

Analysis of HSF4 Binding Regions Reveals Its Necessity for Gene Regulation during Development and Heat Shock Response in Mouse Lenses*^[5]

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Heat shock transcription factors (HSFs) regulate gene expression in response to heat shock and in physiological conditions. In mammals, HSF1 is required for heat-mediated induction of classic heat shock genes; however, we do not know the molecular mechanisms by which HSF4 regulates gene expression or the biological consequences of its binding to chromatin. Here, we identified that HSF4 binds to various genomic regions, including the introns and distal parts of protein-coding genes *in vivo* in mouse lenses, and a substantial numbers of the regions were also occupied by HSF1 and HSF2. HSF4 regulated expression of some genes at a developmental stage when HSF1 and HSF2 expression decreased. Although HSF4 binding did not affect expression of many genes, it induces demethylated status of histone H3K9 on the binding regions. Unexpectedly, a lot of HSF4 targets were induced by heat shock treatment, and HSF4 is required for induction of a set of non-classic heat shock genes in response to heat shock, in part by facilitating HSF1 binding through chromatin modification. These results suggest novel mechanisms of gene regulation controlled by HSF4 in non-classic heat shock response and in lens development.

Heat shock response is characterized by induction of a set of heat shock proteins (Hsps)³ and is a fundamental adaptive response that maintains protein homeostasis (1–3). This response is regulated mostly at the level of transcription by heat shock transcription factors (HSF1–4) (4). The classic heat shock genes contain a heat shock element (HSE) that is composed of at least three inverted repeats (typically six to nine) of

the consensus sequence nGAAAn (5). Heat shock triggers conversion of an HSF1 monomer that is negatively regulated by heat shock proteins into a trimer that can bind to HSE with a high affinity, and bound HSF1 rapidly induces robust activation of the heat shock genes (6). Among HSF family members, HSF1 is required for induction of heat shock genes in mammals when cells or tissues are exposed to heat shock (7–9). This HSF1-mediated induction of Hsps is required for acquisition of thermotolerance (7, 8, 10) and protection of cells from various pathophysiological conditions such as neurodegenerative diseases (11–13) and other degenerative diseases (14, 15). Inversely, HSF1 also induces cell death by up-regulating a proapoptotic gene in response to stress in some cells such as male germ cells (16, 17).

Even in physiological conditions, all three HSFs, including HSF2 and HSF4, must regulate gene expression (18). HSF2 is highly expressed in early developmental stages and stays mostly a dimer (19, 20). Although we do not know what triggers HSF2 activation, it is converted to an active HSE-binding trimer when erythroleukemia cells are differentiated (21). HSF4 is highly expressed in the lens (22, 23), and basically remains as an HSE-binding trimer, because it uniquely lacks an inhibitory domain of trimerization (24, 25). HSFs play critical functions in developmental processes such as gametogenesis and neurogenesis (26–31), in maintenance of sensory organs and ciliated tissues (22, 32–35), and in immune response (36, 37). Furthermore, HSF1 plays major roles in lifespan (11, 12) and in progression and maintenance of cancer (38, 39). It has been revealed that, in these physiological and pathological processes, HSFs not only maintain protein homeostasis by regulating constitutive expression of Hsps but are also involved in cell growth and differentiation by regulating expression of genes such as *IL-6*, *FGFs*, *LIF*, and *p35* (9, 22, 31, 35, 40). However, all reports have analyzed a limited set of classic heat shock genes and some development-related genes. We do not know how HSF2 and HSF4, as well as HSF1, regulate gene expression in physiological conditions, or the relationship between regulation of HSF1-mediated heat shock response and regulation of genes by HSFs during development. To answer these questions, it is necessary to identify HSF-target genes comprehensively and to analyze regulation of these genes.

The lens is composed of only two cell types, epithelial cells and fiber cells (41). HSF4, as well as HSF1 and HSF2, is expressed in both cell types at early stage of lens development

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^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S7 and Tables S1 and S2.

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³ The abbreviations used are: Hsp, heat shock protein; ChIP, chromatin immunoprecipitation; HSF, heat shock transcription factor; HSE, heat shock element; PBS, phosphate-buffered saline; GST, glutathione S-transferase; RT, reverse transcription.

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and is required for normal cell growth and differentiation of the two cells (22). Therefore, the lens tissue is suitable for comprehensive identification of HSF4-target genes and their analysis. Such analysis revealed unexpected roles of HSF4 in the regulation of gene expression during development and in the regulation of heat shock-mediated gene expression.

MATERIALS AND METHODS

Cell Culture and Adenovirus Infection—Two lenses from a 2-day-old wild-type or HSF4-null mouse (22) were treated with 0.5 ml of 0.1% trypsin/phosphate-buffered saline (PBS) at 37 °C for 30 min, and incubated for more 30 min after being added with 0.5 ml of 1% collagenase/PBS. The lens cells were dissected by using a vortex, collected by centrifugation, and maintained in a 48 well plate at 37 °C in 5% CO₂ in Dulbecco's modified eagle medium containing 10% fetal bovine serum. Immortalized wild-type LEWd2 and HSF4-null LE4Nd2 cells were established by maintaining the cells for 24 h with a retroviral vector expressing a large T antigen of SV40 (42), which was produced by using PLAT-E packaging cells (43).

To overexpress HSF4 in cultured lens cells, we generated adenovirus expressing human HSF4b isoform (Ad-hHSF4) by inserting an XbaI/KpnI fragment of pGEM7-hHSF4b (25) into a pShuttle vector (Clontech) as described previously (13). Immortalized lens cells were infected with Ad-hHSF4 at a titer of $2 \sim 10 \times 10^5$ plaque-forming units/ml. At 24 h after infection of viruses, cells were treated with heat shock and then harvested for analysis.

Western Blot Analysis—To examine protein levels of HSFs, the lenses were homogenized in Nonidet P-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, 1 μg/ml pepstatin) by sonication and centrifuged at $12,000 \times g$ for 10 min (22). Aliquots containing 300 μg of protein were loaded on SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blotted with α-mHSF4t (25). To detect HSF1 and HSF2, we used an antiserum α-mHSF1J that was raised against recombinant mouse HSF1 (amino acids 130–503) fused to GST, and α-mHSF2–4 that was raised against recombinant mouse HSF2 (amino acids 107–517) fused to GST. To detect Hsps and crystallins, aliquots containing 20 μg of protein were subjected to Western blot analysis (22).

Gel Shift Assay and Gel Filtration—The lenses were dissected and stored at –80 °C until use. They were homogenized in buffer C by using a Dounce homogenizer, frozen, and then thawed (22). After centrifuging at $100,000 \times g$ for 5 min at 4 °C, the supernatants were frozen in liquid nitrogen and stored at –80 °C. Aliquots containing 80 μg of proteins were subjected to gel shift assay using an ideal HSE oligonucleotide as a probe in the presence or absence of antiserum for each HSF, α-HSF1γ, α-HSF2d, or α-HSF4b (2.0 ml of 1:10 diluted antiserum in PBS) (24, 44). Random oligonucleotide selection was performed essentially as described previously (45). Recombinant GST-hHSF4 were induced in *Escherichia coli* by treating it with 0.4 mM isopropyl thiogalactosidase for 3 h, purified by using glutathione-Sepharose 4B (GE Healthcare Bio-Sciences Ltd.), and aliquots containing 2 μg of recombinant protein were subjected to gel shift assay. To compare the DNA binding activ-

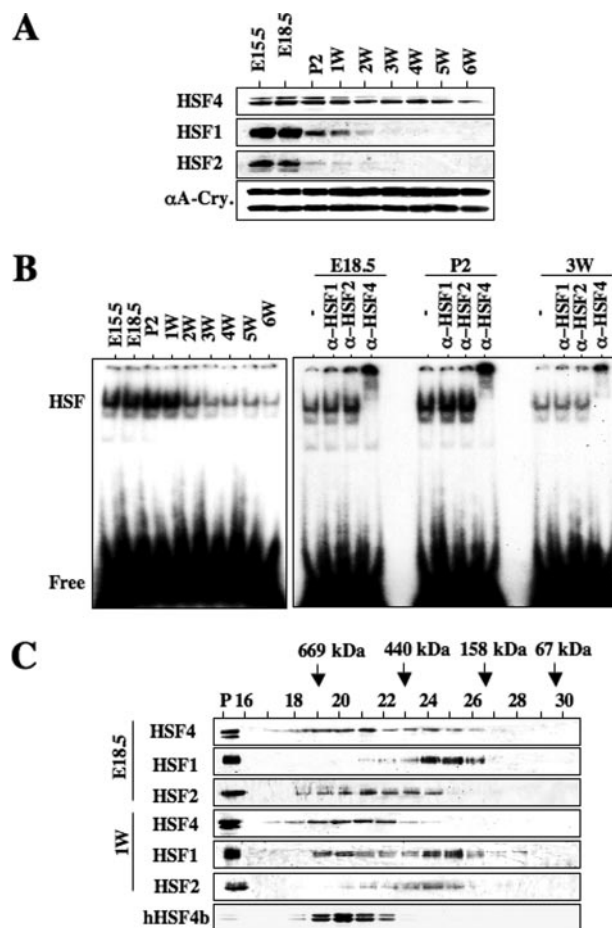


FIGURE 1. Temporal profiles of HSF expression during lens development. A, protein levels of HSFs and Hsps. Western blot analysis was performed using the lens extracts isolated from embryos (E15.5 and E18.5), mice at 2 days after birth (P2), and 1-week-old (1W) to 6-week-old (6W) mice. B, DNA binding activities in the lens extracts from developing mice. Gel shift assay was performed using a ³²P-labeled oligonucleotide as a probe in the absence (left) or presence of antiserum for each HSF (right). C, gel-filtration analysis of HSFs in the lens extracts from E18.5 embryos and 1-week-old mice. Fractionation of a human HSF4b isoform overexpressed in Cos7 cells, which forms a trimer, is shown at the bottom. Numbers of fractions are indicated, and P indicates blots using total lens extracts. The protein standards are: thyroglobulin, 669 kDa; ferritin, 440 kDa; aldolase, 158 kDa; and albumin, 67 kDa.

ity of HSFs, aliquots containing equal amounts of recombinant GST-hHSF1, GST-hHSF2, and GST-hHSF4 were subjected to gel shift assay. Gels were dried and exposed to HR-HA30 film (Fujifilm) with an intensifying screen. The DNA-binding activities were quantified by using NIH Image. Gel-filtration analysis was performed as described previously (25, 35) by using the lens extracts in buffer C.

Chromatin Immunoprecipitation—Immortalized lens epithelial LEW2d cells established from the lenses of a 2-day-old wild-type mouse in a 100-mm dish were treated with 10 ml of 1% formaldehyde/Dulbecco's modified eagle medium containing 10% fetal bovine serum at 37 °C for 10 min. After washing with PBS two times, the cell were suspended in 1 ml of PBS and centrifuged at 2000 rpm for 2 min. The pellet fractions were suspended in 200 μl of SDS-lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)), incubated at 4 °C for 10 min, sonicated, mixed with 4 μl of an rabbit antiserum for HSF4 (α-hHSF4–3 that was raised against recombinant human HSF4

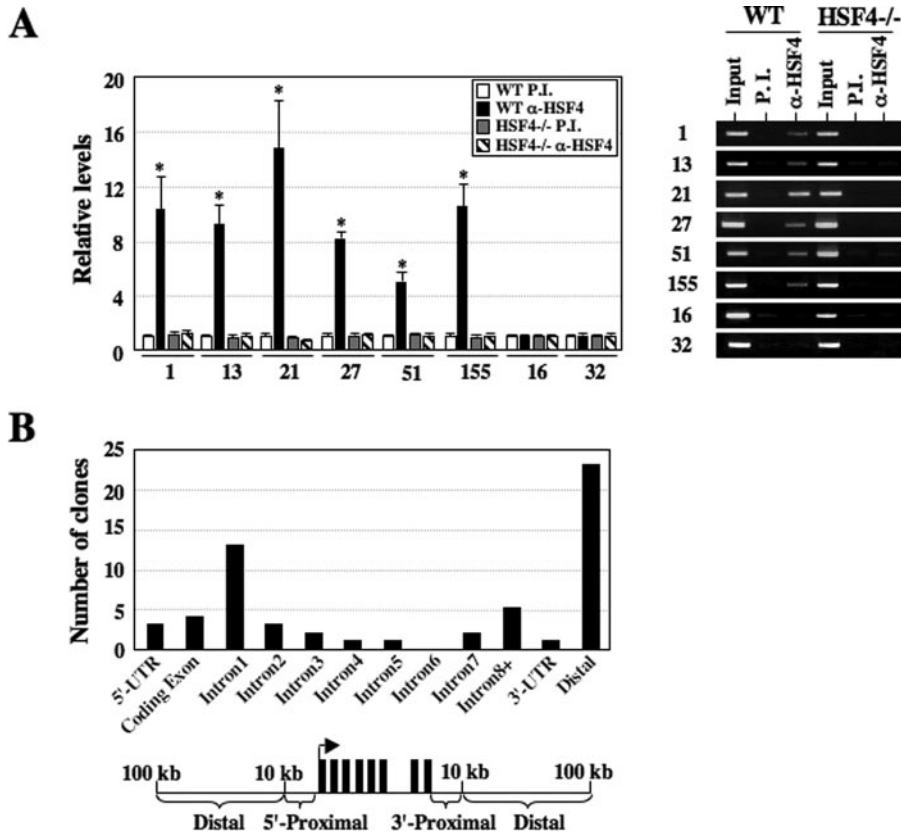


FIGURE 2. Identification of HSF4 binding regions *in vivo*. A, confirmation of HSF4 binding regions in the wild-type (WT) and HSF4-null (HSF4^{-/-}) P2 lenses by ChIP analysis using preimmune serum (P.I.) or anti-HSF4 serum (α -HSF4). To amplify DNA fragments, 35–40 cycles of PCR reactions were performed. A total of 58 of 71 regions are specifically bound by HSF4, and the other 13 regions are not. The amplified DNA bands were quantified, and some of them are shown. Means and standard deviations are shown from three independent experiments. Numbers of the binding regions are indicated. B, the locations of the 58 HSF4 binding regions are shown in relation to a gene structure model. The number of binding regions at each particular location is shown. The binding regions of coding exons or intron 8 to intron 14 are collectively shown as Coding Exon or Intron8+, respectively. The 5'- or 3'-proximal regions (Proximal) and distal parts (Distal) are shown.

fused to GST), and incubated at 4 °C for 16 h. Chromatin immunoprecipitation (ChIP) was performed using a ChIP assay kit (Upstate, New York) essentially according to the manufacturer's instructions. The precipitated DNA fragments were inserted into a pCR-Blunt II-TOPO vector (Invitrogen) after they were treated with T4 DNA polymerase. Sequencing reactions of the cloned DNA fragments were performed with an ALFexpress AutoRead sequencing kit, and the sequences were analyzed using an ALFexpress sequencer (GE Healthcare Bio-Sciences Ltd.) and mapped to the mouse genome using the BLAST algorithm. To confirm specific binding of HSF4 in LEWd2 cells, 35–40 cycles of PCR reactions were performed to amplify DNA using primers that hybridize to the end of each precipitated DNA (supplemental Table S1). Primers used to amplify Hsp70-1 promoter were described previously (46). ChIP analysis of the lens was performed exactly as reported previously (22), by using 12 lenses from E18.5 embryos, 8 lenses from 2-day-old mice, and 4 lenses from 3-week-old mice. Antibodies used for ChIP assay were a rabbit anti-serum for HSF1 (α -cHSF1c) (40), for HSF2 (α -mHSF2-4), or for acetyl-histone H3 (Lys9) (#07-352, Upstate), a rabbit IgG for dimethyl-histone H3 (Lys9) (#07-212, Upstate), and a mouse IgG for phospho-RNA polymerase II (#05-623,

Upstate). The amplified DNA was stained with ethidium bromide, photographed, scanned, and quantified by NIH Image.

RT-PCR Analysis—RT-PCR analysis of mRNA levels of genes on and near the HSF4 binding regions was performed essentially as described previously (22), using specific primers (supplemental Table S2). The amplified DNA was stained with ethidium bromide and photographed using Epi-Light UV FA1100 (Aisin Cosmos R&D Co., Japan).

Lens Organ Culture—Lenses isolated from 2-day-old wild-type, HSF4-null (22), and HSF1-null (9) mice were incubated in 35-mm dishes at 37 °C in 5% CO₂ in 2 ml of serum-free medium 199 (Invitrogen) (47). To treat the lenses with heat shock, culture dishes were submerged in a water bath for 30 min at 37, 41, 42, 43, and 45 °C, and then lens extracts in an Nonidet P-40 lysis buffer were prepared for Western blot analysis. Alternatively, the heat-shocked lenses were recovered at 37 °C at 6 h, and total RNAs were isolated for RT-PCR analysis.

Statistical Analysis—Significant values were determined by analyzing data with the Mann-Whitney *U* test using StatView version 4.5J for

Macintosh (Abacus Concepts, Berkeley, CA). A level of $p < 0.05$ was considered significant.

RESULTS

Temporal Profiles of HSF Expression during Mouse Lens Development—We previously showed that HSF4 mRNA starts to be expressed at embryonic day 13.5 in both lens epithelial and fiber cells. Also, disruption of HSF4 gene results in abnormal expression of *FGF* genes in lens epithelial cells and of *Hsp27* and γ -*crystallin* genes in fiber cells (22). These results indicate that HSF4 plays major roles in both lens cell types during development and maintenance. Therefore, we examined the relative expression levels of HSF family members in whole mouse lenses in detail, and found that the level of HSF4 protein in the lenses was high even at embryonic day 15.5, reaching a peak at 2 days after birth. Then, it gradually decreases, but continues to be expressed at substantial levels even at 6 weeks (Fig. 1A). In marked contrast, levels of HSF1 and HSF2 proteins in the lenses were relatively high at embryonic days 15.5 and 18.5, but they decreased quickly after birth. Consequently, we detected a small amount of HSF1 and HSF2 proteins at 2 days after birth, but hardly detected them at 3 weeks (Fig. 1A). Similar expres-

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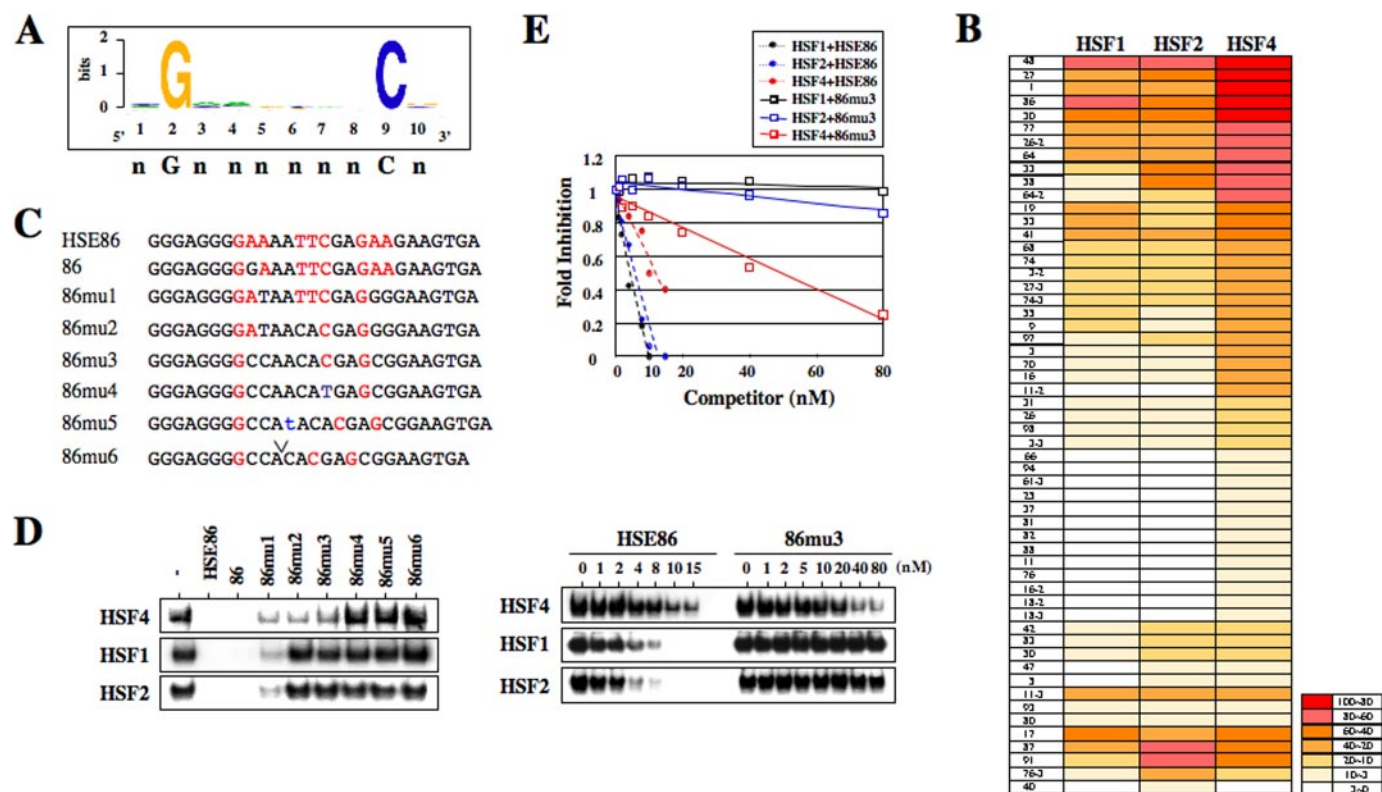


FIGURE 3. Sequence properties of HSF4 binding sites *in vitro*. *A*, DNA consensus sequences for HSF4 binding *in vitro* were generated with WebLogo by using DNA sequences of 108 HSF4 binding DNA fragments (supplemental Fig. S2). The height of each letter represents the relative frequency of nucleotides at different positions in the consensus. *B*, DNA binding activities of HSFs to the HSF4-bound oligonucleotides were compared by gel shift assay. The activities were quantified and shown by relative quantities to the HSF4 binding activity to an oligonucleotide (#48) as 100%. The upper 43 rows represent oligonucleotides to which HSF4 bound stronger than HSF1 and HSF2. *C*, sequences of an oligonucleotide (#86) and its mutants. Nucleotides that matched the nGAAn sequence are shown as red characters and an inserted nucleotide as blue. A nucleotide deletion position is indicated by a triangle. HSE86 has three perfect nGAAn sequences. *D*, gel shift assay was performed by using a ^{32}P -labeled HSE86 oligonucleotide as a probe and extracts from Cos7 cells overexpressing each HSF, in the absence or presence of the indicated cold oligonucleotides as competitors. *E*, gel shift assay was performed by using a ^{32}P -labeled HSE86 oligonucleotide and extracts from Cos7 cells overexpressing each HSF, in the presence of increasing amounts of cold HSE86 and HSE86mu3 oligonucleotides as competitors (lower). Relative DNA binding activities compared with the activities in the absence of competitors are shown (upper).

sion profiles of HSFs in developing rat lenses were reported previously (23).

We next examined DNA-binding activity with gel shift assay using an consensus HSE probe and found that HSE-binding activity in the lenses was high even at embryonic day 15.5, reaching a peak at 2 days after birth, and then gradually decreasing. A supershift experiment using each specific antiserum demonstrated that HSE-binding activities in the lenses at embryonic day 18.5, at day 2 after birth, and at 3 weeks were mostly composed of HSF4 (Fig. 1B). To clarify why HSF1 and HSF2 do not have major HSE-binding activities in the lenses, we examined oligomeric forms by gel-filtration analysis. As we expected (24, 25), a major population of HSF4 was an HSE-binding trimer, whereas HSF1 was a monomer and HSF2 could be a dimer in the lenses at embryonic day 18.5 (Fig. 1C). A substantial population of HSF1 forms a trimer in the lenses at 1 week, but the level of whole HSF1 protein was quite low at this developmental stage (Fig. 1A).

Identification of HSF4 Binding Regions—To identify HSF4 binding regions in the lens tissue, we first performed ChIP analysis by using immortalized lens LEWd2 cells established from the lenses of 2-day-old wild-type mice to diminish false-positive clones. The precipitated DNA fragments were cloned into a plasmid vector, and their sizes were 120 bp to 1.6 kb (161

clones). Among the DNA fragments from 120 to 500 bp in size (125 clones, 78%), 100 fragments were sequenced and 71 sequences were mapped to the mouse genome using a BLAST search, although other 29 sequences were repetitive sequences or could not be determined. We generated primers specifically hybridized to the 71 DNA fragments (supplemental Table S1) and confirmed binding of HSF4 on the identified DNA in the lenses from 2-day-old mice by ChIP analysis (Fig. 2A). We concluded that HSF4 binds specifically to 58 of 71 DNA regions (82%), because a preimmune serum did not precipitate the DNA fragments and they were not precipitated in the HSF4-null lens. We failed to show binding of the other 13 DNA regions in the lens, but in the LEWd2 cells we were able to detect the binding of HSF4 to 6 of 13 DNA regions (total of 64 HSF4-binding DNA regions, 90%) (data not shown).

We next determined the locations of HSF4 binding in 58 regions relative to gene coding sequences along the genome in the lens (Fig. 2B). It appeared that 31 regions (53%) located on the introns and exons of protein-coding genes and 23 regions (40%) were on distal parts of the genes. Among 31 regions on the introns and exons, HSF4 preferentially bound to the first intron (13 regions, 22%). Unexpectedly, a number of HSF4 binding regions on the promoter, which is a 5'-proximal region within 10 kb from each transcription start site, was quite low (3

regions, 5%). These results demonstrated the importance of genomic regions distinct from the promoter-proximal regions as HSF4 binding sites, and highlighted the introns of protein-coding gene and distal parts.

Sequence Properties of HSF4 Binding Sites—To understand the molecular mechanisms of HSF cooperation on the HSF4 binding regions, we first examined sequences of HSF4 binding regions, which is composed of at least three inverted repeats of nGAA n pentameric units (“G” should be conserved) (48, 49). Among them, only six HSE-like sequences contained two to four perfect GAA sequences, but the others had none or only one GAA sequence (supplemental Fig. S1). To identify binding site sequences of HSF4 more clearly, we performed *in vitro* random oligonucleotide selection by using purified recombinant GST-HSF4 as described previously to identify binding sequences for HSF1 and HSF2 (50). Sequence analysis of 108 HSF4-bound clones revealed that they contain HSE-like sequences (supplemental Fig. S2) for which conservation at each position was indicated as the height of the stack in a sequence logo generator, WebLogo (weblogo.berkeley.edu). We found that HSF4 binds to inverted repeats of nGnnn pentameric units (Fig. 3A). Ten clones (9%) were composed of only two inverted repeats but may use another distantly located unit (51). Because the HSF4 binding consensus sequence was more ambiguous than that of HSF1 and HSF2, we compared their binding activity to the HSF4-bound DNA fragments by gel shift assay (Fig. 3B). This revealed that recombinant HSF4 bound at higher levels to 43 of 56 DNA fragments (77%) than HSF1 and HSF2, and HSF1 or HSF2 bound to 10 DNA fragments (18%) at similar levels to HSF4. HSF4 bound less to only three fragments (5%) than did HSF2. These results indicate that HSF4 prefers sequences of the isolated DNA fragments.

We next examined the binding activity to an isolated DNA fragment (#86) and to its mutants by competition assay (Fig. 3, C and D). HSE86 contains three perfect nGAA n pentameric units, and “A” nucleotides were substituted to other nucleotides in mutants 1, 2, and 3. Remarkably, HSF4 binding activity to the ³²P-labeled HSE86 was competed with cold mutants 2 and 3, whereas HSF1 or HSF2 binding activity was not (Fig. 3D). The binding activity was not competed with mutants in which “G” was substituted with T (mutant 4), or one nucleotide was deleted or inserted (mutant 5 or 6, respectively). We had the same results by using another DNA fragment (#26) and its mutants, and only HSF4 binding activity was competed with randomly selected HSF4-bound DNA fragments (#18 and #41), in which none of the A nucleotides were conserved in the consensus nGAA n sequence (data not shown). We further performed a competition assay by increasing the amounts of competitor oligonucleotides (Fig. 3E) and found that HSF1 and HSF2 bound to HSE86 with higher affinity than HSF4. HSF4 still bound to mutant 3, whereas HSF1 and HSF2 failed to bind to it.

To determine the apparent dissociation constant (K_d) for recombinant HSFs, we then performed saturation binding (supplemental Fig. S3). As reported previously (50), HSF1 and HSF2 had similar K_d values of 2.9 and 2.3 nM, respectively, to HSE86, whereas HSF4 had a higher K_d value of 11 nM. By

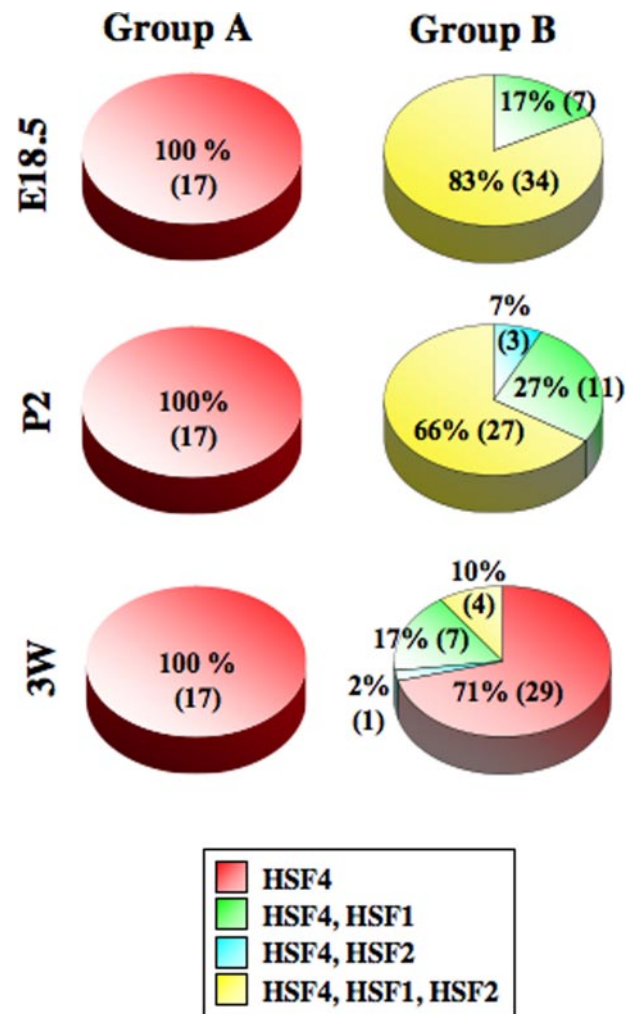


FIGURE 4. Developmental profiles of HSF occupancy in the two groups of HSF4 binding regions. Only HSF4 bound to the regions in the lens of E18.5 embryos (E18.5) in group A (17 regions), whereas multiple HSFs bound to the regions in group B (41 regions). As expression of HSF1 and HSF2 decreased, they tended to lose binding to the HSF4 binding regions. Percentages of fractions in each group in the lenses of E18.5 embryos, 2-day-old mice (P2), and 3-week-old mice (3W) are shown.

increasing the concentration of HSF4, we still detected saturation of HSF4 binding to the HSE86 mutant 3, in which none of the A nucleotides were conserved in the nGAA n sequence, and the value was 384 nM. Altogether, these results demonstrate that HSF4 uniquely binds to inverted repeats of a consensus nGnnn pentameric unit.

Temporal Profiles of Occupancy of HSF Family Members—To clarify developmental profiles of occupancy of HSF family members on the identified HSF4 binding regions, we first examined HSF binding in the lenses of E18.5 embryos. We found that 17 regions (group A) were occupied by only HSF4, although all three HSFs were abundantly expressed in the E18.5 lenses (Fig. 4A, supplemental Fig. S4). Among 41 other regions (group B), 34 regions (83%) were occupied by all of three HSFs and 7 regions (17%) by HSF4 and HSF1 (Fig. 4A and supplemental Fig. S5). The HSF4 binding regions in group A continued to be occupied by only HSF4 in the lenses of 2-day-old and 3-week-old mice (P2 and 3W lenses, respectively). In contrast, HSF1 and HSF2 tended to lose binding on the HSF4 binding

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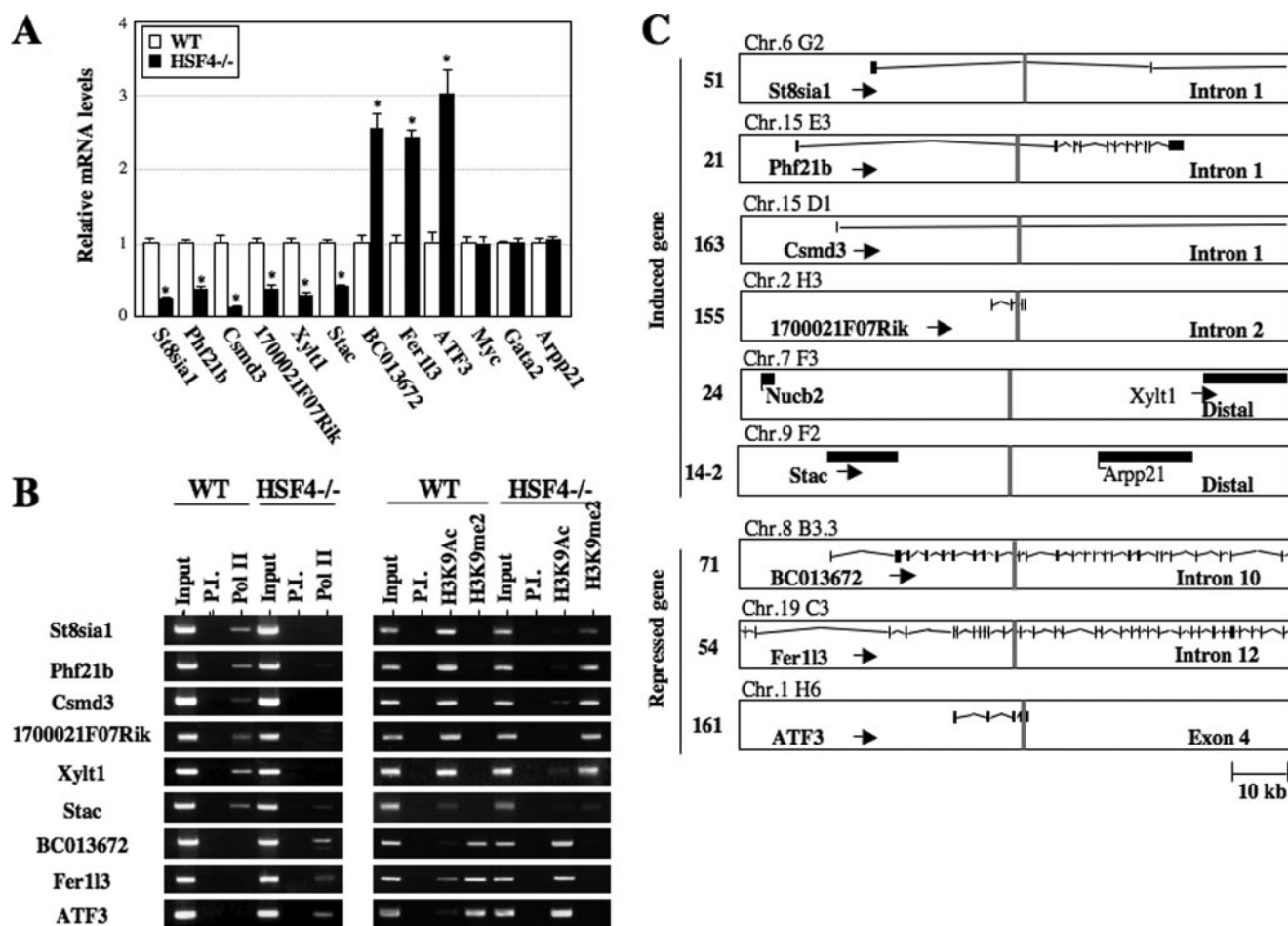


FIGURE 5. Expression of genes on and near the HSF4 binding regions in the lenses. *A*, mRNA levels of nine genes related to the HSF4 binding regions were analyzed by RT-PCR using total RNAs isolated from wild-type (*W*) and HSF4-null (*4K*) 3W lenses. Relative mRNA levels were quantified in each genotype, and means and standard deviations are shown from three independent experiments. mRNAs of the other three genes were shown as a controls. *B*, ChIP analysis of 3W lenses was performed using antibodies for RNA polymerase II, acetylated histone H3K9, dimethylated histone H3K9, and preimmune serum (*P.I.*). Precipitated DNAs were amplified using primers specific for each promoter region. *C*, HSF4 binding regions relative to specific gene targets. Gray bars at the center of each column represent HSF4 binding regions. Arrows indicate the 5'-3' orientation of the genes. Expression of five genes (*upper*) was induced by HSF4, and expression of the other three genes (*lower*) was repressed.

regions in group B during development as their expression significantly decreased (Fig. 1), and 29 regions (71%) were occupied by only HSF4 in the 3W lenses.

Expression of Genes Located On and Near the HSF4 Binding Regions during Development—To correlate HSF4 binding with transcription of genes located on and near the HSF4 binding regions, we examined their mRNA levels in the wild-type and HSF4-null 3W lenses by RT-PCR. mRNAs levels of 17 genes on and near the 13 group A binding regions were constant in the HSF4-null lenses, whereas mRNA levels of 9 genes (22%) on and near the 41 group B binding regions decreased or increased in the HSF4-null lenses (Fig. 5*A*). Activation or repression of gene expression was confirmed by analysis of the promoter regions of the nine genes, in which expression levels correlated with levels of RNA polymerase II recruitment, with levels of histone H3K9 acetylation, and reversely with levels of histone H3K9 dimethylation (Fig. 5*B*). As was expected from the fact that HSF4-null lenses are morphologically normal until 2 weeks after birth (22), mRNA levels of the nine genes were constant in the HSF4-null E18.5 and P2 lenses, except ATF3 mRNA (supplemental Fig. S6). These developmental profiles of gene

expression coincide with disappearance of the expression and DNA binding of HSF1 and HSF2 (Figs. 1 and 4). The HSF4 binding regions on and near five of the nine genes were bound by only HSF4 in the 3W lenses, whereas the others were bound by HSF4 and HSF1 (supplemental Fig. S5). Therefore, HSF1 and HSF4 could compete with each other or act cooperatively on the same genomic regions.

By determining the locations of HSF4 binding regions in relation to gene structure, we found that seven of nine HSF4 binding regions were located on the introns (six regions) and exons (one region) (Figs. 5*C*). Promoter regions within 500 bp from the transcription start sites of the seven genes were not bound by any HSF (data not shown). It was unclear whether two distantly located other regions directly affect gene expression as HSF4 bound to the promoter region of the *Stac* gene (data not shown). These analyses revealed that HSF4 binds to the intron much more frequently than to the promoter, and the binding to the intron is highly correlated with its expression.

HSF4 Binding Promotes Demethylation of Histone H3K9—Our results showed that HSF4 binding on the genomic regions is not associated with regulation of constitutive expression of

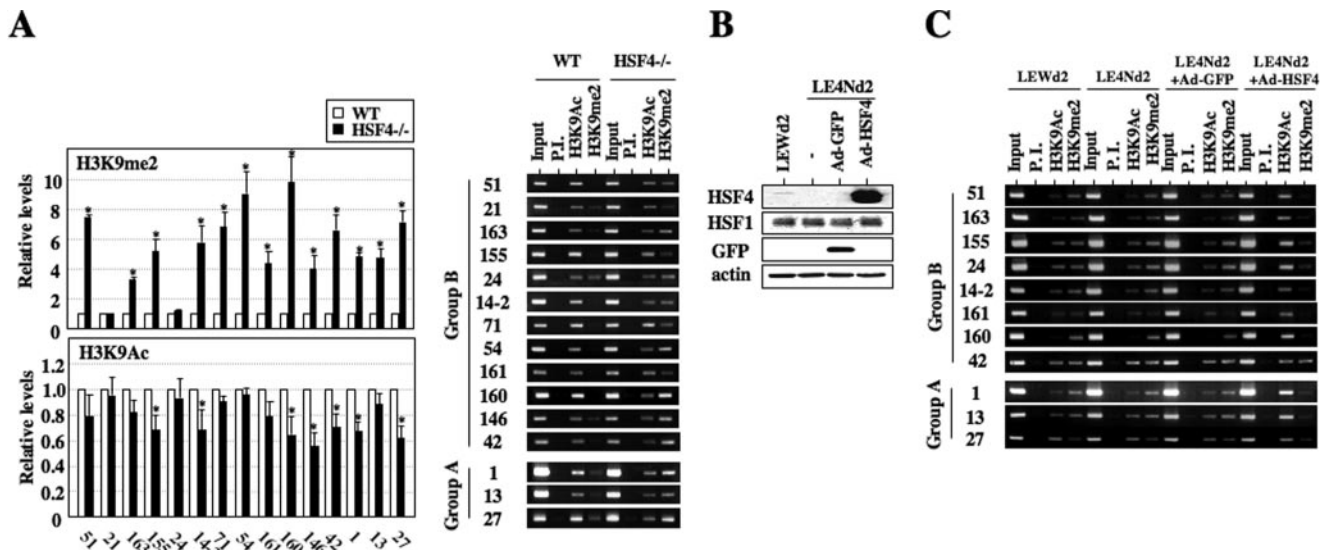


FIGURE 6. HSF4 binding is associated with demethylation of histone H3K9. A, ChIP analysis was performed using antibodies for acetylated histone H3K9 and dimethylated histone H3K9, and preimmune serum (P.I.). Precipitated DNAs were amplified using primers specific for HSF4 binding regions in groups A and B (right). Representative data are shown. Amplified DNAs were quantified, and means and standard deviations are shown from three independent experiments (left). Stars indicate statistical significance ($p < 0.05$). B, wild-type LEWd2 and HSF4-null LE4Nd2 cells infected with Ad-GFP or Ad-hHSF4 for 36 h, and Western blot analysis was performed. C, ChIP analysis was performed using adenovirus-infected LEWd2 and LE4Nd2 cells as described in A. Representative data are shown.

many genes. Therefore, we would like to know the biological consequences of the HSF4 binding. Because HSF1 binds to the promoter of the interleukin-6 gene in unstressed cells and partially opens its chromatin by recruiting histone acetylase and chromatin remodeling complexes (40), it is possible that HSF4 may affect chromatin structure. We first analyzed status of histone H3K9 acetylation by ChIP analysis on three HSF4-bound genomic regions in group A (see below) and randomly picked 12 regions, including that related to developmental regulation in group B (Fig. 6A). Although levels of H3K9 acetylation on many HSF4-binding regions in both groups A and B decreased by 20% to 40% in the HSF4-null P2 lenses, the -fold changes were modest. Remarkably, we found that levels of histone H3K9 dimethylation significantly increased on most HSF4 binding regions in the HSF4-null P2 lenses, even though HSF1 and HSF2 still bind to the regions (data not shown).

We next analyzed chromatin modification of immortalized lens cells, wild-type LEWd2 cells, and HSF4-null LE4Nd2 cells. Remarkably, overexpression of HSF4 promoted demethylation of histone H3K9 and its acetylation in many HSF4 binding regions in both groups A and B (Fig. 6, B and C). These results indicate that in the lenses HSF4 promotes demethylated status of histone H3K9 on its binding regions, and that chromatin modification caused by HSF4 is not always associated with regulation of constitutive gene expression during development.

HSF4 Is Required for Induction of a Set of Non-classic Heat Shock Genes—Although we did not identify classic heat shock genes as preferential HSF4 targets, we wondered whether the identified genes could be induced in response to heat shock. To test this, we cultured the extracted lenses in medium at 37 °C (47) and treated them with heat shock at 43 °C. We confirmed that the lens cells respond to heat shock by phosphorylating HSF1 (Fig. 7A) and inducing mRNA of a classic heat shock gene,

Hsp70-1 (Fig. 7C). Unexpectedly, a large number of genes on and near the HSF4 binding regions (total 33%) are induced in response to heat shock in the lenses (Fig. 7B). Among 17 group A genes, we found mRNAs of three genes (18%) that are induced by heat shock (Fig. 7C), and mRNAs of 17 genes (39%) of 44 group B genes were induced by heat shock (supplemental Fig. S7).

Previous works showed that HSF1 is required for induction of classic heat shock genes in many tissues and cells (7, 8, 9). Furthermore, overexpression of HSF4 did not restore the classic heat shock response in HSF1-null mouse embryo fibroblasts (data not shown). Therefore, we doubt the involvement of HSF4 in the heat shock response. To our surprise, we found that mRNAs of three heat-inducible genes in group A (*C330019L16Rik*, *2310046K01Rik*, and *Cetn1* genes) were not induced by heat shock in both HSF4-null and HSF1-null P2 lenses, although *Hsp70* mRNA was induced even in the absence of HSF4 (Fig. 7C). Furthermore, expression of 8 of 17 heat-inducible genes in group B was much less induced in the HSF4-null lenses (supplemental Fig. S7). Interestingly, among them, 4 genes (*Fer1l3*, *St8sia1*, *Xylt1*, and *Stac* genes) were identical to the genes that are regulated by HSF4 during development (Fig. 5A). Moreover, we analyzed induction of three group A heat-inducible genes in cultured lens cells. We found that mRNAs of two genes (*C330019L16Rik* and *2310046K01Rik* genes) were modestly induced in wild-type LEWd2 cells in response to heat shock at 42 °C but were not induced in HSF4-null LE4Nd2 cells (Fig. 7D). Re-expression of HSF4 at high levels restored the induction strongly. Induction of *Hsp70* mRNA was not affected by the absence or presence of HSF4. These results clearly demonstrate that HSF4 is required for induction of a set of non-classic heat shock genes in the lenses, and suggest a close connection of regulatory mechanisms between heat shock response and developmental regulation.

Regulation of Gene Expression by HSF4

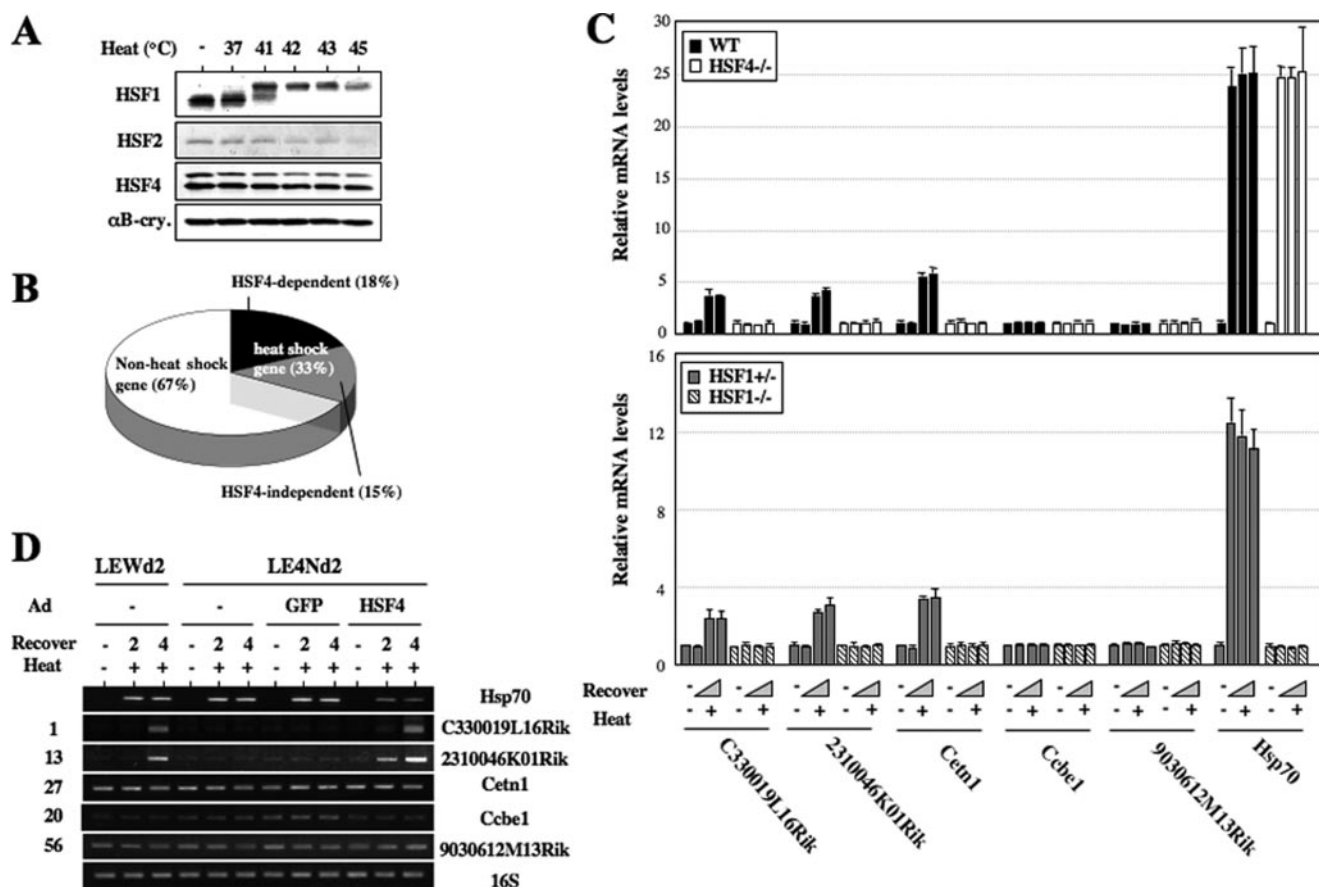


FIGURE 7. HSF4 is required for induction of a set of non-classic heat shock genes in response to heat shock. *A*, P2 Lenses were incubated in medium at 37 °C and then submerged in a water bath for 30 min at the indicated temperatures. Lens extracts were prepared and subjected to Western blot analysis of each HSF and α B-crystallin. *B*, proportion of heat-inducible genes and genes of which induction depends of HSF4 among the 61 HSF4 targets. *C*, wild-type and HSF4-null P2 lenses incubated at 37 °C were submerged in a water bath at 43 °C for 30 min, and then recovered for 1, 2, and 4 h. RT-PCR analysis of the genes in group A was performed using total RNAs isolated from the lenses. Means and standard deviations are shown from three independent experiments (upper). HSF1^{+/-} and HSF1^{-/-} P2 lenses were similarly analyzed (lower). *D*, wild-type LEWd2 and HSF4-null LE4Nd2 cells infected with Ad-GFP or Ad-hHSF4 were heat-shocked at 42 °C for 1 h, and then recovered at 37 °C for 2 or 4 h. mRNA levels were analyzed by RT-PCR analysis.

Because lack of HSF4 leads to methylation of histone H3K9, which is associated with densely packed chromatin (52, 53), we examined binding of HSF1 on the HSF4 binding regions after heat shock. HSF1 appeared to bind to the regions 13 and 27 in the group A after heat shock, but it bound less to the same regions in the absence of HSF4 (Fig. 8), although HSF1 acquired HSE-binding activity after heat shock in both wild-type and HSF4-null lens cells (data not shown). HSF4 binding regions in group B were bound by HSF4 even before and after heat shock independent of HSF4 (supplemental Fig. S7). These results indicate that HSF4 facilitates HSF1 to bind to at least some HSF4 binding sites.

DISCUSSION

Molecular mechanisms of heat shock response have been extensively studied. HSF1 is activated by heat shock, binds to consensus HSEs located on proximal promoters of classic heat shock genes, and robustly induces their transcription (4, 6). In contrast, HSF2 and HSF4 have much less potential to activate transcription than HSF1 and can bind to the HSEs of classic heat shock genes under normal growth conditions (18, 54). Although HSF2 is implicated to be involved in heat shock response through direct interaction with HSF1 (46, 55) or in

keeping chromatin uncompacted during mitosis (56), we still do not know molecular mechanisms that regulate gene expression or the biological consequences of their binding. Because HSF4 is uniquely a trimer that can bind to HSE with a high affinity, it regulates gene expression at a step distinct from binding to the HSE. To this end, we first identified 58 genomic regions that are preferentially bound by HSF4 in the whole genome of mouse lenses and found that none of these regions is related to classic heat shock genes (supplemental Fig. S1).

By mapping the relative location of the HSF4 binding regions, we found that 53% maps to the intron and exons, and 40% to the distal parts (>10 kb) of the genes (Fig. 2). Remarkably, we found that only 5% of HSF4 binding regions maps to 10-kb promoter-proximal regions. This result is in marked contrast to localization of the HSEs on the 5'-proximal promoter of classic heat shock genes. Actually, genome-wide analysis of HSF1 binding regions was performed by a ChIP-on-chip experiment using a DNA microarray corresponding to human promoter (57, 58). It would be better to use a tiling DNA array for genome-wide ChIP-on-chip analysis of HSF binding regions in the future.

We previously showed that HSF1 and HSF4 competitively or additively regulate gene expression in the lens (22). To understand cooperation mechanisms during development, here we

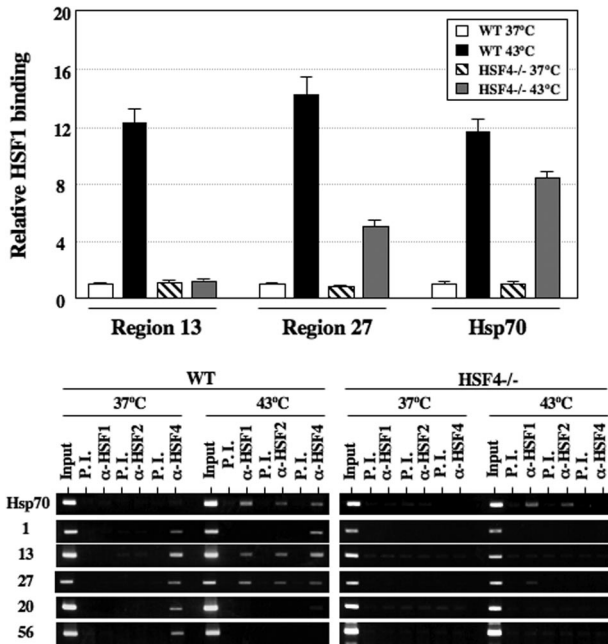


FIGURE 8. Inducible binding of HSF1 to the HSF4 binding region is less efficient *in vivo*. CHIP analysis of HSF4, HSF1, and HSF2 occupancy on the HSF4 binding regions in group A was analyzed in the wild-type (WT) and HSF4-null (HSF4^{-/-}) P2 lenses treated with or without heat shock at 43 °C for 30 min. Representative data are shown (lower). Amplified DNAs were quantified, and means and standard deviations of relative HSF1 binding are shown from three independent experiments (upper).

examined sequence properties of HSF4 binding sites *in vitro* and revealed a consensus sequence of at least three inverted repeats of nGnnn pentameric units as a HSF4 binding site (Fig. 2). Interestingly, HSF1 and HSF2 do not bind to this less conserved sequence *in vitro*, but a substantial number of HSF4 binding regions is occupied also by HSF1 and HSF2, suggesting that *in vivo* binding of HSFs may depend on sequence properties of the binding sites and interacting factors that stabilize HSFs on the sites. They might compete with each other or recruit on the same genomic regions.

It is a matter of great interest that HSF binding to genomic regions in stressed and unstressed conditions is linked to gene regulation. In *Drosophila*, HSF1 binds to multiple chromosome loci, including non-heat shock loci on both conditions (59, 60), and genome-wide analysis showed that HSF1 binding to the promoter does not necessarily induce gene expression in yeast (61, 62) and in human (57, 58). We previously showed that even in an unstressed condition HSF1 binds to the promoter of non-heat shock interleukin-6 gene and opens its chromatin structure through recruitment of histone acetylase and nucleosome remodeling complexes (40) like its roles during heat shock (63, 64, 65). Here, we showed that HSF4 binding to genomic regions is closely associated with reduced histone H3K9 methylation, irrespective of the relative location of the HSF4 binding regions (Fig. 6) and is not correlated with transcription of genes on or near the HSF4 binding regions (Fig. 5). This is a first semi-comprehensive analysis demonstrating that HSF binding modulates chromatin modification and suggests that HSF4 plays roles on the regulation of chromatin structure.

In general, methylation of Histone H3K9 results in generation of a heterochromatic environment and repression of tran-

scription (52, 53). Actually, we showed that histone H3K9 methylation in the promoter is associated with reduced expression (Fig. 5B). However, lack of HSF4 does not affect transcription of the genes on and near the HSF4 binding regions in the P2 lens, although it markedly promotes histone H3K9 methylation. To our surprise, methylation of histone H3K9 on the HSF4 binding regions promoted by HSF4 deficiency is associated with reduced occupancy of HSF1 on the same regions after heat shock (Fig. 8), probably by generating packed chromatin (52, 53), and with a lack of induction of all three heat shock genes in group A. Thus, HSF4 facilitates HSF1 to bind to chromatin. Although HSF4-dependent 5 genes in group B are bound by HSF1 even in the absence of HSF4, these may have other key regions around the genes to which heat-induced HSF1 binding depends on HSF4 occupancy.

Our results reveal a novel mechanism of non-classic heat shock response and developmental gene expression regulated by cooperation of HSF family members. The results also suggest that there are many HSF4 targets on the whole genome, and heat shock induces expression of substantial numbers of these genes that may play roles in maintaining homeostasis of cells. Because HSF4 is abundantly expressed in the lenses, it is necessary to understand whether similar mechanisms work in other cell types in the future.

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