Chapter 2
Structure and Function of the HSF Family Members

Ryosuke Takii and Mitsuaki Fujimoto

Abstract The heat shock response is a fundamental mechanism to adapt against various proteotoxic stresses in all living organisms. This response is characterized by the induction of heat shock proteins (HSPs) and regulated mainly at the level of transcription by heat shock factor (HSF). Vertebrate cells possess four *HSF* genes, which are located in the conserved syntenic regions among species. The amino acid sequences of the DNA-binding domain (DBD) and oligomerization domain (HR-A/B) located in the N-terminal region are highly conserved. The DBD interacts with genomic DNA, and HR-A/B is required for the formation of an HSF trimer that binds to DNA with high affinity. The HR-A/B is flanked by two nuclear localization signals. There are some activation or regulatory domains in the C-terminal region. Among HSF family members, HSF1 is a master regulator of the expression of *HSP* gene in mammalian cells, while that of *non-HSP* genes is also regulated by HSF2, HSF3, and HSF4. Furthermore, the HSF family members cooperatively or competitively regulate the expression of some common targets.

Keywords Amino acid • Consensus sequence • DNA binding • Evolution • Hydrophobic heptad repeat • Structure • Transcriptional activity

2.1 Introduction

When cells are exposed to elevated temperatures, cellular proteins are denatured and aggregated. Cells have the ability to adapt to this proteotoxic stress by inducing the expression of heat shock proteins (HSPs) or molecular chaperones, which facilitate protein folding and suppress protein aggregation. This response is called the heat shock response and is a universal mechanism to maintain protein homeostasis and to protect cell from protein-damaging insults (Lindquist 1986). This response is well conserved from bacteria to human. In eukaryotes, the heat shock response is regulated mainly at the level of transcription by heat shock factor (HSF).

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HSF stays as an inert monomer in normal growth condition. In response to heat shock, HSF is quickly converted to a trimer that binds to the heat shock response element (HSE) located in the promoters of heat shock genes encoding HSPs including HSP70. The consensus sequence of the HSE is at least three inverted repeats of a pentanucleotide nGAAn (Fernandes et al. 1994). As a result, the expression of HSPs is rapidly induced.

HSF is required not only for the heat shock response but also cell growth and differentiation and normal lifespan in yeast, *C. elegans*, and *Drosophila* (Hsu et al. 2003; Morano et al. 1999; Morley and Morimoto 2004). There is a single HSF in these invertebrates, whereas four *HSF* genes (*HSF1* to *HSF4*) exist in higher animals (Nakai et al. 1997; Nakai and Morimoto 1993; Rabindran et al. 1991; Schuetz et al. 1991). In mammals, HSF1 is required for the heat shock response, whereas HSF3 is required for this response in birds (McMillan et al. 1998; Tanabe et al. 1998). Both mammal HSF1 and chicken HSF3 are necessary for acquisition of the thermotolerance, which is correlated with the induction of HSPs. In addition, members of HSF family are involved in various developmental processes including gametogenesis and neurogenesis and maintenance of sensory organs (Chang et al. 2006; Kallio et al. 2002; Takaki et al. 2006; Wang et al. 2004). Thus, the *HSF* gene has duplicated and acquired multifaceted functions during evolution. In this review, we describe the structure of HSF family members; e.g., the DNA-binding domain, oligomerization domain, and activation domain. We also review the transcriptional activity, biochemical characters, and target sequences of HSF family members and discuss about evolution of the *HSF* gene family.

2.2 Structure of the HSF Family Members

The sizes and amino acid sequences of eukaryotic HSF family members are different, except for some conserved domains (Fig. 2.1) (Clos et al. 1993). Especially, amino acid sequences of the DNA-binding domain (DBD) and oligomerization domain (HR-A/B, hydrophobic heptad repeat-A/B) are highly conserved in eukaryotic species (Nakai et al. 1997; Nakai and Morimoto 1993; Sarge et al. 1991). In addition, there are several short sequences including the nuclear localization signal, which are conserved in all or parts of the HSF family members. The location and sequences of the transcriptional activation domain are different in the HSF family members.

2.2.1 DNA-Binding Domain

The DBD is located near the N-terminus of all HSFs. Amino acid sequence of the DBD of human HSF1 (hHSF1) is 46 % identical to that of yeast HSF (ScHSF) (Fig. 2.1). The crystal structure and NMR solution structure of the DBD in ScHSF
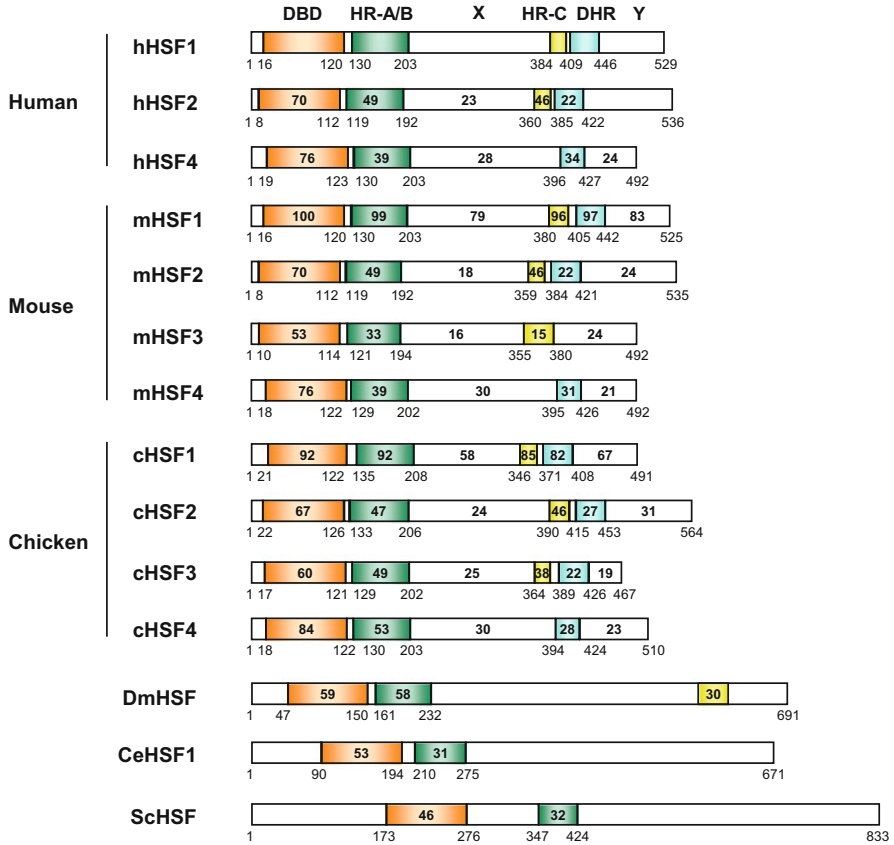
Fig. 2.1 Structures of the HSF family members. Diagrammatic representation of structures of the HSF family members. The percentage identities between amino acid sequences in regions of human HSF1 and those corresponding regions in other HSFs are established using the computer program GENETYX-WIN. The number of amino acids of each HSF is shown at the N-terminus. *DBD* DNA-binding domain; *HR* hydrophobic heptad repeat; *DHR* downstream of HR-C; *h* human; *m* mouse; *c* chicken; *Dm Drosophila melanogaster*; *Ce C. elegans*; *Sc Saccharomyces cerevisiae*. hHSF1 (an isoform hHSF1α) (Rabindran et al. 1991); hHSF2 (hHSF2α) (Schuetz et al. 1991); hHSF4 (hHSF4b) (Nakai et al. 1997); mHSF1 (mHSF1α) and mHSF2 (mHSF2α) (Sarge et al. 1993); mHSF3 (mHSF3a) (Fujimoto et al. 2010); mHSF4 (mHSF4b) (Tanabe et al. 1999); cHSF1, cHSF2, and cHSF3 (Nakai and Morimoto 1993); cHSF4 (cHSF4b) (Fujimoto et al. 2010); DmHSF (Clos et al. 1990); CeHSF1 (Swiss-P accession no Q9XW45); ScHSF (Wiederrecht et al. 1988). The HR-C is not conserved in HSF4, CeHSF1, and ScHSF

show that HSF belongs to winged helix-turn-helix DNA-binding proteins containing mammalian ETS and HNF-3/forkhead proteins (Gajiwala and Burley 2000) and consisted of three helical bundles (H1, H2, and H3) and four-stranded, antiparallel β-sheets (Fig. 2.2) (Damberger et al. 1995; Harrison et al. 1994; Vuister et al. 1994a, b). The central α-helix H3 interacts with the major groove of DNA. In contrast, the α-helix H1 and wing (loop) region are exposed outside and interact
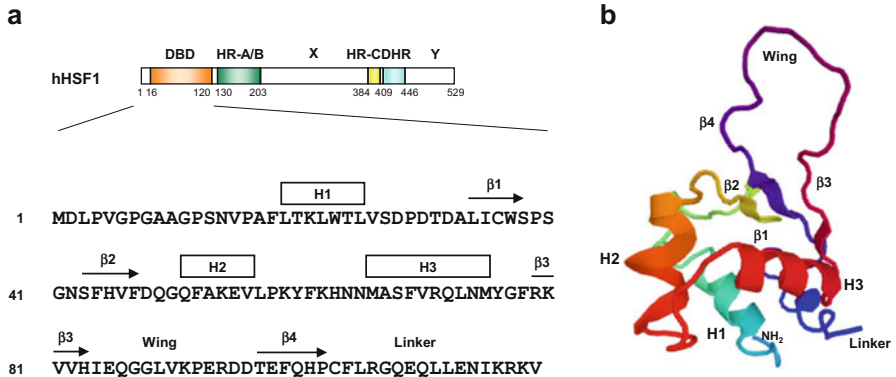
Fig. 2.2 Structure of the DNA-binding domain in human HSF1. (**a**) Sequences of the DBD in hHSF1. *Boxes* indicate α-helices and *arrows* indicate β-sheets. (**b**) A model to predict the structure of the DBD in hHSF1 by comparison with that in *Drosophila* HSF1 using SWISS-MODEL (http://swissmodel.expasy.org/). Each absolute structure is indicated by rainbow color. It shows a winged helix-turn-helix motif, which consisted of H2 (*orange*) and H3 (*red*). The α-helix H3 interacts with the major groove of DNA. The α-helix H1 and wing (loop) region are exposed outside

with regulatory factors such as RPA1 and ATF1 (Fujimoto et al. 2012; Takii et al. 2015). The wing is not necessary for the trimer formation of HSF but is required for full activity of HSF (Cicero et al. 2001; Littlefield and Nelson 1999).

2.2.2 Oligomerization Domain

The HR-A/B is connected to the DBD by a flexible linker of 10–20 amino acids (Flick et al. 1994). It contains two α-helices, HR-A and HR-B, which consist of a repeating pattern of seven amino acids. Especially, amino acids at positions a and d of the heptad repeat, a-b-c-d-e-f-g, are hydrophobic amino acids such as leucine (L), isoleucine (I), and valine (V) (Fig. 2.3a). HSF uniquely forms a trimer that binds to DNA with a high affinity through the hydrophobic interaction between three HR-A/B domains located in parallel when HSF is activated (Clos et al. 1990; Peteranderl et al. 1999) (Fig. 2.3a). The electrostatic interactions also occur primarily between positions e and g, which are often charged residues such as arginine (R), lysine (K), aspartic acid (D), and glutamic acid (E) (Fig. 2.3b) (Creighton 1993). The HR-A/B is required for the formation of not only a trimer but also a dimer (Nakai et al. 1995; Sistonen et al. 1994). Therefore, the HR-A/B is also called the trimerization domain or oligomerization domain. The linker domain modulates the trimerization of HSF (Liu and Thiele 1996). There is another hydrophobic heptad repeat HR-C near the C-terminus of HSF. The HR-C inhibits the oligomerization of the HR-A/B by forming an intramolecular coiled coil with the HR-A/B and keeps HSF as a monomer that cannot bind to DNA in control conditions. Therefore, the point mutation of hydrophobic amino acids in the HR-C of hHSF1
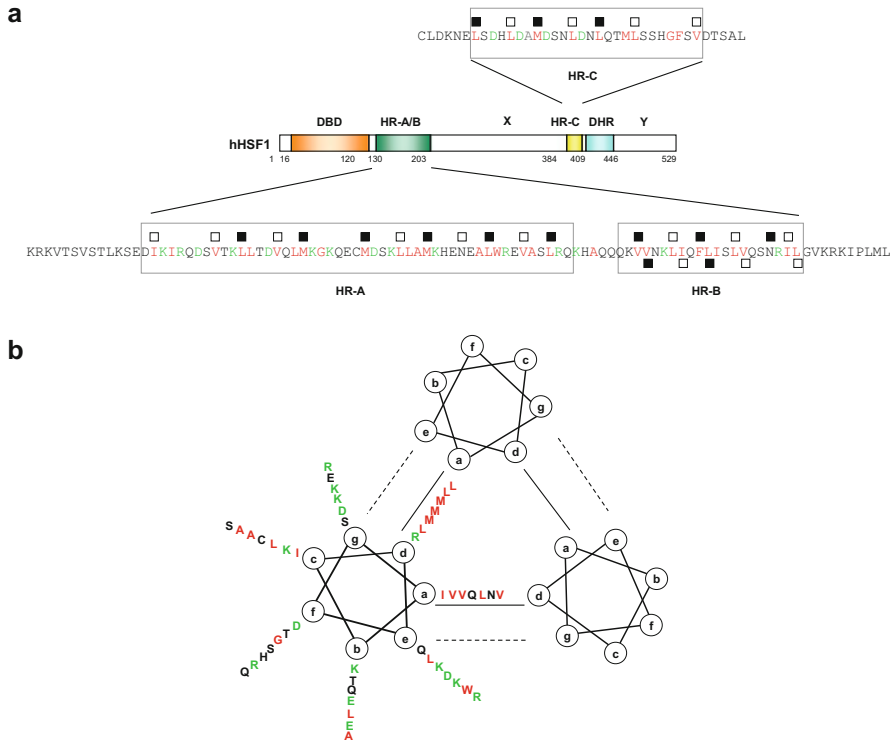
a

b

Fig. 2.3 Trimer formation through interaction between the hydrophobic heptad repeats. (**a**) Amino acid sequences of the HR-A/B and HR-C in hHSF1. The *open* and *solid squares* indicate amino acids at positions a and d, respectively, in the repeating seven amino acids. Hydrophobic amino acids are marked by *red* and charged amino acids by *green*. (**b**). Trimer formation of the HR-A/B. The HR-A/B forms a trimer through the interaction between hydrophobic amino acids at positions a and d (*solid lines*) in the HR-A/B. The electrostatic interactions also occur primarily between positions e and g (*dotted lines*), which are often charged residues

results in the formation of a DNA-binding trimer (Rabindran et al. 1993; Zuo et al. 1995). Because HSF in yeasts and HSF4 lack the HR-C, they exist mostly as DNA-binding trimers in control conditions (Giardina et al. 1995; Jakobsen and Pelham 1988; Nakai et al. 1997; Sorger and Nelson 1989; Sorger and Pelham 1988; Wiederrecht et al. 1988). Another hydrophobic heptad repeat DHR exists downstream of the HR-C in most vertebrate HSFs (Nakai et al. 1997), but its function is unknown yet.

2.2.3   Nuclear Localization Signal

The HR-A/B domains in the HSF family members are surrounded by two putative nuclear localization signals (NLSs), NLS1 and NLS2 (Fig. 2.4). These sequences fit

Fig. 2.4 Nuclear localization signals in vertebrate HSFs. Sequences of the NLS1 and NLS2 in human and chicken members of the HSF family are shown. These sequences fit the consensus for a bipartite NLS, which consisted of two clusters of basic amino acids (*red*), separated by about ten amino acids

the consensus for a bipartite NLS, which consisted of two clusters of basic amino acids, separated by a spacer of about ten amino acids (Sheldon and Kingston 1993). Human HSF2 (hHSF2) partly accumulates to the nucleus upon heat shock (Shinkawa et al. 2011), but a mutated hHSF2 lacking a cluster of the basic amino acids in the NLS1 or NLS2 does not translocate to the nucleus (Sheldon and Kingston 1993). Thus, both NLS1 and NLS2 are required for the nuclear translocation of HSF2. Human HSF1 (hHSF1) is a master regulator of the HSP expression during heat shock. The NLS2 of hHSF1 is required for the nuclear translocation, whereas the NLS1 is dispensable for that (Vujanac et al. 2005). Lysine amino acids in the NLSs are acetylated and related with the formation of nuclear stress bodies in response to heat shock (Raychaudhuri et al. 2014). Chicken HSF3 (cHSF3) is a master regulator of the HSP expression in birds, and the NLS2, but not the NLS1, is required and sufficient for the nuclear translocation of cHSF3 upon heat shock (Nakai and Ishikawa 2000). *Drosophila* HSF also has a bipartite NLS located downstream of the HR-A/B which is required for its nuclear translocation in response to heat shock and during development (Fang et al. 2001; Orosz et al. 1996). In plants, HSFs have a nuclear export signal (NES) in the C-terminal activation domain (Kotak et al. 2004), but there is no report about the NES in vertebrate HSFs.

In addition to the NLS, there are at least seven conserved sequences, termed sites a to g in the regions X and Y, in vertebrate HSF members (Fujimoto et al. 2010; Nakai et al. 1997; Nakai and Morimoto 1993; Tanabe et al. 1999). The function of these conserved sequences should be revealed in the future.

2.2.4 Transcriptional Activation Domain

HSF from a budding yeast *Saccharomyces cerevisiae* possesses the N-terminal and C-terminal transcriptional activation domains (Sorger 1990). In contrast, HSF from another budding yeast *Kluyveromyces lactis* does not have the N-terminal activation domain but has the C-terminal activation domain, whose sequence is completely divergent from that of *S. cerevisiae* HSF (Jakobsen and Pelham 1991). Thus, the sequences of transcriptional activation domains in HSFs are not evolutionarily conserved. However, transcriptional activity of the activation domain is repressed in unstressed condition by a conserved motif CE2, which is located near the activation domain.

Mammalian HSF1 is functionally analogous to yeast HSF and robustly induces the expression of HSPs during heat shock. It has a potent transcriptional activation domain (AD) in the C-terminus, which is divided into two regions (Green et al. 1995; Shi et al. 1995; Zuo et al. 1995) (Fig. 2.5a). The AD1 (a.a. 372–431)
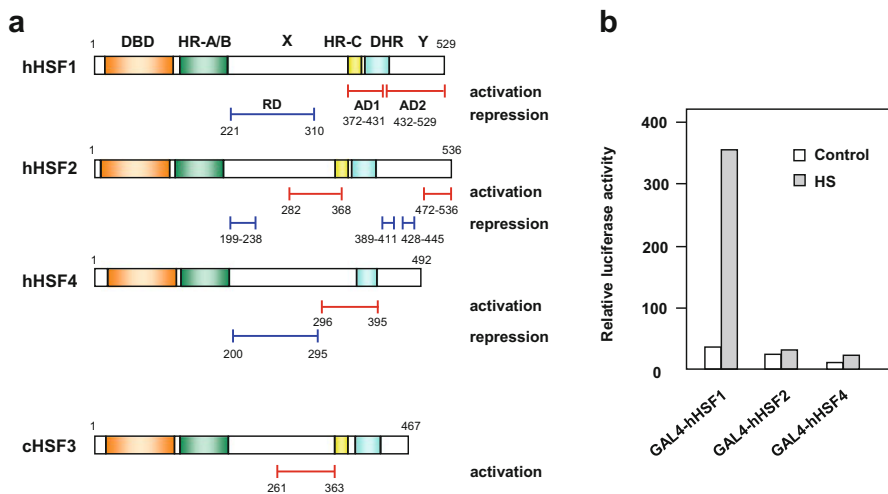
Fig. 2.5 Transcriptional activation domains of human HSFs. (**a**) Localization of activation domains in human HSF1, HSF2, and HSF4 and chicken HSF3. *Red bars* indicate transcriptional activation domains (*ADs*), and *blue bars* indicate repression domains of the activation domains. Human HSF1 possesses two activation domains in the C-terminus (AD1, a.a. 372–431; AD2, a.a. 432–529) (Green et al. 1995). The AD1 is predicted to form an α-helix, while AD2 is rich in proline and glycine. The regulatory domain (*RD*, a.a. 221–310) represses the two activation domains (Green et al. 1995). In human HSF2, two activation domains (a.a. 282–386 and a.a. 472–536) and three negative regulatory domains (a.a. 199–238, a.a. 389–411, and a.a. 428–445) are present (Yoshima et al. 1998b). Human HSF4 also has an activation domain (a.a. 296–395) and a negative regulatory domain (a.a. 200–295) (Nakai et al. 1997). Chicken HSF3 has at least a strong activation domain (a.a. 261–363) (Tanabe et al. 1997). (**b**) Human HSF1, hHSF2, and HSF4 have different potential to activate transcription. Reporter analysis in human cells treated without (control) and with heat shock (*HS*) (42 °C for 1 h and then recovery at 37 °C for 6 h) (Nakai et al. 1997; Yoshima et al. 1998a)

contains the HR-C and is predicted to form an α-helix, while AD2 (a.a. 432–529) is rich in proline (13 %) and glycine (8 %). Hydrophobic residues in the AD1 and AD2 are involved in the elongation step of the transcription processes, and acidic residues are in the initiation steps (Brown et al. 1998). The regulatory domain (RD, a.a. 221–310) located downstream of the HR-A/B represses the two activation domains in unstressed condition and confers heat shock inducibility of the transcriptional activity upon heat shock (Green et al. 1995; Newton et al. 1996).

In contrast to the strong potential of mammalian HSF1 to activate transcription upon heat shock, the potential of HSF2 and HSF4 is weak in both unstressed and heat-shocked conditions (Tanabe et al. 1999; Yoshima et al. 1998a) (Fig. 2.5b). Nevertheless, HSF2 and HSF4 have a potential to activate the reporter gene constitutively, and HSF4 induces the gene expression in response to proteotoxic stresses including heat shock. In human HSF2, two activation domains (a.a. 282–386 and a.a. 472–536) and three negative regulatory domains (a.a. 199–238, a.a. 389–411, and a.a. 428–445) are present (Yoshima et al. 1998b). Human HSF4 also has an activation domain (a.a. 296–395) and a negative regulatory domain (a.a. 200–295) (Nakai et al. 1997). Chicken HSF3, like human HSF1, has a strong potential to activate transcription upon heat shock and possesses at least a strong activation domain (a.a. 261–363) (Tanabe et al. 1997).

2.3 Vertebrate *HSF* Gene Family

In contrast to invertebrate cells, vertebrate cells have four *HSF* genes. The human, mouse, and chicken genome sequences have become available (International Chicken Genome Sequencing Consortium 2004; International Human Genome Sequencing Consortium 2001; Mouse Genome Sequencing Consortium 2002); therefore, it is possible to compare the syntenic regions, where the same genes occur in a similar order along the chromosomes of different organisms (Koonin et al. 2000). The orthologues of each *HSF* gene are located in the same syntenic regions, which are derived from the same ancestral genomic region (Fujimoto et al. 2010) (Fig. 2.6). For example, *HSF2* is flanked by the *SERINCI* gene in human, mouse, and chicken orthologous segments. *HSF4* was located in a region between the *TRADD-FBXL8* and *NoL3* in these genomes, and *HSF3* was located between the *Vsig4* and *HEPH*. The exon-intron structures of each *HSF* gene are also evolutionarily conserved in three species (Fujimoto et al. 2010).

Four HSF family members are expressed in mouse and chicken cells, whereas human HSF3 is not expressed since human *HSF3* is a pseudogene (Fujimoto et al. 2010). Because all of human, mouse, and chicken *HSF* genes have been molecularly cloned and functionally characterized, phylogenetic tree is generated from predicted amino acid sequences of the HSF family members in these endotherms (Fig. 2.7). It shows that the sequence of each HSF (HSF1, HSF2, HSF3, or HSF4) in a species is more related with those of orthologous ones in other species than those of paralogous ones. Furthermore, the sequences of HSF1 orthologues are
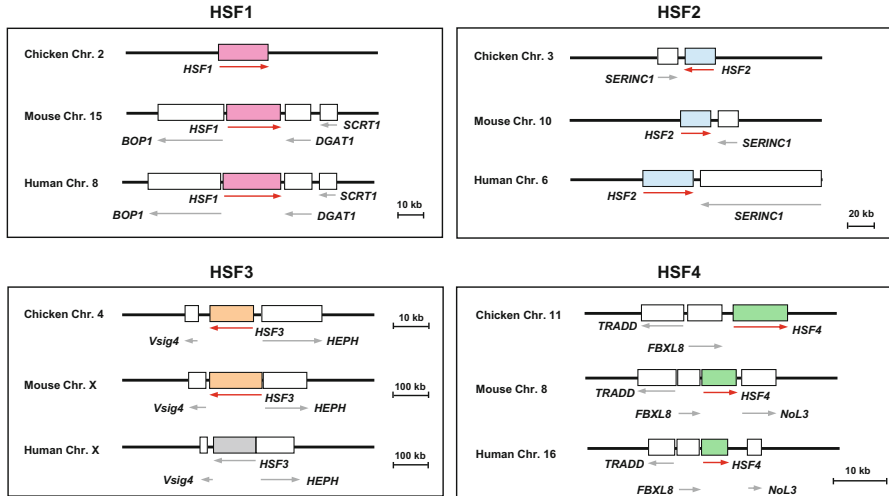
Fig. 2.6 Syntenic regions containing the vertebrate *HSF* genes. The location of each segment is as follows: *HSF1*, human Chr. 8 q24.3 and mouse Chr. 15 D3; *HSF2*, human Chr. 6 q22.31, mouse Chr. 10 B4, and chicken Chr. 3 63.95–63.98 Mb; *HSF3*, human Chr. X q12, mouse Chr. X B4, and chicken 0.252–0.265 Mb; *HSF4*, human Chr. 16 q22.1, mouse Chr. 8 D3, and chicken 2.44–2.45 Mb. A genomic sequence corresponding to chicken HSF1 cDNA has not yet been identified. *Arrows* indicate the 5′ to 3′ orientation of each gene. *Colored boxes* indicate the *HSF* genes, which are flanked by genes shown as *white boxes*. Human *HSF3* is a pseudogene. Note that size markers differ in human, mouse, and chicken HSF3 loci

more related with those of HSF4 orthologues than those of HSF2 or HSF3 orthologues, whereas those of HSF2 orthologues are more related with those of HSF3 orthologues. It has been suggested that two rounds of whole-genome duplication have occurred in vertebrate ancestral cells more than 440 million years (Myr) ago (Holland et al. 1994; Ohno 1970; Putnam et al. 2008), which resulted in polyploidization. Thereafter, avian and mammalian cells evolved differently from an ancestral cell 310 Myr ago, and human and mouse cells did 75 Myr ago. Taken together, four *HSF* genes may have been generated through the two rounds of whole-genome duplication, and thereafter the sequences and functions have been conserved or diverged during vertebrate evolution (Semon and Wolfe 2007). For example, mammal HSF1 is required for the induction of HSPs during heat shock, whereas HSF3, but not HSF1, is required for this response in birds (McMillan et al. 1998; Tanabe et al. 1998). In contrast, HSF4 is dominantly expressed in the lenses of the human, mouse, and chicken eyes (Bu et al. 2002; Fujimoto et al. 2004; Fujimoto et al. 2010). The phylogenetic tree also shows that the relatedness of chicken HSF3 with mammalian HSF3 is much weaker than that of chicken HSF1 with mammalian HSF1, that of chicken HSF2 with mammalian HSF2, or even that of chicken HSF4 with mammalian HSF4. These estimations indicate that nucleic acid sequences of HSF3 diverged most quickly during evolution, whereas those of
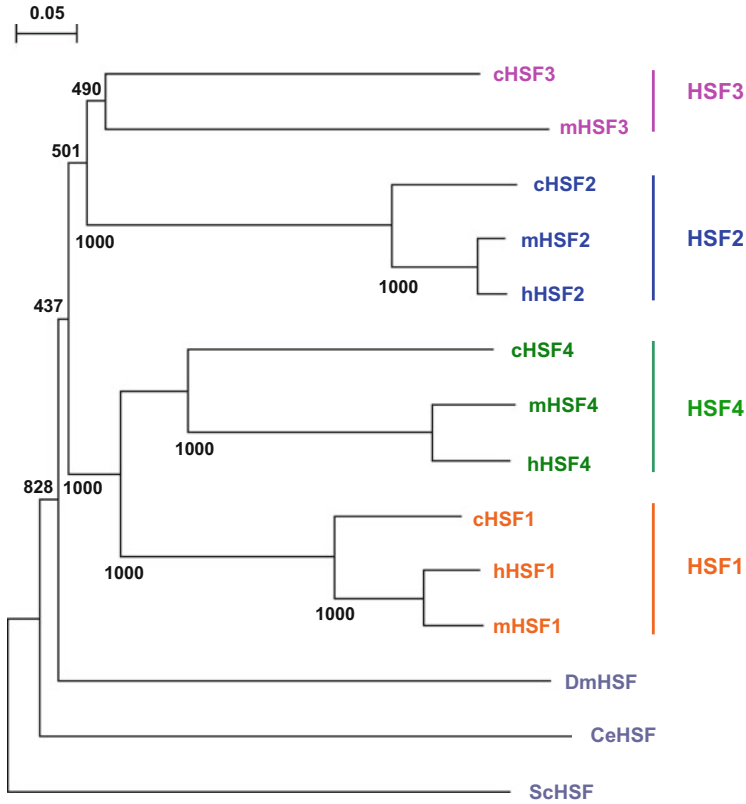
Fig. 2.7 The phylogenetic tree of HSF family members. Phylogenetic tree generated in CLUSTAL X (Thompson et al. 1997), using amino acid sequences of human (*h*), mouse (*m*), and chicken (*c*) HSF family members as well as *Drosophila melanogaster* HSF (*DmHSF*), *C. elegans* HSF1 (*CeHSF1*), and *Saccharomyces cerevisiae* HSF (*ScHSF*) (Fujimoto et al. 2010; Inouye et al. 2003). The number represents bootstrap values (1000 bootstrap replicates were performed). The unrooted tree was drawn using program TreeView (Page 1996). The bar represents 0.05 substitutions per site

HSF1 and HSF2 were similarly conserved. The divergence rates of the HSF4 sequence might also be relatively high.

In contrast to the HSF family members in the endotherms, genes of the HSF members in ectotherms, such as frog, lizard, and fish, have been molecularly cloned and functionally characterized only partially (Hilgarth et al. 2004; Råbergh et al. 2000; Stump et al. 1995; Swan et al. 2012; Yeh et al. 2006; Zatsepina et al. 2000) (Fig. 2.8). Analysis using the gene database (http://www.ensembl.org/index.html) suggests that *HSF1*, *HSF2*, and *HSF4* genes are evolutionarily conserved in all vertebrate species. In contrast, *HSF3* is conserved in rodents, avians, reptiles, and amphibians, while *HSF3* seems to be lost in some mammalian and fish species. In plants, 252 HSF family members, which have similar structures and
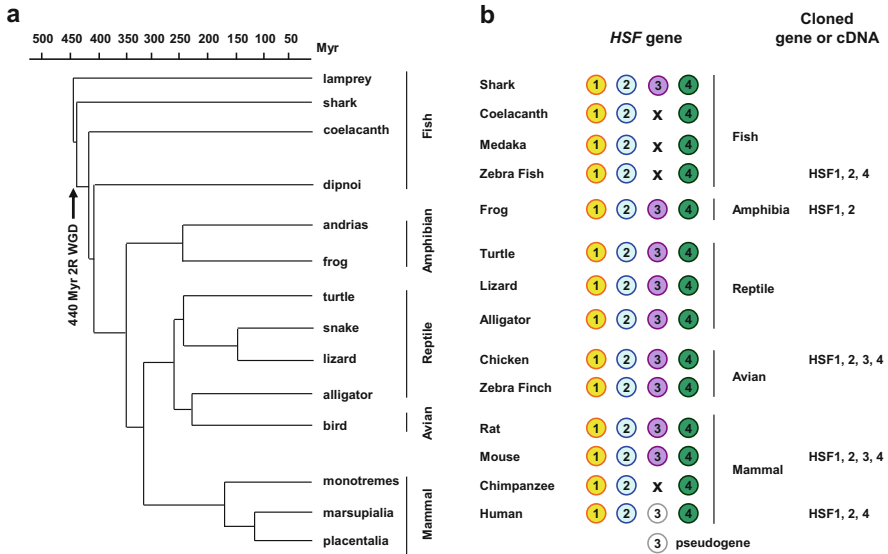
Fig. 2.8 Members of vertebrate *HSF* gene family. (**a**). Evolution of the vertebrates. Phylogenetic tree among main groups of the vertebrates is shown (Patterson 2001). Two rounds (*2R*) of whole-genome duplication (*WGD*) may have occurred in vertebrate ancestral cells more than 440 million years (Myr) ago. (**b**) *HSF* genes in vertebrate species. HSF genes, whose products are registered in NCBI database (http://www.ncbi.nlm.nih.gov/), are shown. *1 HSF1*; *2 HSF2*; *3 HSF3*; *4 HSF4*. HSF3 protein is not registered in chimpanzee, zebra fish, medaka, and coelacanth (x). Human *HSF3* is a pseudogene (*white circle*). Cloned cDNAs are zebra fish HSF1 (Hsu and Yeh 2002; Råbergh et al. 2000), HSF2 (Yeh et al. 2006), and HSF4 (Swan et al. 2012); frog HSF1 (Mercier et al. 1997) and HSF2 (Hilgarth et al. 2004); chicken HSF1, HSF2, HSF3 (Nakai and Morimoto 1993), and HSF4 (Fujimoto et al. 2010); mouse HSF1, HSF2 (Sarge et al. 1991), HSF3 (Fujimoto et al. 2010), and HSF4 (Tanabe et al. 1999); and human HSF1 (Rabindran et al. 1991), HSF2 (Schuetz et al. 1991), HSF3 (Fujimoto et al. 2010), and HSF4 (Nakai et al. 1997). The genes for shark HSF3 (XP_007891631), frog HSF3 (NP_001039053), turtle HSF3 (XP_006136197), lizard HSF3 (XP_008118582), alligator HSF3 (XP_006258300), and zebra finch HSF3 (XP_004177297.1) are located on the same syntenic regions in the genomes of these species

features, are identified from nine plant species (Scharf et al. 2012). It is necessary to examine the function of each HSF member in ectotherms in the future.

2.4 Potential to Activate *HSP* and *Non-HSP* Genes During Heat Shock

The heat shock response is characterized by the induction of *HSP* gene expression during heat shock. After the identification of multiple *HSF* genes in vertebrate genomes (Fujimoto et al. 2010; Nakai et al. 1997; Nakai and Morimoto 1993; Rabindran et al. 1991; Sarge et al. 1991; Schuetz et al. 1991), researchers have

investigated which HSF activates the *HSP* genes. Biochemical analyses showed that human and mouse HSF1, but not HSF2, acquires DNA-binding activity and translocated to the nucleus in response to heat shock, suggesting that HSF1 is involved in the induction of *HSP* genes (Baler et al. 1993; Sarge et al. 1993). Subsequently, disruption of *HSF* genes in mouse embryonic fibroblasts (MEFs) demonstrated that mouse HSF1 is required for the HSP induction during heat shock (McMillan et al. 1998; Zhang et al. 2002), while HSF2 is not (McMillan et al. 2002). Overexpression of human HSF1 restored the induction of HSPs during heat shock (Inouye et al. 2003). HSF1 is also responsible for the induction of the HSP expression in response to other proteotoxic stresses including a blockade of proteasome and an incorporation of amino acid analogues. Thus, HSF1 is a master regulator of the HSP expression in mammalian cells. HSF2 modulates the expression of HSPs to some extent, probably through the direct interaction with HSF1 (Ostling et al. 2007).

In addition to the expression of *HSP* genes, the expression of many non-*HSP* genes is induced during heat shock in mammalian cells (Trinklein et al. 2004). HSF1 plays a major role in the induction of these non-*HSP* genes, but HSF2, HSF3, and HSF4 are also involved in the induced expression of some non-*HSP* genes in MEF cells (Fujimoto et al. 2008; Fujimoto et al. 2010; Shinkawa et al. 2011).

Avian *HSF* genes have functionally diverged during evolution. Both HSF1 and HSF3 acquired the DNA-binding activity during heat shock in chicken cells (Nakai et al. 1995). Unexpectedly, disruption of chicken *HSF3* in chicken B-lymphocyte DT40 cells resulted in a severe reduction in the induction of HSP70 expression during heat shock, and the expression of HSP110, HSP90α, HSP90β, and HSP40 was not induced at all (Tanabe et al. 1998). The disruption of chicken *HSF1* had no effect on the induction of HSP expression during heat shock in DT40 cells, and overexpression of chicken HSF1 did not restore the induction of HSPs during heat shock in HSF1-null MEF cells (Inouye et al. 2003). These results demonstrate that HSF3, but not HSF1, is a master regulator of the HSP expression in chicken cells.

2.5 Oligomeric State

HSFs are defined by its ability to bind to the heat shock response element (HSE) that is composed of three inverted repeats of an nGGAn pentanucleotide. Therefore, activated HSFs form homotrimers that bind to the HSE with high affinity, through the interaction between the HR-A/B domains (Fig. 2.9). In budding yeast *Saccharomyces cerevisiae*, HSF is constitutively a DNA-binding trimer because it does not have the HR-C domain that inhibits oligomerization of the HR-A/B (Sorger and Pelham 1988; Wiederrecht et al. 1988). It acquires elevated potential to activate transcription via phosphorylation upon heat shock (Sorger 1990). In contrast, HSF from fission yeast *Schizosaccharomyces pombe* or *Drosophila melanogaster* stays an inert monomer in unstressed condition and is converted to a DNA-binding trimer in heat shock condition (Clos et al. 1990; Gallo et al. 1993). Thus, a monomer-to-
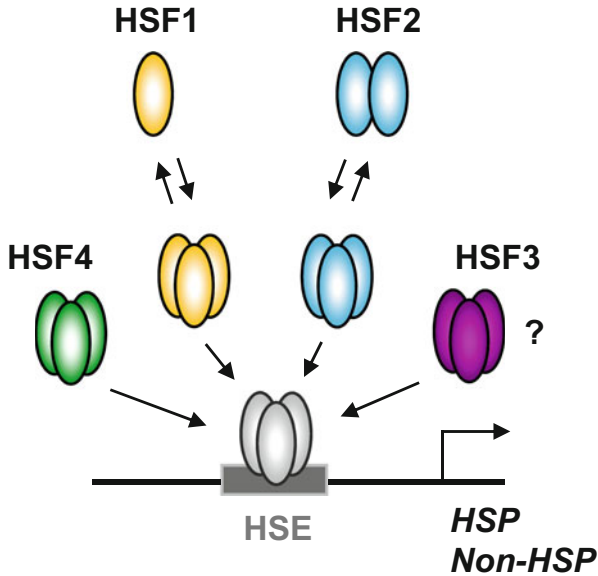
Fig. 2.9 Oligomeric states of mammalian HSFs. HSF1 remains mostly as an inert monomer in unstressed condition and is converted to a DNA-binding trimer. The activated HSF1 binds to the HSE located in the promoters of *HSP* and non-*HSP* genes. HSF2 stays as a dimer and a trimer and is converted to a trimer in response to proteasome inhibition or mild heat shock. HSF4 exists as a DNA-binding trimer constitutively because it lacks the HR-C. HSF3 may be a trimer in unstressed mouse cells. Mouse HSF3 may exist in trimer in unstressed condition

trimer transition of HSF is a fundamental mechanism of HSF activation in response to heat shock (Rabindran et al. 1993).

Oligomeric status of vertebrate HSFs involves a monomer, dimer, and trimer (Fig. 2.9). Mammalian HSF1, like *Drosophila* HSF, mostly stays as an inert monomer in unstressed condition and is converted to a DNA-binding trimer (Baler et al. 1993; Sarge et al. 1993). Mammalian HSF2 stays as both an inert dimer and a DNA-binding trimer in unstressed condition, and the latter increased during the treatment with cells with proteasome inhibitors such as MG132, lactacystin, and hemin (Sistonen et al. 1994; Mathew et al. 1998). Although HSF2 is unstable in severe heat shock conditions such as 42 °C (Sistonen et al. 1994), it is stable and acquires a DNA-binding activity through trimerization in febrile-range, mild heat shock conditions such as 40 °C (Shinkawa et al. 2011). Mammalian HSF4 lacks the HR-A/B domain and therefore forms a DNA-binding trimer constitutively in the lens and other tissues including the brain and lung (Fujimoto et al. 2004; Tanabe et al. 1999). HSF3 may exist as a trimer in unstressed mouse cells, because sequences of the heptad repeats of hydrophobic amino acids in the HR-C domain are not well conserved (Fujimoto et al. 2010). It is worth noticing that chicken HSF3, a master regulator of HSP expression, stays mostly as an inert dimer in control condition and is converted to a DNA-binding trimer upon heat

shock (Nakai et al. 1995). Therefore, the dimer-to-trimer transition of HSF2 and HSF3 is also one of HSF activation mechanisms in vertebrates.

2.6 Recognition Sequences

Extensive studies have revealed that *HSP* genes in *Drosophila* and other eukaryotic species have a 14 bp conserved promoter element, CnnGAAnnTTCnnG (n is any nucleic acid), termed the heat shock response element (HSE) (Pelham 1985). Furthermore, inverted repeats of a 5 bp sequence nGAAn are shown to be key features of the HSE (Xiao and Lis 1988; Amin et al. 1998). In fact, *Drosophila* HSF trimer stably binds to the HSE composed of three contiguous inverted 5 bp units (Perisic et al. 1989; Xiao et al. 1991). Analysis of crystal structure of the DBD of yeast HSF reveals that the second G and ninth C of 5′-nGAAnnTTCn-3′ in major groove of DNA are essential for the interaction with the α-helix H3 of the winged helix-turn-helix motif (Littlefield and Nelson 1999).

Recognition sequences of mammalian HSFs have been analyzed using in vitro random oligonucleotide selection. As is expected from the fact that sequences of the DBD in all of eukaryotic HSFs are highly conserved, both mouse HSF1 and HSF2 recognize inverted repeats of a pentameric consensus nGAAn (Kroeger and Morimoto 1994) (Fig. 2.10). Each HSF has some preference for the nucleotides

Fig. 2.10 In vitro consensus sequence of mammalian HSF binding sites. Consensus recognition sequences for mammalian HSF1, HSF2, and HSF4 binding in vitro were generated with WebLogo (http://weblogo.berkeley.edu/) by using DNA sequences of HSF1, HSF2 and HSF4 binding fragments (Fujimoto et al. 2008; Kroeger and Morimoto 1994). The height of each letter represents the relative frequency of nucleotides at different positions in the consensus
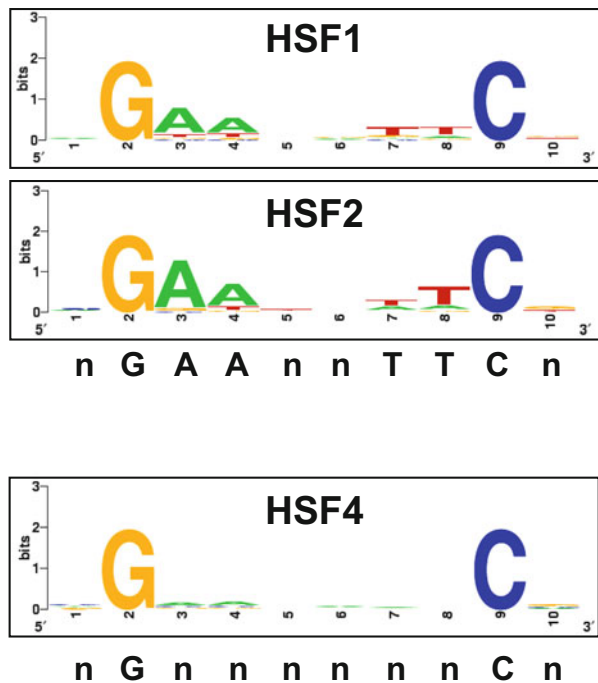
flanking the core GAA motif. In contrast, human HSF4 uniquely recognizes inverted repeats of an ambiguous nGnnn sequence in vitro (Fujimoto et al. 2008). Furthermore, most of the HSF4 binding region contains none or only one GAA sequence even in vivo. The numbers of consensus pentamers per binding sites are high (four to five pentamers) in only HSF1-selected oligonucleotide, suggesting that cooperative binding between trimers could affect the binding of HSF1 (Bonner et al. 1994; Kroeger and Morimoto 1994).

2.7   Cooperativity and Competition Between Distinct HSFs

Vertebrate HSF family members typically form a homotrimer when they are activated and bind to the HSEs in the promoters of target genes. Cooperativity of HSF1 and HSF2 has been shown in spermatogenesis (Wang et al. 2004). Spermato-genesis is normal in HSF1-null mice (Izu et al. 2004) and is partially impaired in HSF2-null mice (Kallio et al. 2002; Wang et al. 2003). These male mice are still fertile, although the number of sperm is markedly reduced in HSF2-null mice. In marked contrast, spermatogenesis is completely blocked in mice lacking both HSF1 and HSF2, and the double-null mice are infertile (Wang et al. 2004). Analysis of target genes shows that the expression of some genes, including *HSP70-2*, *HSC70t*, and *acrosin*, is significantly reduced only in double-null testis. Thus, HSF1 and HSF2 cooperatively upregulate target genes and support spermatogenesis. In addi-tion, both factors are required for the constitutive and inducible expression of CRYAB in mouse embryonic fibroblasts (Shinkawa et al. 2011), and HSF2 enhances the HSF1-mediated inducible expression of HSP70 (Östling et al. 2007). Mechanisms of these cooperativities are unclear yet. HSF1 and HSF2 trimers may simultaneously occupy the target gene promoters, or HSF1 trimer may interact with HSF2 trimer. Alternatively, HSF1 and HSF2 may form heterotrimers (Sandqvist et al. 2009).

On the contrary, there is evidence that HSF1 and HSF4 compete with each other. In lens fiber cells of the mouse lens, both HSF1 and HSF4 cooperatively upregulate the expression of *γ-crystallin* genes. In contrast, in lens epithelial cells, HSF4 downregulates the expression of *FGF* genes, while HSF1 upregulates it (Fujimoto et al. 2004). Therefore, increased proliferation and premature differentiation due to the elevated expression of FGFs in the HSF4-null lens are alleviated in the lens lacking both HSF4 and HSF1. Furthermore, HSF1 and HSF4 have opposing effects on the expression of *LIF* gene in the mouse olfactory epithelium (Takaki et al. 2006). Thus, HSF4 competes with HSF1 for the expression of genes such as cytokine genes.

In chicken B-lymphocyte DT40 cells, disruption of *HSF1* or *HSF3* gene does not affect the expression of HSP90α (Nakai and Ishikawa 2001). However, disruption of both genes results in the marked reduction of the constitutive expression of HSP90α and blockade of cell cycle progression at elevated temperature. Thus, avian HSF1 and HSF3 redundantly regulate the expression of HSP90α, which is

required for cell cycle transition under stress condition (Morano et al. 1999; Zarzov et al. 1997).

2.8   Future Perspectives

In this chapter, we described mainly the structure and function of four *HSF* genes in vertebrates. The crystal structure and NMR solution structure of the DBD in yeast HSF revealed the precise structure in the DBD. It is proposed that an inert HSF1 monomer is folded for the HR-A/B and HR-C to interact with each other in unstressed condition and is converted to three-bundle, rod-shaped structure in response to heat shock. The structure of full-length HSF1 or domains other than the DBD is not analyzed yet, and these analyses should be done in the future. Among domains of HSFs, amino acid sequences of the DBD and oligomerization domains are highly conserved, and functions of these domains are well known. On the other hand, roles of the regions X and Y and the DHR are little known. There are several conserved sequences termed a to g sites in these regions, whose functions should be revealed.

In mammalian cells, HSF1 is required for the heat shock response, while roles of other HSF family members had hardly been known until recently. It is now known that not only HSF1 but also HSF2, HSF3, and HSF4 regulate proteostasis capacity within a cell to adapt to proteotoxic stresses. However, target genes and activation mechanisms of each HSF are not well known at present. In addition, mechanisms of cooperative and competitive regulation by the HSF family members are still unclear and should be clarified in the future.

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