nucleus in unstressed cells, but is converted to a trimer that can bind to a heat-shock element (HSE), translocated into the nucleus in response to heat shock (Sarge *et al.,* 1993; Baler *et al.,* 1993) and induces a robust activation of heat-shock genes (McMillan *et al.,* 1998; Zhang *et al.,* 2002). This HSF1-mediated induction of Hsp expression is required for the acquisition of thermotolerance (McMillan *et al.,* 1998; Tanabe *et al.,* 1998; Zhang *et al.,* 2002), protection of cells from various pathophysiological conditions such as neurodegenerative and other degenerative diseases (Fujimoto *et al.,* 2005; Cohen *et al.,* 2006; Sakamoto *et al.,* 2006; Tanaka *et al.,* 2007; Steele *et al.,* 2008), and lifespan extension (Hsu *et al.,* 2003; Morley and Morimoto, 2004). HSF1 is also required for the development and maintenance of tissues consisting of neuronal cells (Santos and Saraiva, 2004; Homma *et al.,* 2007), germ cells (Nakai *et al.,* 2000; Izu *et al.,* 2004; Wang *et al.,* 2004), ciliated cells (Takaki *et al.,* 2007), and immune cells (Inouye *et al.,* 2004; Zheng and Li, 2004), but it is not always associated with the regulation of major Hsps.

In contrast to the function of HSF1, the roles other members of the HSF family play in gene expression are unclear. HSF2 remains mainly as a dimer in unstressed cells, and for the most part neither forms a trimer or is translocated into the nucleus during heat shock (Sistonen *et al.,* 1994). HSF2 is considered to have roles in development (Pirkkala *et al.,* 2001). In fact, HSF2 is associated with development of the brain and reproductive organs (Kallio *et al.,* 2002; Wang *et al.,* 2003; Chang *et al.,* 2006). However, some part of HSF2 associates with HSF1 in the nucleus and modulates expression of the classical heat-shock genes (He *et al.,* 2003; Ostling *et al.,* 2007) and a nonclassical heat-shock gene, satellite III DNA (Sandqvist *et al.,* 2009). HSF4 remains a trimer in the nucleus in unstressed cells as it uniquely lacks the HR-C domain that suppresses the trimer formation (Nakai *et al.,* 1997; Tanabe *et al.,* 1999). The expression of HSF4 is ubiquitous among cell types (Tanabe *et al.,* 1999) and dominates in the lenses at a postnatal stage (Somasundaram and Bhat, 2000). Also, HSF4 is required for maintenance of the lenses (Fujimoto *et al.,* 2004; Min *et al.,* 2004; Shi *et al.,* 2009), and mutations of its gene are associated with human cataracts (Bu *et al.,* 2002). Recently, a semicomprehensive analysis revealed that HSF4 binds to various genomic regions, and substantial numbers of genes (33%) in and near the HSF4-binding regions are nonclassical heat-shock genes (Fujimoto *et al.,* 2008). Remarkably, HSF4 is required for the expression

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Abbreviations used: GFP, green fluorescence protein; HSE, heat-shock element; HSF, heat-shock transcription factor; Hsp, heat-shock protein; MEF, mouse embryo fibroblast.

of half of these genes, implying that not only HSF1, but also other HSF family members, play significant roles in the induction of nonclassical genes in response to heat shock. The regulation of these genes would be important for protection of cells as the expression of tremendous numbers of genes is up-regulated after heat shock in yeast cells (Hahn *et al.*, 2004) and mammalian cells (Trinklein *et al.*, 2004; Page *et al.*, 2006).

Avian cells have orthologues of mammalian *HSF1* and *HSF2* genes, but chicken HSF1 is functionally different from mammalian HSF1 as it is dispensable to the expression of major Hsps (Nakai, 1999). Rather, chicken HSF3 (cHSF3) is required for their activation (Nakai and Morimoto, 1993; Tanabe *et al.*, 1998). cHSF3, which remains as a dimer in the cytoplasm in unstressed cells, forms a trimer and is translocated into the nucleus in response to heat shock (Nakai *et al.*, 1995). However, HSF3 is considered an avian-specific factor, as human HSF1 can compensate for a lack of cHSF3 in terms of the induction of major Hsps (Inouye *et al.*, 2003). Here, we found the *HSF3* gene in silico in the mouse genome and characterized its unique features.

MATERIALS AND METHODS

Cloning of Mouse HSF3

In the chicken, *HSF3* was located in a region between the genes *Vsig4* and *HEPH* on chromosome 4 using Ensenbl genome databases (http://www.ensembl.org/index.html). We found the *B230358A15Rik* gene flanked by two orthologous genes in the mouse genome. Its EST clones, AK046229.1 and BC117805.2, were also identified in the midbrain. Sequencing of *B230358A15Rik* revealed it to be a mouse orthologue of the chicken *HSF3* gene. We amplified mHSF3b cDNA from the brain by RT-PCR using mHSF3-RT5, 5′-GTG GAT CCC TCC GGA GGA ATT ACA AGG-3′ and mHSF3-RT3, 5′-GTC TCG AGA CCC TTT AGC AGG TAG GA-3′ and cloned them into a pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA; pCR-mHSF3b). As its gene product lacks a part of the DNA-binding domain, we searched corresponding sequences and found exon 1 located between exon 1a and exon 2 (see Figure 2). cDNA of mHSF3a, which was amplified using the primers mHSF3a-5, 5′-AGC AGT GAA ATC CCA AAA CAT GGA G-3′, and mHSF3a-STOP, 5′-TCA AAA AAT ATT AGT AAC AGG GTT GAG AAT-3′, was also cloned into the vector (pCR-mHSF3a). The cDNAs were sequenced using an ALFexpress sequencer (GE Healthcare, Waukesha, WI). Sequencing reactions were performed using an ALFexpress AutoRead sequencing kit (GE Healthcare), ALFexpress dATP labeling mix, and each synthetic oligonucleotide.

We also found the *LOC100129692* gene flanked by *Vsig4* and *HEPH* in the human genome. This region contains at least 10 exons, which correspond to exons 2–4 and exons 6–12 of the mouse *HSF3* gene. However, its EST clone was not identified in the genome databases, and we could not amplify its transcripts by RT-PCR using various sets of primers corresponding to sequences of the exons (data not shown).

Construction

Expression vectors for GST-hHSF1 and GST-hHSF4b in *Escherichia coli* were described previously (Fujimoto *et al.*, 2008). The mHSF3b cDNA was modified using PCR mutagenesis to introduce BamHI and EcoRI sites and ligated into the pGEX-2T vector (GE Healthcare) to create pGEX2T-mHSF3b (Nakai *et al.*, 1997). Primers used were glutathione *S*-transferase (GST)-mHSF3b-5, 5′-GT**G GAT CC**A TGC TAC GCC CTG TTG AGA-3′ and GST-mHSF3b-3, 5′-GT**G AAT TC**T CAT ATG ATT GTC TCA C-3′ (boldface nucleotides are BamHI and EcoRI recognition sites, respectively). The pGEX2T-mHSF3a vector was generated similarly using GST-mHSF3a-5, 5′-GT**G GAT CC**A TGG AGC AAT TTC GC-3′, and GST-mHSF3a-3, 5′-GT**G AAT TC**T CAA AAA ATA TTA GTA ACA GGG-3′.

An expression vector pEGFP-mHSF3a was constructed by inserting a cDNA fragment, which was amplified using the primers listed in Supplementary Table 1 into the pEGFP2-C1 vector (Nakai and Ishikawa, 2000) at EcoRI/BamHI sites. pEGFP-mHSF3b, pEGFP-mHSF3a-NT1, and pEGFP-mHSF3a-NT2 were generated similarly. To generate pEGFP-mHSF3b-3a, a 1247-base pair PstI/BamHI fragment of pEGFP-mHSF3a and a 199-base pair XhoI/PstI fragment of pEGFP-mHSF3b were inserted into the pEGFP2-C1 vector at XhoI/BamHI sites. To generate pEGFP-mHSF3a-NLSmu, we used mutated primers: mHSF3a-NLSmu-3: 5′-CGC TGC CGC CGC TGC TAG TAC TGT ATG GTT TTC AC-3′ and mHSF3a-NLSmu-5: 5′-GCA GCG GCG GCA GCG TCA TTA TCA TTC ATA TCT GA-3′ (substituted nucleotides are underlined).

RT-PCR Analysis

We isolated total RNA of tissues from 6-wk-old mice (ICR background) using TRIzol (Invitrogen). The total RNA was reverse-transcribed using avian myeloblastosis virus reverse transcriptase (AMV-RT) and oligo $(dT)_{20}$ according to the manufacturer's instructions (Invitrogen). PCR was carried out for 35–40 cycles of 94°C denaturing (1 min), 65°C annealing (2 min), and 72°C extension (3 min) by using Ex Taq polymerase (Takara, Kyoto, Japan) in an automated thermal cycler (Takara). Primers used in Figure 2B are listed in Supplementary Table 2. Primers used to amplify PDZK3 and PROM2 cDNAs were as follows: PDZK3-5: 5′-GGC TCC ATC ATC TTA GTT CTG TGG CAG C-3′, PDZK3-3: 5′-CGC GTT AAC TAG CTT CAG AGA GAG GTG ACC-3′, PROM2-5: 5′-CGC GGA TTC GCC ACC ATG GTC CAG GAG GAG GAA AGC-3′, and PROM2-3: 5′-CGC GTT AAC TAG CTT CAG AGA GAG GTG ACC-3′. Other primers for cDNAs of Hsp70-1 and S16 ribosomal protein were described previously (Fujimoto *et al.*, 2008). After each reaction mixture was electrophoresed on a 2% agarose gel, and the amplified DNA was stained with ethidium bromide, photographed using Epi-Light UV FA1100 (Aisin Cosmos R&D, Kariya, Japan), scanned, and quantified by using NIH images. To determine the sequences of the amplified products, an appropriate band was cut out and subcloned into a pCR2.1-TOPO vector (Invitrogen). The sequencing reaction was performed using M13 reverse and forward primers as described above.

Gel Mobility Shift Assay and DNase I Footprint Analysis

A culture of *Escherichia coli* transformed with each expression vector was treated with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 3 h. Crude cell extracts were prepared and gel mobility shift assay was performed using ^{32}P-labeled ideal HSE probe (Nakai *et al.*, 1997). To show specificity of the binding, we performed competition assay using cold HSE86 oligonucleotides containing three perfect nGAAn units and its mutants (Fujimoto *et al.*, 2008) as competitors. DNase I footprint analysis were performed as described (Nakai *et al.*, 1997).

Analysis of Subcellular Localization

COS7 cells were maintained at 37°C in 5% CO_2 in DMEM containing 5% fetal calf serum. Cells were cultured on 35-mm dishes for 16 h and transfected with each expression vector by the calcium phosphate transfection method. At 4 h after the transfection, the dishes were washed three times with PBS, fed with fresh medium, and incubated further for 24 h. Then, the cells were fixed with 4% paraformaldehyde for 30 min at 4°C. Photomicrographs were taken using fluorescence microscopy (Axiovert 200, Zeiss, Thornwood, NY).

Western Blot Analysis

Cells transfected or infected with expression vectors were lysed in a NP-40 lysis buffer {150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin}, and centrifuged at 12,000 × *g* for 10 min. Aliquots of protein were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blotted with mouse antibodies for green fluorescent protein (GFP; GF200, Nakalai), β-actin (AC-15, Sigma), and Hsp70 (W27, Santa Cruz Biotechnology, Santa Cruz, CA), rat IgG for BRG1 (a gift from Dr. Y. Okawa, Kyushu University; Ohkawa *et al.*, 2006), and rabbit antisera for mHSF1 (α-mHSF1J; Fujimoto *et al.*, 2008), cHSF1 (α-cHSF1x; Fujimoto *et al.*, 2005), and cHSF3 (α-cHSF3γ; Inouye *et al.*, 2003). To detect mHSF3, we used an antiserum (α-mHSF3-1) raised against a recombinant mouse HSF3b fused to GST. Antisera for major Hsps were shown previously (Fujimoto *et al.*, 2005).

Generation of Adenoviral Vectors

Adenoviral vectors encoding chimeric HSFs were constructed by a PCR-mediated method using the primers listed in Supplementary Table 3 as described previously (Inouye *et al.*, 2003). Chimeric cDNAs amplified by PCR were digested with KpnI and XhoI and inserted into a pShuttle-CMV vector (Stratagene, La Jolla, CA) at a KpnI/XhoI site (Fujimoto *et al.*, 2005). Viral DNA was generated according to the manufacturer's instructions for the AdEasy adenoviral vector system (Stratagene). To generate Ad-mHSF3aR65A, we used mutated primers: mHSF3aR65A-5: 5′-ATA ACA AGC TTT ATT GCA CAA CTC AAC ATG TAT-3′ and mHSF3aR65A-3: 5′-ATA CAT GTT GAG TTG TGC AAT AAA GCT TGT TAT-3′ (substituted nucleotides are underlined) in addition to 5′- and 3′-terminal primers; pshuttle-m3a-5: 5′-CGA **GGT ACC** GCC ACC ATG GAG CAA TTT CGC AAG ACA ATG GTT-3′ and pshuttle-m3a-3, 5′-GT**C TCG AG**T CAA AAA ATA TTA GTA ACA GG-3′ (boldface nucleotides are KpnI and XhoI recognition sites, respectively). Ad-mHSF3aR65G was similarly constructed. Infectious adenovirus was generated by transfecting adenoviral DNA into HEK293 cells, and viral titers were determined as plaque forming units (pfu). Immortalized mouse embryonic fibroblast (MEF) cells deficient in HSF1 (Inouye *et al.*, 2003) were plated on 60-mm dishes and then incubated in 2 ml of serum-free DMEM containing adenovirus (2×10^7 pfu/ml) for 2 h. After 2 ml of DMEM containing 20% fetal bovine serum was added, cells were maintained for 48 h, treated with heat shock at 42°C for 1 h, and recovered at 37°C for 3 h. Cell extracts were prepared and the expression of Hsps was examined by Western blotting. Ectopically expressed mRNA for mHSF3a increased during heat shock for un-

known reasons, but the endogenous mHSF3 mRNA level did not (see Supplementary Figure 7).

To generate an expression vector for shRNA, a fragment of the human U6 promoter was amplified by PCR using humanU6-5: 5′-CCC CAG TGG AAA GAC GCG CAG GCA AAA CGC A-3′ and humanU6-3: 5′-GGA TCC CGC GTC CTT TCC ACA AGA TAT ATA AAC CCA AGA AAT CG-3′ and inserted into a pCR2.1-TOPO vector (Invitrogen; pCR2.1-hU6). shRNA target sequences for the human *BRG1* and mouse *HSF3* genes were determined using BLOCK-iT RNAi Designer (Invitrogen), and corresponding sense and antisense oligonucleotides (Supplementary Table 4) were annealed and inserted into pCR2.1-hU6 at an BamHI/HindIII site (pCR2.1-hU6-shRNA). An XhoI/HindIII fragment containing hU6-shRNA was inserted into a pShuttle vector (Stratagene) at an XhoI/HindIII site (pShuttle-hU6-shRNA). Viral DNA was generated as described above.

Chromatin Immunoprecipitation

Immortalized MEF cells deficient in HSF1 were infected with an adenovirus expressing mHSF3a, hHSF1, c389/m, or h383/m as described above, and chromatin immunoprecipitation (ChIP) was performed as detailed previously (Fujimoto *et al.*, 2008). Antibodies used for ChIP assays were rabbit antibodies for human and chicken HSF1 (α-cHSF1c; Inouye *et al.*, 2007), for mouse HSF3a (α-mHSF3-1), and for BRG1 (α-SNF2b/BRG1, 07-478, Millipore, Bedford, MA). Primers used to amplify the Hsp70-1 promoter (Inouye *et al.*, 2007) and its downstream region (Mariner *et al.*, 2008) were shown previously. Primers used to amplify the PDZK3 promoter were as follows: PDZK3-CHIP-5: 5′-CGC AAG CTT GGC TTA ATT GAA GGA TTA ACA GCT CTG AGG-3′ and PDZK3-CHIP-3: 5′-CGC GTC GAC CGC TGC GCA GCG CAG GGA TGC TCC G-3′. The amplified DNA was stained with ethidium bromide and photographed.

Immunoprecipitation

HSF1-null MEF cells plated in 100-mm dishes were infected with an adenovirus expressing mHSF3a, hHSF1, c389/m, or h383/m as described above. Cells were washed with PBS, harvested, and then suspended in 500 μl of NP-40 lysis buffer for 10 min on ice. The supernatant was collected after centrifugation at 15,000 rpm for 10 min, added to 1 μl of antiserum for hHSF1 (α-cHSF1c), mHSF3 (α-mHSF3–1), or BRG1 (rat IgG) at 4°C for 2 h, and mixed with 40 μl of protein A-Sepharose beads (1:1 suspension in PBS; Amersham Biosciences, Piscataway, NJ) by rotating at 4°C for 1 h. The complexes were washed five times with NP-40 lysis buffer, suspended in SDS-sample buffer, and boiled for 3 min. The samples were subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and blotted using each specific antibody.

Estimation of Cell Death

Primary cultures of wild-type and HSF1-null MEF cells (Inouye *et al.*, 2003) were plated on 60-mm dishes in DMEM containing 10% FBS at 37°C, and incubated in 1 ml of serum-free DMEM containing adenovirus (1×10^6 pfu/ml) for 2 h. After 4 ml of DMEM containing 10% fetal bovine serum was added, cells were maintained for 24 h, then treated with heat shock at 42°C for the indicated periods. The numbers of adherent cells were counted, and MTT assays were performed as described previously (Hayashida *et al.*, 2006).

Statistical Analysis

Significant values were determined by analyzing data with Mann-Whitney's U test using StatView version 4.5J for Macintosh (Abacus Concepts, Berkley, CA). A level of $p < 0.05$ was considered significant.

Nucleotide Sequence Accession Numbers

The DDBJ accession numbers for mHSF3a, mHSF3b, and cHSF4b mRNAs are AB510446, AB510447, and AB510448, respectively.

RESULTS

Mouse HSF3 *Genes Identified by Comparative Genomic Analysis*

After isolation of the chicken *HSF3* gene (Nakai and Morimoto, 1993), we tried to isolate orthologous mammalian genes by screening human HeLa cell, heart, pancreas, and bone marrow cDNA libraries and mouse mammary carcinoma FM3A cell, heart and brain cDNA libraries with a chicken HSF3 (cHSF3) cDNA as a probe under low-stringency nucleic acid hybridization conditions. We isolated novel human and mouse *HSF4* genes in addition to *HSF1* and *HSF2* (Nakai *et al.*, 1997; Tanabe *et al.*, 1999), but failed to isolate a mammalian orthologue of the chicken *HSF3* gene. Although the human and mouse genome sequences were available (International Human Genome Sequencing Consortium, 2001; Mouse Genome Sequencing Consortium, 2002), we were still unable to identify *HSF3* gene-related sequences in silico from the genome database (Inouye *et al.*, 2003). However, sequence analysis of the chicken genome enabled us to compare synthetic regions (International Chicken Genome Sequencing Consortium, 2004), where the same genes occur in a similar order along the chromosomes of different organisms (Koonin *et al.*, 2000). In fact, the *HSF2* gene was flanked by the *SERINCI* gene in human, mouse, and chicken orthologous segments (Figure 1). As we found that the *HSF4* gene was located in a region between the *TRADD-FBXL8* genes and *NoL3* gene in the human and mouse genome, we analyzed sequences in the chicken genome flanked by the orthologous genes and a BLAST search revealed an orthologous segment containing the chicken *HSF4* (*cHSF4*) gene (Figure 1).

We then tried to identify the mammalian *HSF3* gene. In the chicken, *HSF3* was located in a region between *Vsig4* and *HEPH* on chromosome 4 and orthologous segments containing the two orthologous genes were found on the human

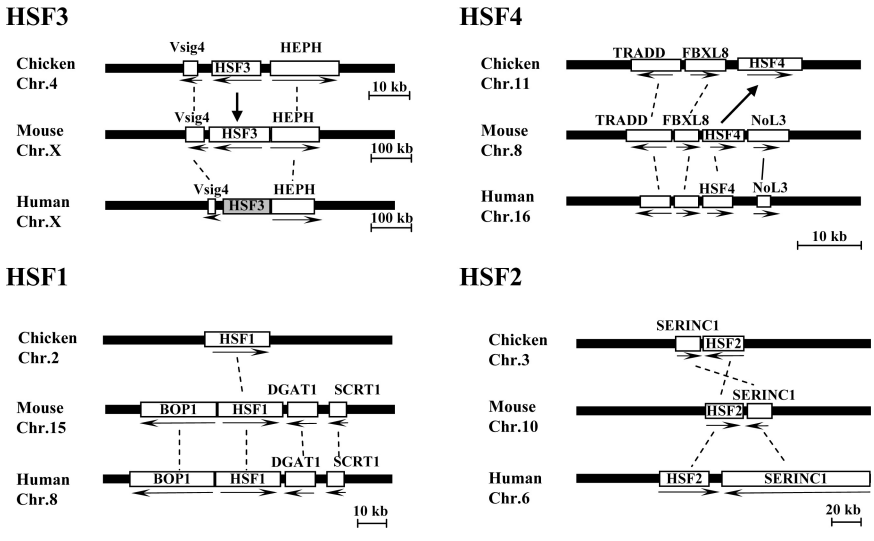

Figure 1. Comparative genomic analysis of orthologous segments containing *HSF* genes. The location of each segment is as follows: human Chr.8 q24.3 and mouse Chr.15 D3 for *HSF1* genes; human Chr.6 q22.31, mouse Chr.10 B4, and chicken 63.95–63.98 Mb for *HSF2* genes; human Chr. X q12, mouse Chr. X B4, and chicken 0.252–0.265 Mb for *HSF3* genes; human Chr.16 q22.1, mouse Chr.8 D3, and chicken 2.44–2.45 Mb for *HSF4* genes. A genomic sequence corresponding to chicken HSF1 cDNA has not been identified yet. Arrows indicate the 5′ to 3′ orientation of each gene. The gray box in human chromosome X is likely to be an *HSF3* pseudogene.

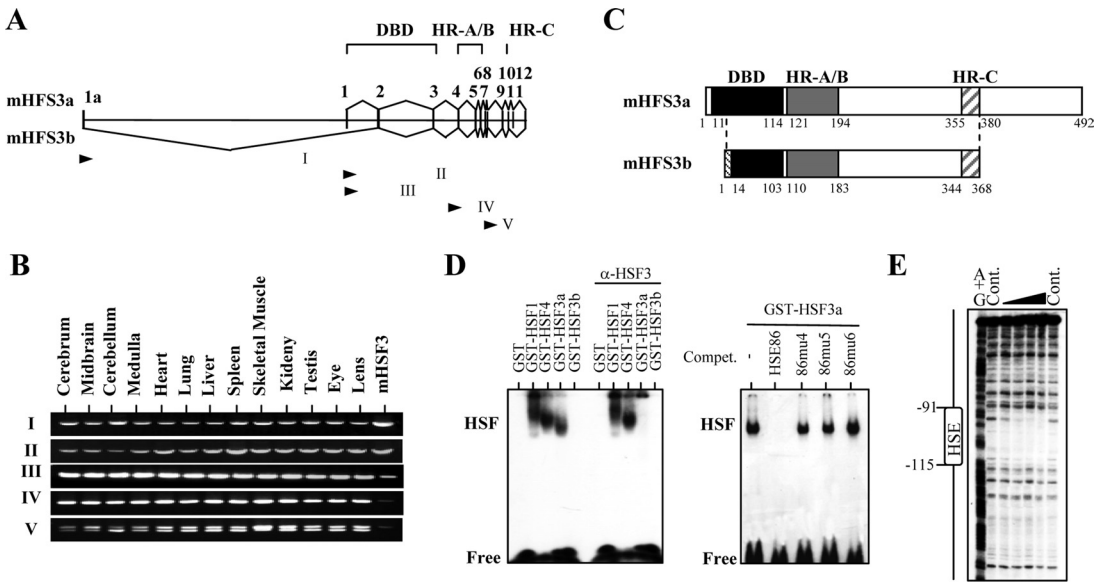

Figure 2. Structure of the *mHSF3* gene and DNA-binding activity of its products. (A) Exons and introns are denoted by boxes and lines. Exon 1 and exon 1a encode the translation start sites of mHSF3a and mHSF3b, respectively. The exons coding for the functional domains are indicated at the top. DBD, DNA-binding domain; HR-A/B, heptad repeat of hydrophobic amino acids A and B; DHR, downstream of the heptad repeat. The positions and directions of the primers used to amplify the cDNA fragments I–V by RT-PCR are indicated at the bottom. (B) RT-PCR was performed using total RNA from various tissues of 6-wk-old ICR mice. The plasmid pcDNA3.1-mHSF3a or b was used as a template for the control PCR (mHSF3). cDNA fragments I–V were sequenced. The fragment II and the upper fragment V contain exon 11, whereas the fragment I and the lower fragment V do not. (C) Comparison of structure between mHSF3a and mHSF3b. Numbers of amino acids are indicated. mHSF3b contains a part of the DBD domain of mHSF3a (amino acids 34–114). (D) mHSF3a binds specifically to HSE. GST and mHSF3a, mHSF3b, hHSF1, and hHSF4 fused to GST were expressed in *E. coli*. Bacterial cell lysate was incubated with ^{32}P-labeled ideal HSE oligonucleotides and loaded on a 4% native gel (left). HSF, HSF:HSE complex; Free, free probe. Competition assay was performed using cold HSE86 oligonucleotides containing three perfect nGAAn units and its mutants as competitors. (E) DNase I footprinting analysis of GST-mHSF3a binding to the promoter of the human *Hsp70* gene. Increasingly large amounts of lysate containing GST-mHSF3a (0, 0.5, 1, 2, and 4 μl of cell lysate) were assayed. Cont., zero microliters of lysate; A+G, A and G ladders. The HSE contains five inverted nGAAn units located at −91 to −115.

and mouse X chromosome (Figure 1). Sequence analysis of a region between the two genes revealed mouse *HSF3* genes (see *Materials and Methods*). Sequences related to *HSF3* genes were also observed in a human orthologous region. However, this genomic region is likely to be an *HSF3* pseudogene as no transcript was identified (see *Materials and Methods*).

Structure and DNA-binding Activity of mHSF3

Vertebrate *HSF1, HSF2,* and *HSF4* consist of 13 exons, the first exon containing a translation start site (Zhang *et al.*, 1998; Tanabe *et al.*, 1999; Nykänen *et al.*, 2001), whereas the chicken *HSF3* gene consists of 12 exons (Supplemental Figure 1). We performed RT-PCR by using primers corresponding to the first exon and the last exon and isolated a cDNA for full-length mHSF3 (mHSF3a isoform) from all the tissues examined (Figure 2, A and B, fragment II). A comparison of *mHSF3* gene sequences with those of its cDNA revealed that there were 12 exons, and most introns were much longer in the *mHSF3* gene than in the *cHSF3* gene (Supplemental Figure 1). There are 492 predicted amino acids of mHSF3a, and sequences of its DNA-binding and HR-A/B domain are conserved like those of other HSF family members (Figure 2C, Supplemental Figure 2). However, the amino acid sequence of the DNA-binding domain of mHSF3a was only 60% identical to that of cHSF3, whereas the sequences of mHSF1 and mHSF2 were almost identical to the corresponding domains of cHSF1 and cHSF2 (92 and 86%, respectively). Furthermore, the identity of amino acid sequence in the DNA-binding domain of mHSF1 was much lower for mHSF3 (53%) than mHSF2 or mHSF4 (70 and 76%, respectively; Figure 3A). Moreover, a phylogenetic tree, which was generated from full-length amino acid sequences of HSF family members including cHSF4, showed the relatedness of mHSF3 with cHSF3 to be much weaker than that of mHSF1 with cHSF1, that of mHSF2 with cHSF2, or even that of mHSF4 with cHSF4 (Figure 3B). These results suggest that nucleic acid sequences of HSF3 diverged quickly during evolution. This phylogenetic tree also demonstrated the amino acid sequence of HSF1 to be most closely related with that of HSF4 among the HSF family, and the amino acid sequence of HSF2 to be most closely related with that of HSF3 (Figure 3B). These findings are consistent with the assertion that two rounds of whole-genome duplication occurred in the vertebrate lineage (Ohno, 1970; Holland *et al.*, 1994; Putnam *et al.*, 2008). Alignment of the human and chicken *HSF* genes with the mouse *HSF* gene showed that sequences of the exons are well conserved, whereas those of introns are not (Supplemental Figure 1B, data not shown), suggesting that four duplicated *HSF* genes have been conserved during evolution under selective pressure (Sémon and Wolfe, 2007).

Even though nucleic acid sequences of HSF3 diverged quickly during evolution, recombinant mHSF3a specifically bound to HSE, and an anti-mHSF3 antibody supershifted the complex (Figure 2D). A DNase I footprint analysis of the coding strand in the human Hsp70 promoter showed that mHSF3a protects regions corresponding to inverted repeats of the nGAAn unit (Figure 2E; Kroeger and Morimoto,

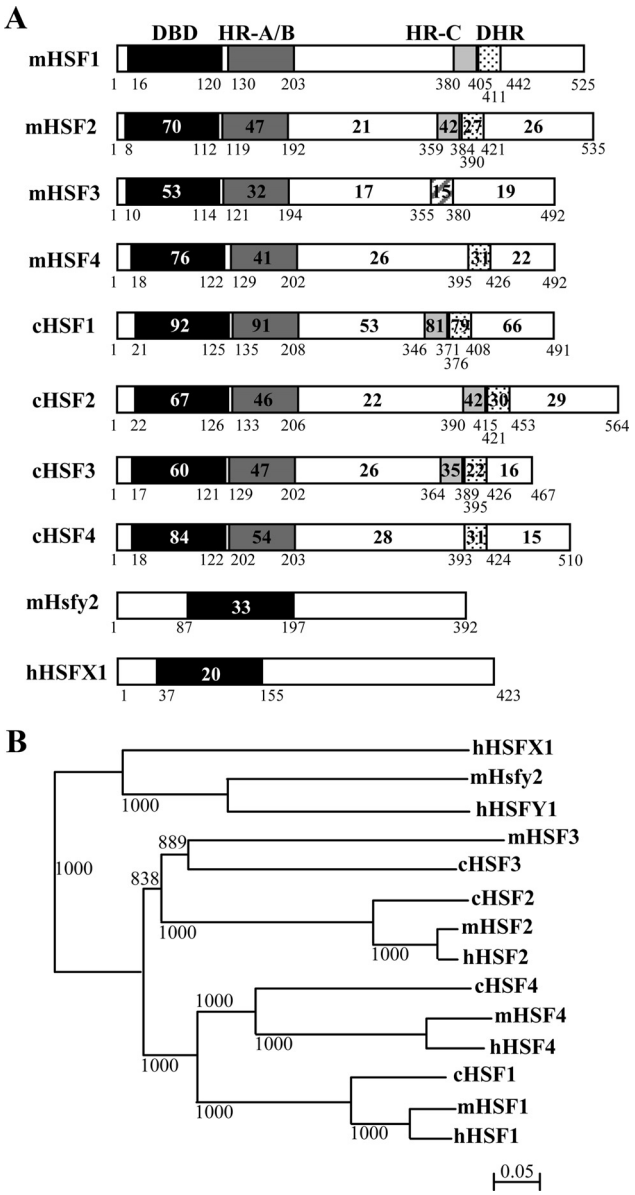

Figure 3. Members of the HSF superfamily. (A) Diagrammatic representation of mouse and chicken HSF family members and HSF-related gene products. The percent identity between mouse HSF1 and each HSF was established using the computer program GENETYX-MAC. The number of amino acids of each HSF is shown at the amino-terminal end. DBD, DNA-binding domain; HR, hydrophobic heptad repeat; DHR, downstream of HR-C. mHSF1 and mHSF2 (Sarge *et al.*, 1993); mHSF4 (Tanabe *et al.*, 1999); cHSF1, cHSF2, and cHSF3 (Nakai and Morimoto, 1993); mHsfy2 (originally identified as mHSFY, but its gene is not located on the mouse Y chromosome; Shinka *et al.*, 2004; Tessari *et al.*, 2004); hHSFX1/LW-1 (Swiss-Prot accession no. Q9UBD0). mHSF3 (mHSF3a) and cHSF4 (cHSF4b) were identified in this study. The dotted box indicates an HR-C-like domain, in which hydrophobic amino acids are not well conserved (Supplemental Figure 2). A part of the DBD domain of HSF family members is conserved with one region in mHsfy2 and hHSFX1. (B) The phylogenetic tree generated in CLUSTAL W (Thompson *et al.*, 1994) for vertebrate members of the HSF superfamily. Gaps were excluded from all phylogenetic analyses. The numerals represent bootstrap values (1000 bootstrap replicates were performed). The unrooted tree was drawn with the program TREEVIEW (Page, 1996). Bar, 0.05 substitutions per site. The amino acid sequences used in the tree's construction are hHSFY1 (*HSFY1* gene and its paralogous *HSFY2* gene are located on the human Y chromosome; Swiss-Prot accession no. Q96LI6), mHsfy2 (SP accession no. Q80Y37), and hHSFX1 (SP accession no. Q9UBD0). Amino acid sequences of other HSF family members were shown previously (Inouye *et al.*, 2003).

1994). Taken together, these results suggest that HSF3a exhibits sequence-specific interaction with the HSE. As the EST database also suggested the existence of another short transcript that contains exon 1a sequences, we used a primer corresponding to it and isolated cDNA for a short form of mHSF3 (mHSF3b isoform; Figure 2, A and B, line I), which lacks the N-terminal part of the DNA-binding domain and a C-terminal domain (Figure 2C). Recombinant mHSF3b could not bind to the HSE (Figure 2D), like hHSFY1 and hHSFX1 (LW1), sequences of which were partially conserved with those of the DNA-binding domain of HSFs (Figure 3, A and B; Shinka *et al.*, 2004; Tessari *et al.*, 2004).

mHSF3a Is Translocated to the Nucleus in Response to Heat Shock

To examine the localization of mHSF3, we overexpressed the GFP-mHSF3a fusion protein in the COS7 cells (Figure 4, A and B), and found that it stayed mostly in the cytoplasm (Figure 4C, a and b). When cells were heat-shocked at 42°C for 1 h, it moved into the nucleus like mammalian HSF1 and cHSF3 (c and d; Sarge *et al.*, 1993; Baler *et al.*, 1993; Nakai *et al.*, 1995). However, a truncated GFP-mHSF3b, which lacks N- and C-terminal regions, stayed in the cytoplasm even after heat shock (e–h). Deletion analysis showed that the N-terminal region of mHSF3a (amino acids 1-33) is necessary for nuclear translocation in the heat-shocked cells (i–t), although this region does not possess a typical nuclear localization signal such as the KKRK sequence (u–x), suggesting that it may modulate unmasking of the nuclear localization signal. These results strongly imply that mHSF3a plays a role in the protection of cells from detrimental stress.

mHSF3a Fails to Activate the Hsp70 *Gene*

We next determined its potential to induce major Hsps in MEF cells. Although adenovirus-mediated overexpression of cHSF3 elevated the expression of Hsp70, mHSF3a overexpression did not affect expression of major Hsps including Hsp70 at all even after heat shock, which caused the accumulation of ectopically expressed mHSF3a for unknown reasons (Figure 5, A and B). We substituted domains of mHSF3a with corresponding domains of cHSF3 and found that the chimeras, m194/c, m114/c, m9/c, and c389/m, which possess the cHSF3 activation domain (Supplemental Figure 3), induced Hsp70 expression at least in the heat-shock condition. In contrast, Hsp70 expression was not induced by the chimeric proteins m355/cDH and m355/cHR, which do not have any activation domain, or by c194/m, in which the DNA-binding domain and HR-A/B domain of mHSF3a were substituted with those of cHSF3 (Figure 5A). Furthermore, we substituted a C-terminal activation domain of human HSF1 (Green *et al.*, 1995; Shi *et al.*, 1995; Zuo *et al.*, 1995) with the activation domain of mHSF3a (Supplemental Figure 3) and found that the resultant protein h383/m did not induce expression of Hsp70 in the heat-shock condition (Figure 5A). These results indicate that the activation domain of mHSF3a does not have the potential to activate classical heat-shock genes.

mHSF3a Does Not Recruit BRG1

Why is mHSF3a unable to induce the expression of classical heat-shock genes? We overexpressed mHSF3a or hHSF1 in

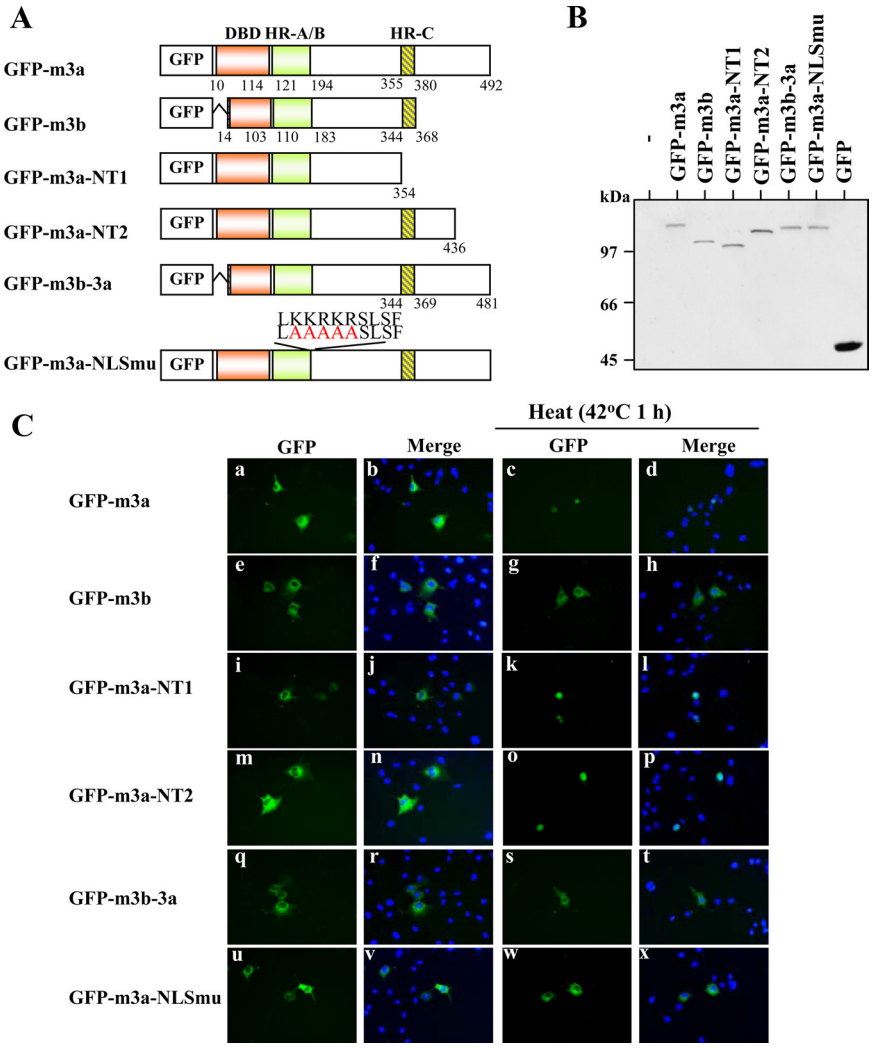

Figure 4. mHSF3a is translocated to the nucleus in response to heat shock. (A) Schematic representation of mHSF3a, mHSF3b, and mutants fused with GFP. A nuclear localization signal was mutated in GFP-m3a-NLSmu. (B) COS7 cells were transfected with expression vectors. Cell extract was prepared and subjected to Western blotting using an antibody for GFP. (C) COS7 cells transfected with expression vectors for 48 h were treated with heat shock at 42°C for 1 h and then fixed with 4% paraformaldehyde. Fluorescence was examined under an Axiovert microscope.

immortalized HSF1-null MEFs, in which the expression of classical heat-shock genes is not induced during heat shock (Inouye *et al.*, 2003). We found that both mHSF3a and hHSF1 bound to the promoter of the *Hsp70* gene in vivo in cells exposed to heat shock at 42°C for 1 h (Figure 6A). Although hHSF1 recruited RNA polymerase II to the Hsp70 promoter and its downstream region, the binding of mHSF3a to the Hsp70 promoter did not induce its recruitment (Figure 6B). Previous studies showed that hHSF1 recruits BRG1 to the Hsp70 promoter through direct interaction (Sullivan *et al.*, 2001) and expression of an HSF1 mutant, which cannot interact with BRG1, did not restore the induction of Hsp70 mRNA expression in HSF1-null MEF cells during heat shock (Corey *et al.*, 2003). Therefore, we examined the interactions of HSFs with BRG1 and found that hHSF1 and c389/m interacted with BRG1, whereas mHSF3 and h383/m did not (Figure 6C). Also, overexpression of hHSF1 and c389/m recruited BRG1 to the Hsp70 promoter and its downstream region in heat-shocked HSF1-null MEF cells, whereas mHSF3 and h383/m did not (Figure 6D). Furthermore, we examined the effects of reduced expression of BRG1 on the HSF1- and c389/m-mediated induction of Hsp70 expression in response to heat shock. When wild-type MEF cells were infected with adenovirus expressing shBRG1, the expression of Hsp70 was partially reduced (Supplemental Figure 4). In HSF1-null cells, overexpression of hHSF1 and c389/m restored the expression of Hsp70, but levels were much lower in the presence of shBRG1 (Figure 6E, Supplemental Figure 5). These results demonstrate that mHSF3a does not induce the expression of classical heat-shock genes partly due to its inability to interact with BRG1.

mHSF3a Has the Potential to Activate Nonclassical Heat-Shock Genes

It is possible that mHSF3a activates nonclassical heat-shock genes as it translocates into the nucleus in response to heat shock (Figure 4). We examined a set of up-regulated mRNAs in HeLa cells overexpressing cHSF1 by DNA microarray analysis (N. Hayashida, M. Fujimoto, H. Ichikawa, and A. Nakai, unpublished data) and found that the expression of some of the mouse orthologues are induced in wild-type MEF cells during heat shock (Supplemental Figure 6). Therefore, we examined the expression of two nonclassical heat-shock genes in more detail. HSF1-null MEF cells were infected with or without adenovirus expressing GFP, mHSF3a, or cHSF1 and then heat-shocked. Levels of mRNA for a PDZ domain–containing protein PDZK3/PDZD2/PAPIN (Deguchi *et al.*, 2000) increased greatly during heat shock in wild-type cells, but the induced levels were markedly reduced in HSF1-null cells (Figure 7A). mRNA levels for a membrane glycoprotein PROM2 (Fargeas *et al.*, 2003) were also increased in wild-type cells, but not at all in HSF1-null cells.

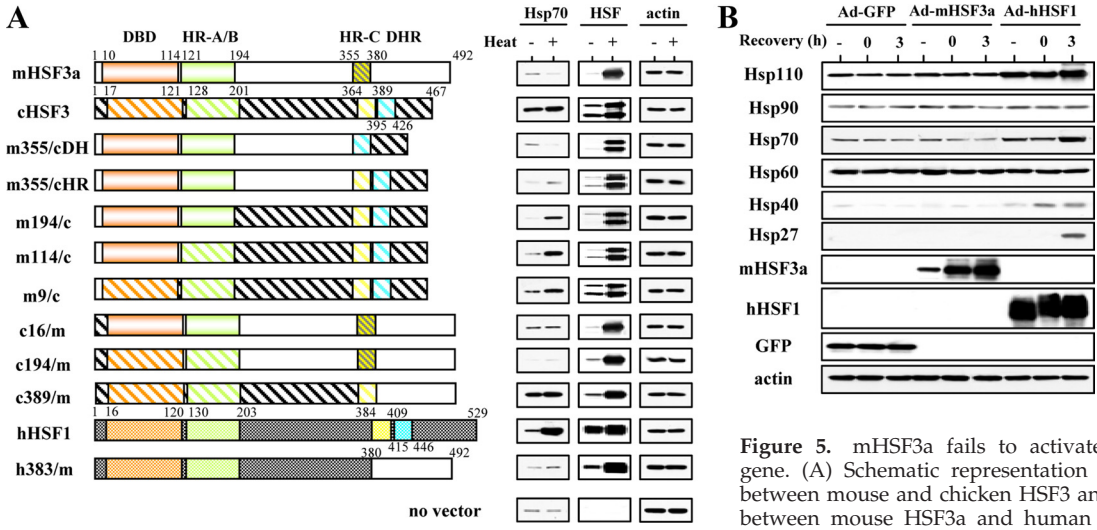

Figure 5. mHSF3a fails to activate the *Hsp70* gene. (A) Schematic representation of chimeras between mouse and chicken HSF3 and a chimera between mouse HSF3a and human HSF1 (left). Positions of amino acids at the terminals of each domain are shown. The h383/m chimera comprises human HSF1 (amino acid 1-384) fused to mouse HSF3a (amino acid 380-492). HSF1-null MEF cells were infected with each expression vector or with no vector, heat-shocked at 42°C for 1 h, and then allowed to recover at 37°C for 3 h. Cell extracts were prepared and subjected to Western blotting by using antibodies for Hsp70, mHSF3, cHSF3, HSF1, and β-actin (right). (B) HSF1-null MEF cells infected with Ad-GFP, Ad-mHSF3a, and Ad-hHSF1 were heat-shocked at 42°C for 1 h, and then allowed to recover at 37°C for 0 or 3 h. Cell extracts were prepared and subjected to Western blotting using antibodies for Hsps, HSF1, GFP, and β-actin.

Remarkably, overexpression of mHSF3a, as well as cHSF1, restored the expression of both PDZK3 and PROM2 mRNAs (Figure 7B). To examine the effects of endogenous mHSF3, wild-type and HSF1-null cells were infected with adenovirus expressing shRNA for mHSF3 (shHSF3-R1 and shHSF3-R2; Figure 7C). Levels of mHSF3a protein were markedly and partially reduced in cells expressing shHSF3-R1 and shHSF3-R2, respectively. The expression of PDZK3

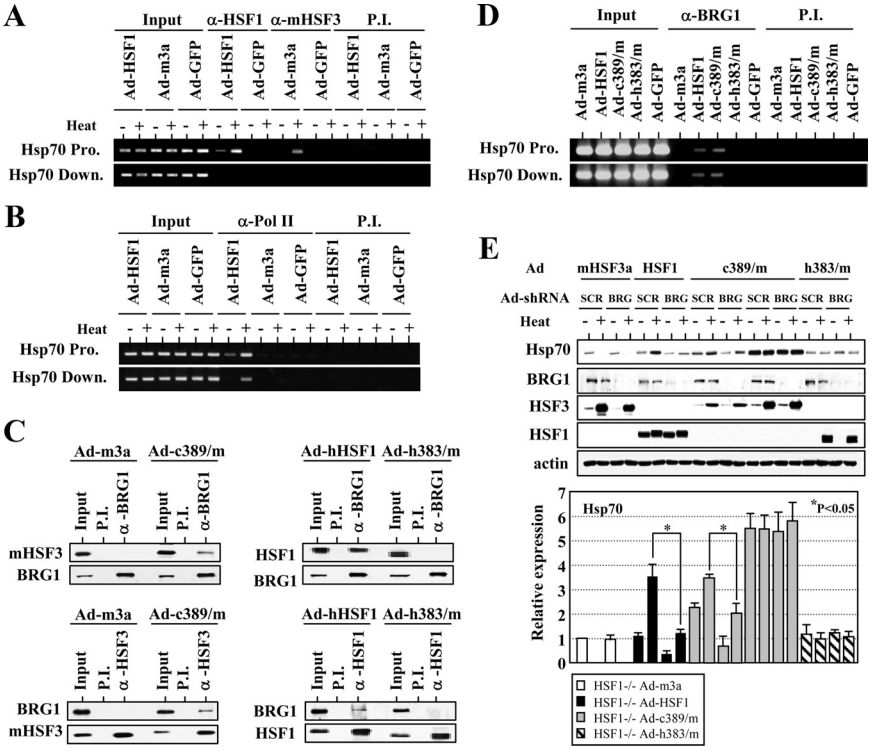

Figure 6. mHSF3a does not recruit BRG1. (A) Immortalized HSF1-null MEF cells were infected with an adenovirus expressing GFP, mHSF3a, or hHSF1, and then heat-shocked at 42°C for 1 h. ChIP was performed using antibodies for mHSF3 (α-mHSF3-1), hHSF1 (α-cHSF1c), and preimmune serum (P.I.). Precipitated DNA was amplified using primers for the Hsp70 promoter (−272 to +47) and its downstream region (+353 to +613). (B) HSF1-null MEF cells infected with each adenovirus were heat-shocked at 42°C for 1 h, and chromatin precipitation and DNA amplification were performed using primers for the Hsp70 promoter and its downstream region. (C) HSF1-null MEF cells were infected with an adenovirus expressing mHSF3a, hHSF1, or chimeras c389/m and h383/m. Cell extracts were prepared in an NP-40 lysis buffer, and immunoprecipitation was performed using antibodies for BRG1 (rat IgG), mHSF3 (α-mHSF3-1), and hHSF1 (α-cHSF1x). Precipitated complexes were subjected to Western blotting by using the same antibodies. (D) HSF1-null MEF cells infected with each adenovirus were heat-shocked at 42°C for 1 h. ChIP was performed using BRG1 and preimmune antibodies, and DNA amplification was conducted using primers for the Hsp70 promoter and a downstream region of its gene. (E) Decreased BRG1 expression results in a reduction in hHSF1- and c389/m-mediated Hsp70 induction. HSF1-null MEF cells were coinfected with an adenovirus expressing mHSF3a, hHSF1, c389/m (low and high levels), or h383/m and shBRG1 (shBRG1-R3) or scrambled RNA (SCR). Cells were then treated with (heat +) or without (heat −) heat shock at 42°C for 1 h with recovery at 37°C for 3 h. Expression levels of Hsp70 were examined by Western blotting (top), and quantified (bottom). Means and SDs are shown from three independent experiments.

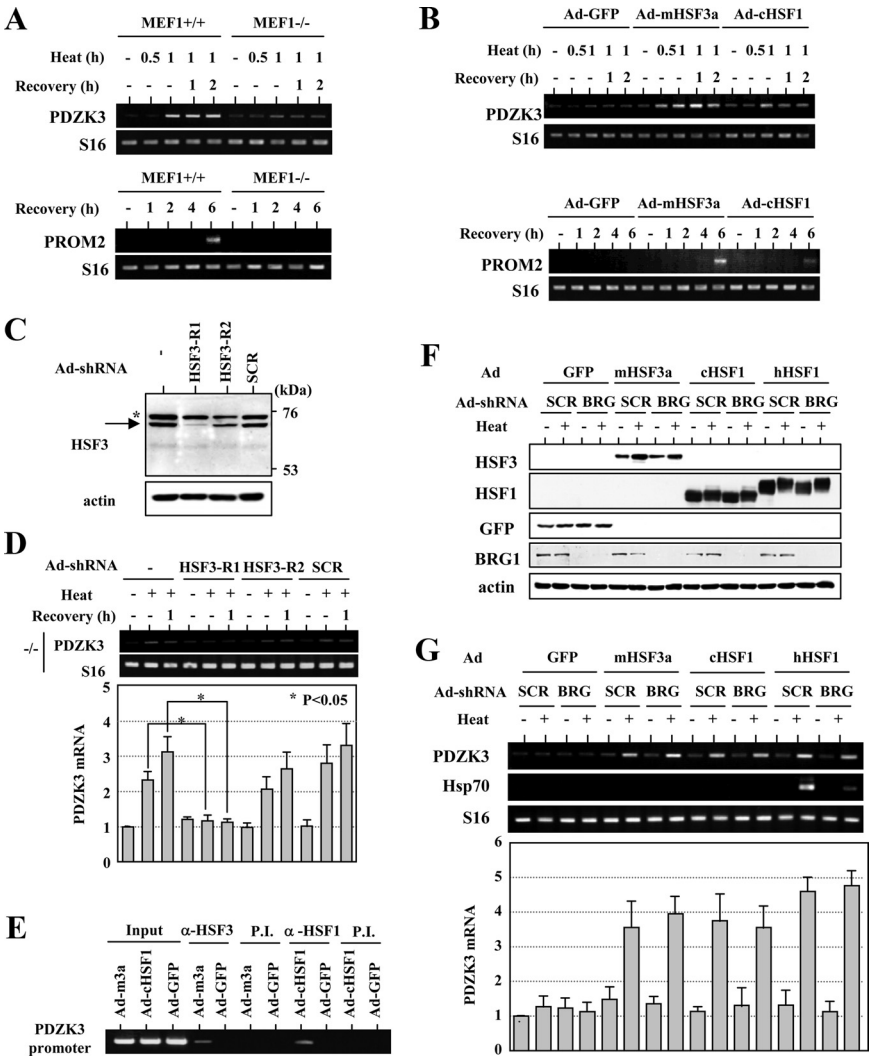

Figure 7. mHSF3a activates nonclassical heat-shock genes. (A) Wild-type (+/+) and HSF1-null (−/−) MEF cells were heat-shocked at 42°C and allowed to recover at 37°C for the indicated periods, and RT-PCR was performed to examine the mRNA levels of PDZK3, PROM2, and S16 ribosomal protein. (B) HSF1-null MEF cells infected with an adenovirus expressing GFP, mHSF3a, or cHSF1 were heat-shocked at 42°C and then left to recover at 37°C for the indicated periods, and RT-PCR was performed. (C) Wild-type MEF cells were left uninfected (−) or infected with an adenovirus expressing shHSF3-R1, shHSF3-R2, or scrambled RNA. A Western blot analysis was performed using an antibody for mHSF3 or β-actin. An arrow and an asterisk indicate mHSF3 and a nonspecific band, respectively. (D) HSF1-null MEF cells were infected as in C. Cells were heat-shocked at 42°C for 1 h (+), and left to recover for 0 or 1 h. RT-PCR was performed (top), and PDZK3 mRNA levels were quantified (bottom). Means and SDs are shown from three independent experiments; significance at *$p < 0.05$. (E) Adenovirus-infected HSF1-null MEF cells were heat-shocked at 42°C for 1 h. ChIP analysis was performed using preimmune serum (P.I.) and antibodies for mHSF3 and cHSF1, whereas DNA amplification was performed using primers for the PDZK3 promoter (−529 to + 24). (F) HSF1-null MEF cells were coinfected with an adenovirus expressing each HSF and shBRG1 or scrambled RNA. Western blotting was performed using each specific antibody. (G) Cells prepared in C were then treated with (heat +) or without (heat −) heat shock at 42°C for 1 h with recovery at 37°C for 3 h. mRNA levels of PDZK3, Hsp70, and S16 ribosomal protein were examined by RT-PCR (top), and PDZK3 mRNA levels were quantified (bottom). Means and SDs are shown from three independent experiments.

mRNA was not affected in wild-type cells expressing shHSF3-R1 (Supplemental Figure 7), but its moderate induction was abolished in HSF1-null cells expressing shHSF3-R1 (Figure 7D), whereas it was observed in cells expressing shHSF3-R2 and scrambled shRNA. We found that mHSF3a, as well as cHSF1 and hHSF1, bound to the PDZK3 promoter in the heat-shock condition (Figure 7E, Supplemental Figure 8). As expected, the induction of PDZK3 mRNA expression by mHSF3a, cHSF1, or hHSF1 was not impaired even though BRG1 expression was downregulated (Figure 7, F and G). These results demonstrate that mHSF3a has the potential to induce the expression of some nonclassical heat-shock genes in response to heat shock.

mHSF3a Can Protect Cells from Heat Shock and Proteotoxic Stress

We next examined whether mHSF3a can protect cells from detrimental stress. HSF1-null primary cultures of MEF cells were more sensitive to high temperature stress than wild-type cells (Inouye *et al.*, 2003). We found that overexpression of mHSF3a, as well as cHSF1, partially restored resistance to high temperature stress, whereas overexpression of GFP had no effect (Figure 8A). Furthermore, overexpression of mHSF3aR65A or mHSF3aR65G, which cannot bind to DNA because the DNA-contacting arginine at position 65 was substituted with alanine or glycine (data not shown; Inouye *et al.*, 2003), did not restore the resistance (Figure 8B). A reduction in mHSF3 expression increased sensitivity to high temperature in HSF1-null cells, but not in wild-type cells (Figure 8C). Overexpression of mHSF3a also inhibited the formation of polyglutamine aggregates in HeLa cells without the induction of major Hsps like overexpression of cHSF1 (Supplemental Figure 9; Fujimoto *et al.*, 2005). These results indicate that mHSF3a can protect cells from detrimental stress, probably through the induction of nonclassical heat-shock genes.

DISCUSSION

Mouse HSF3, especially mHSF3a, is a new member of the mammalian HSF family that possesses highly conserved DNA-binding and trimerization domains at its N-terminal, by which it binds to a consensus HSE with high-affinity. mHSF3 may be functionally more closely related to mammalian HSF1 than HSF2 and HSF4. First, the *mHSF3* gene is a mouse orthologue of the chicken *HSF3* gene (Figures 1 and 3), which is required for the expression of major Hsps during heat shock (Tanabe *et al.*, 1998). Second, both mHSF3 and HSF1 have activation domains at the C-terminal that strongly induce the expression of a GAL4-driven reporter

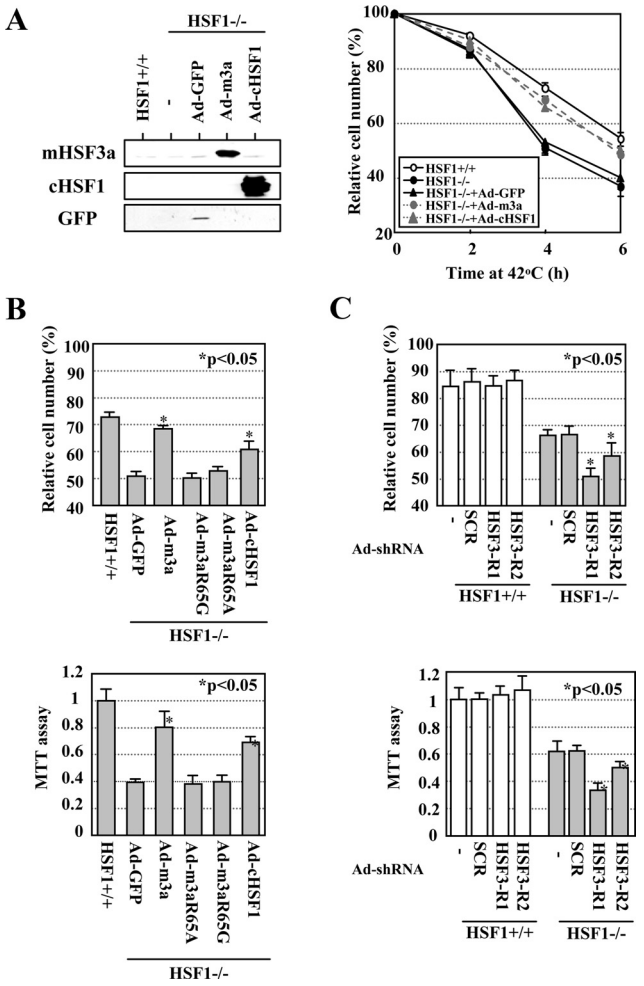

Figure 8. mHSF3a protects cells from high temperature stress. (A) Primary cultures of wild-type and HSF1-null MEF cells were infected with an adenovirus expressing GFP, mHSF3a, or cHSF1. Western blotting was performed using each specific antibody (left). Cells were infected with each adenovirus for 24 h and then incubated at 42°C for 0, 2, 4, and 6 h, and attached cells were counted (right). Means and SDs are shown from three independent experiments. (B) Wild-type and HSF1-null cells were infected with an adenovirus expressing GFP, mHSF3a, mHSF3aR65A, mHSF3aR65G, or cHSF3 for 24 h and then incubated at 42°C for 4 h. Attached cells were counted (left) and an MTT assay was performed (right). Means and SDs are shown from three independent experiments; significance at $^*p < 0.05$. (C) Wild-type and HSF1-null (−/−) cells were coinfected without (−) or with an adenovirus expressing shHSF3-R1, shHSF3-R2, or scrambled RNA for 72 h, and then incubated at 42°C for 6 h. Attached cells were counted (top) and an MTT assay was performed (bottom) as in B. Means and SDs are shown from five independent experiments; significance at $^*p < 0.05$.

gene (Supplemental Figure 3; Green *et al.*, 1995; Shi *et al.*, 1995; Zuo *et al.*, 1995), whereas the activation domains of HSF2 and HSF4 located in internal and C-terminal regions are five to 10 times weaker (Nakai *et al.*, 1997; Yoshima *et al.*, 1998). Third, mHSF3 is rapidly translocated into the nucleus like HSF1 during heat shock (Figure 4; Baler *et al.*, 1993; Sarge *et al.*, 1993). In contrast, only some HSF2 moves into the nucleus (Sheldon and Kingston, 1993; Alastalo *et al.*, 2003), and HSF4 stays in the nucleus even in unstressed cells (Nakai *et al.*, 1997). Last, mHSF3 binds to the promoter of the *Hsp70* gene in vivo in cells exposed to heat shock independent of HSF1 (Figure 6), whereas the binding of HSF2 on the Hsp70 promoter requires HSF1 (Ostling *et al.*, 2007). Even though mHSF3 is closely related to mammalian HSF1, it is unable to induce the expression of major Hsps during heat shock in the absence of HSF1 (Figure 5) and may compete with HSF1 for the HSE as overexpression of mHSF3a suppressed induction of major Hsps (Supplemental Figure 10). Instead, mHSF3 has the potential to induce the expression of heat-shock genes other than classical heat-shock genes independent of HSF1 (Figure 7). Thus, mHSF3 may play a unique role in response to heat shock.

Comprehensive analyses of HSF1-binding regions in the whole genome have revealed that ~3% of genes are direct targets in heat-shocked cells in yeast and *Drosophila* (Hahn *et al.*, 2004; Brich-Machin *et al.*, 2005), in which HSF is encoded by a single gene, and expression of the majority of the target genes is induced during heat shock (Hahn *et al.*, 2004). In mammalian cells, HSF1 binds to many promoters of possible heat-shock genes (Trinklein *et al.*, 2004; Page *et al.*, 2006), and about a half of the target genes are expressed during heat shock (Trinklein *et al.*, 2004). Furthermore, 33% of genes near HSF4-binding regions, which are also occupied by HSF1, are induced during heat shock in the mouse lens (Fujimoto *et al.*, 2008). Thus, there are tremendous numbers of nonclassical heat-shock genes, and it is possible that some of them are induced by mHSF3 in response to stress.

We wondered why mHSF3 failed to induce transcription of the *Hsp70* gene while it bound to its promoter. It is known that bound HSF1 to the Hsp70 promoter recruits BRG1, a component of SWI/SNF chromatin remodeling complexes (Sullivan *et al.*, 2001) and that this recruitment is necessary for the initiation and elongation of Hsp70 transcription in mammalian cells (Corey *et al.*, 2003). BRG1 bound to human HSF1, but not to mHSF3, or a mutant h383/m, in which an activation domain of hHSF1 was substituted with that of mHSF3. This result indicates that the inability of mHSF3 to induce Hsp70 expression is in part due to a lack of interaction of the mHSF3 activation domain with BRG1. As BRG1 is necessary for the expression of a limited number of genes (Kwon and Wagner, 2007), we expected the expression of some nonclassical heat-shock genes to be induced by mHSF3. We found that overexpression of mHSF3 restored levels of PDZK3 and PROM2 mRNAs in HSF1-null cells (Figure 7). BRG1 is not recruited to the PDZK3 promoter even in the presence of HSF1 (data not shown), suggesting complex mechanisms for stabilizing chromatin remodeling complexes in the genome.

We previously showed that chicken HSF1 (cHSF1) is dispensable for the expression of classical heat-shock genes in chicken B lymphocyte DT40 cells (Nakai and Ishikawa, 2001), but played a significant role in the protection of cells from stress including high temperature, irradiation, and UV rays (Inouye *et al.*, 2003). Ectopic expression of cHSF1 in HSF1-null MEFs did not activate classical heat-shock genes, but protected cells from exposure to high temperatures (Inouye *et al.*, 2003). Furthermore, HeLa cells ectopically expressing cHSF1 are more resistant to the toxic effects of polyglutamine aggregates (Fujimoto *et al.*, 2005). Here, we showed that mHSF3 plays a similar role to cHSF1 through the regulation of gene expression (Figure 8), and propose that mHSF3 is a functional orthologue of chicken HSF1, which may induce the expression of genes other than classical heat-shock genes. mHSF3 is not required for the expression of a limited number of genes examined here and for the protection of MEF cells from heat shock in the presence of HSF1, indicating that mHSF3 may play a redundant role in MEF cells. However, it is possible that it uniquely regu-

lates some sets of genes or plays an indispensable role in some types of cells.

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