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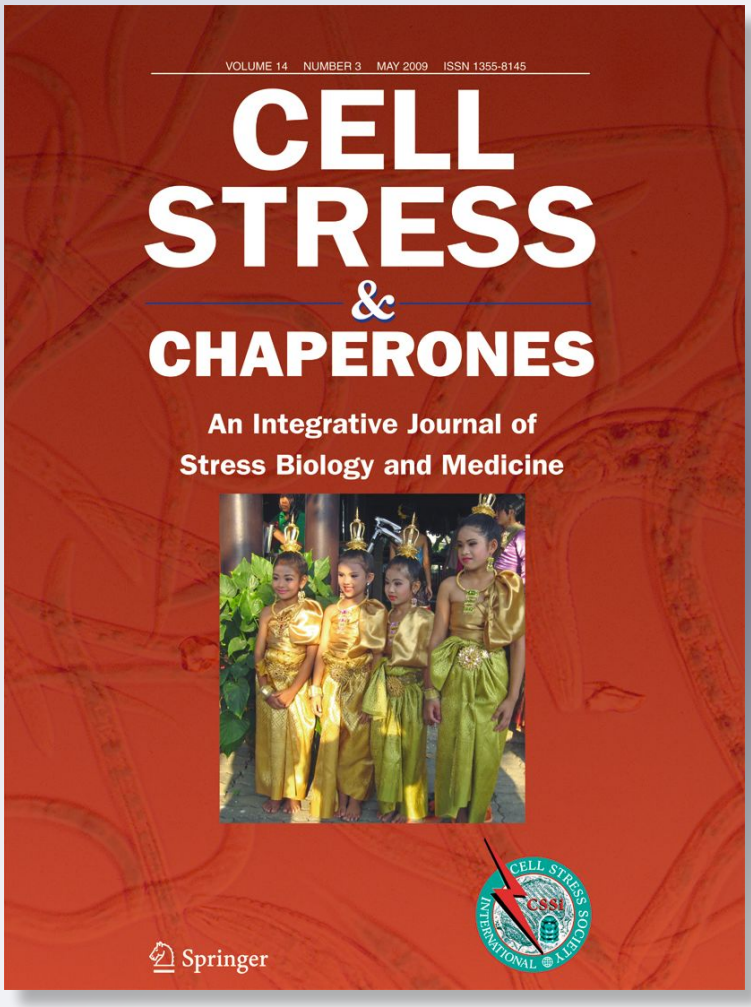

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ORIGINAL PAPER

Zebrafish HSF4: a novel protein that shares features of both HSF1 and HSF4 of mammals

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Abstract Heat-shock proteins (*hsps*) have important roles in the development of the eye lens. We previously demonstrated that knockdown of *hsp70* gene expression using morpholino antisense technology resulted in an altered lens phenotype in zebrafish embryos. A less severe phenotype was seen with knockdown of heat-shock factor 1 (HSF1), suggesting that, while it likely plays a role in *hsp70* regulation during lens formation, other regulatory factors are also involved. Heat-shock factor 4 plays an important role in mammalian lens development, and an expressed sequence tag encoding zebrafish HSF4 has been identified. The deduced amino acid sequence shares structural similarities with mammalian HSF4 including the lack of an HR-C domain. However, the HR-C domain is absent due to a severe C-terminal truncation within zebrafish HSF4 (zHSF4) relative to the mammalian protein. Surprisingly, the amino acid composition of the zHSF4 DNA binding domain shares a greater degree of identity with HSF1 proteins than it does with mammalian HSF4 proteins. Consistent with this, the binding affinity of in vitro synthesized zHSF4 for discontinuous heat-shock response element sequences is more limited, similar to what has been previously observed for HSF1 proteins. *Hsf4* mRNA is expressed in zebrafish adult eye tissue but is only observed in developing embryonic tissue at 60 h post-fertilization or later. This, together with the lack of an observable phenotype following morpholino-based antisense knockdown of *hsf4*, suggests that zHSF4 is unlikely to play a role in regulating early embryonic lens development.

Keywords HSF4 · Zebrafish · Lens · DNA binding domain

Introduction

Heat-shock protein gene expression is regulated by a family of transcription factors called heat-shock factors (HSFs). These proteins regulate heat-shock gene expression in response to cellular stresses and also during different developmental stages in the absence of stress (Åkerfelt et al. 2007; Abane and Mezger 2010; Björk and Sistonen 2010; Fujimoto and Nakai 2010). Heat-shock factors also regulate non-*hsp* genes such as those encoding fibroblast growth factors (FGFs) and c-fos, as well as being involved in chromatin modification (Morano and Thiele 1999; Pirkkala et al. 2001; Xing et al. 2005; Tu et al. 2006; Åkerfelt et al. 2007; Wilkerson et al. 2007; Fujimoto et al. 2008; Shi et al. 2009). HSF proteins exist constitutively within cells in an inactive state as monomers and dimers. These proteins become active when cellular processes signal the monomers and dimers to trimerize, after which they localize to specific DNA sequences to activate target genes (Pirkkala et al. 2001; Björk and Sistonen 2010).

The amino acid sequences for HSFs are highly conserved across a range of organisms from single-celled eukaryotes to mammals. There are several functional domains in HSF proteins that are also highly conserved between species (Rabindran et al. 1993; Pirkkala et al. 2001). All HSFs

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contain a highly conserved amino-terminal winged helix-turn-helix DNA binding domain (DBD) capable of recognizing and binding to a specific sequence of DNA consisting of a series of at least three inverted repeats of the sequence 5′ nGAAn3′ known as a heat-shock response element (HSE) (Amin et al. 1988; Harrison et al. 1994; Morimoto 1998; Pirkkala et al. 2001). The second conserved domain in the amino terminus of HSFs is the transactivation domain (HR-A/B) consisting of three hydrophobic heptad repeats required for the trimerization of HSF monomers and dimers. A third functional domain found in most HSFs also consists of hydrophobic heptad repeats (HR-C) and is located at the carboxyl terminus of the protein. This region has been determined to interact with several other proteins and molecules for the purpose of inhibiting trimerization and maintaining HSFs in their inactive form (Rabindran et al. 1993; Nakai and Morimoto 1993; Morimoto 1998).

In vertebrates, four HSFs have been identified. Heat-shock factor 1 (HSF1) was the first HSF identified and is known to be the primary regulator of heat-shock genes following stress (Pirkkala et al. 2001; Björk and Sistonen 2010). For this reason, it has been the most widely studied. Although HSF1 is the primary HSF that regulates the stress response in vertebrates, it also has roles in development (Åkerfelt et al. 2007; Evans et al. 2007; Abane and Mezger 2010). The role of mammalian HSF2 appears to be largely developmental as it is not activated in response to heat shock, although some evidence has suggested that HSF2 may work in conjunction with HSF1 to regulate the expression of heat-shock genes (Morange 2006; Åkerfelt et al. 2007; Östling et al. 2007; Björk and Sistonen 2010). Heat-shock factor 3 was originally identified as an avian HSF (Nakai and Morimoto 1993), but an ortholog has recently been identified in mice (Fujimoto et al. 2010). The primary role of the HSF3 protein appears to be regulation of the cellular heat-shock response to severe and long-term stresses (Tanabe et al. 1997, 1998; Kawazoe et al. 1999; Fujimoto et al. 2010). Some developmental roles for HSF3 regulation of non-*hsp* genes have also been identified (Kanei-Ishii et al. 1997; Tanikawa et al. 2000; Fujimoto and Nakai 2010).

HSF4 is the fourth HSF to be characterized in mammalian systems (Nakai et al. 1997; Tanabe et al. 1999). The structure of HSF4 has a pronounced difference from other HSFs in that it lacks the inhibition of trimerization domain (HR-C), suggesting that HSF4 may be constitutively bound to HSE. A role for HSF4 in development was first described by Bu et al. (2002) when they identified a mutation in the DBD of HSF4 that is linked to congenital juvenile cataract formation in two different families. The main target of HSF4 appears to be γ-crystallin genes in lens fiber cells (Fujimoto et al. 2004), but HSF4 also has been linked to the regulation of several other genes in the lens including *fgf* and *SKAP2* in epithelial cells, *hsp25* genes in terminally differentiating lens fibers, as well as *α-crystallin* and *Bfsp* genes in lens fiber cells (Fujimoto et al. 2004; Min et al. 2004; Morange 2006; Somasundaram and Bhat 2004; Shi et al. 2009; Enoki et al. 2010; Zhou et al. 2011). Experiments using *hsf4*-null mice demonstrated that the role of HSF4 is tissue-specific, as these mice had no major abnormalities in growth or organ development with the exception of cataracts. In these mice, development of the lens was normal during most of embryogenesis, but abnormalities in lens fiber cells appeared during late embryogenesis and postnatally (Fujimoto et al. 2004; Min et al. 2004; Shi et al. 2009). The level of expression of heat-shock proteins (Hsps) was not affected, suggesting HSF4 interacts predominantly with non-*hsp* target genes in the mammalian lens (Fujimoto et al. 2004; Fujimoto and Nakai 2010).

Recent studies have established that different HSF proteins exhibit different affinities for perfect nGAAn or discontinuous HSE target sequences containing small changes to the nGAAn sequence pattern. Yeast heat-shock factor binds both perfect and discontinuous HSE sequences (Hashikawa et al. 2007). In contrast, human HSF1 (hHSF1) preferentially bound to continuous HSEs, hHSF2 had a higher binding affinity for discontinuous HSEs than hHSF1, and hHSF4 exhibited the highest binding affinity for discontinuous HSEs (Yamamoto et al. 2009). Studies of HSF binding in mouse lens tissue also demonstrated that mouse (m)HSF4 was able to bind to discontinuous and more ambiguous HSE sequences more strongly than mHSF1 and mHSF2 (Fujimoto et al. 2008; Sakurai and Enoki 2010). It has been suggested that this observed preferential binding of HSFs to continuous and discontinuous HSEs may be an important part of the mechanism by which HSFs can differentially regulate gene expression in different tissues or to regulate different biological functions (Yamamoto et al. 2009; Sakurai and Enoki 2010; Enoki and Sakurai 2011).

We have been examining the developmental roles of Hsps and HSFs in zebrafish (*Danio rerio*), a well-established developmental model system. The zebrafish genome contains at least three heat-shock factors; two isoforms of *hsf1* (Råbergh et al. 2000), *hsf2* (Yeh et al. 2006), and *hsf4*. Recent studies by our group have shown that regulation of the *hsp70* gene expression by HSF1 is important for development of the zebrafish eye (Krone et al. 2003; Evans and Krone 2005; Evans et al. 2005, 2007). The knockdown of *hsf1* and *hsp70* by antisense morpholino-modified oligonucleotides resulted in a consistent and reproducible small eye phenotype in microiinjected embryos due primarily to a reduction in lens size. The number of animals exhibiting the small eye phenotype following *hsf1* knockdown was similar to that seen in *hsp70* knockdowns, but the small eye phenotype was less severe in *hsf1* knockdowns (Evans et al. 2007). Similar experiments using

morpholino knockdowns for zebrafish *hsf2* expression showed no effect on eye development (Evans 2006; Evans et al. 2007). The reduced severity of the small eye phenotype in the *hsf1* knockdowns suggested that there are likely other factors involved in regulating *hsp70* expression in the zebrafish lens. Both HSF1 and HSF4 play a role in mammalian lens development (Fujimoto et al. 2004; Min et al. 2004; Smaoui et al. 2004; Mellersh et al. 2006; Fujimoto et al. 2008; Shi et al. 2009; Abane and Mezger 2010), suggesting that the zebrafish *hsf4* recently identified in an expressed sequence tag (EST) library could play a similar role. Here, we have carried out a comparative sequence analysis of zebrafish HSF4 to zebrafish HSF1 and HSF2, as well as HSFs from a wide range of organisms. This analysis, together with an examination of the DNA binding properties and expression of this novel zebrafish HSF4, suggests that it is not functionally equivalent to mammalian HSF4 in all respects.

Materials and methods

Analysis of HSF protein sequences

HSF protein sequences alignments were performed using the ClustalW alignment program (Larkin et al. 2007). Boxshade software was used to outline amino acid similarities between the aligned sequences (Hofmann and Baron 2008; http://www.ch.embnet.org/software/BOX_form.html). Structural domains within the zebrafish HSF2 and HSF4 proteins were identified using NCBI's Conserved Domain Database (Marchler-Bauer et al. 2009) as well as the SMART protein analysis software (Ponting et al. 1999). The location of the protein domains identified using the CDD and SMART software were confirmed by sequence alignment of zebrafish proteins with HSF proteins from other species deposited in Genbank. The percent amino acid identity of protein domains was determined by comparing only the amino acid sequence of the DBD, HR-A/B, and HR-C from each of the three zebrafish HSFs to HSF protein sequences from nine other species found in GenBank using BLAST software available on the NCBI website (Altschul et al. 1990). Phylogenetic analysis was performed for the three zebrafish HSF sequences and 24 other HSF sequences that were identified in GenBank using the Phylogeny.fr software (Dereeper et al. 2008, 2010). Species and gene accession numbers used for protein domain and phylogenetic analysis are shown in Online Resources 1.

Expression of HSF proteins in *Escherichia coli*

Plasmids containing full-length cDNA clones for HSF1 (clone ID 8754667), HSF2 (clone ID 7002403), and HSF4 (clone ID 7143689) were purchased from the I.M.A.G.E. consortium as glycerol stocks. Plasmids for each clone were isolated from transformed bacterial colonies using standard alkaline lysis miniprep procedure (Sambrook and Russel 2001), and the identity of the cDNA sequence from each clone was confirmed by sequencing at the Plant Biotechnology Institute (Saskatoon, SK).

The open reading frame from each of the full-length cDNA sequences was amplified using polymerase chain reaction (PCR). Primers incorporated *Xho*1 and *Bst*B1 restriction enzyme sites on the 5′ and 3′ ends of the PCR products, respectively, to facilitate directional cloning into the pRSET-A expression vector. The sequence for HSF1 was amplified with primers (forward, 5′-CGGGCTCGAGATGGAGTATCACAG-3′; reverse, 5-GGTTTGATAGTATCAAGCTTCCCG-3′) to generate a 1,494-bp fragment; HSF2 was amplified with primers (forward, 5′-CGGGCTCGAGATGAAACACAGCTCG-3′; reverse 5-GCCCTTCGAATCAGATATCAAGCGG-3′) to generate a 1,470-bp fragment, and the HSF4 sequence was amplified with primers (forward, 5′-CGGGCTCGAGATG CAGGAGAACCC-3′; reverse 5′-GCCCTTCGAATCACCTTGATTCC-3′) to generate an 861-bp fragment. All PCR reactions were performed over 40 cycles using annealing temperatures of 50°C for HSF1 and 55°C for both HSF2 and HSF4 reactions.

Products from PCR reactions were visualized by agarose gel electrophoresis and purified using a DNA extraction kit (Fermentas). The isolated PCR fragments were initially cloned into the pCR®4-TOPO vector using the TOPO TA Cloning® Kit following the Invitrogen product manual, and the identity of the PCR fragment was confirmed by DNA sequencing at the Plant Biotechnology Institute (Saskatoon, SK). The PCR fragments were subsequently sub-cloned into the pRSET-A expression vector (Invitrogen). Competent *E. coli* strain BL21(DE3)pLysS cells were then transformed with pRSET-A vectors both with and without PCR fragment inserts (Invitrogen) and incubated at 28°C with agitation. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 mM when cultures reached an $OD_{600\ nm}$ between 0.4 and 0.6. Cultures were then pelleted, resuspended in phosphate buffered saline (PBS), and lysed by homogenizing and sonicating the sample. Cell debris was pelleted by centrifugation, and proteins were purified from the retained supernatant by adding 100 μL of Profinity™ IMAC Ni-charged resin (BioRad)/10 mM imidazole/PBS to 2 mL of supernatant and incubated with gentle rocking at 4°C overnight. The resin pellet was washed in 10 mM imidazole/PBS a total of three times. The purified protein was then eluted from the resin by resuspending the resin in PBS containing 250 mM imidazole. Protein concentration of the purified HSFs was determined by Bradford assay using the Pierce 660 nm protein assay reagent (Thermo Scientific).

Gel mobility shift analysis

A reverse transcriptase fill-in reaction was used to radiolabel 25 pmol of HSE oligo DNA. Sequences of HSE oligo DNA used are listed in Online Resources 2. The reaction was performed using 5 U of mouse leukemia virus reverse transcriptase (Fermentas Inc.), 2 mM each dATP, dGTP, and dTTP; 2 μg BSA, and 20 μCi α^{32}P-dCTP in a final volume of 20 μl and incubated at 37°C for 30 min. Reactions were then diluted to 100 μl with distilled water, extracted once with phenol/chloroform (1:1), and then precipitated with 1/3 volume of 7.5 M ammonium acetate and 2.5 times volume of ethanol.

DNA binding reactions contained 750 ng of in vitro purified HSF. Each sample was combined with 100 cps labeled HSE probe, 0.5 μg poly deoxy-inosine-deoxy-cytosine, 10 mM Tris–HCl pH 7.8, 1 mM EDTA, 1 mM DTT, and 5% glycerol in a final volume of 25 μl. Reactions were incubated for 20 min at room temperature, mixed with 2 μl of 5× loading dye (0.25% bromophenol-blue, 0.25% *w/v* xylene-cyanol, 50% *v/v* glycerol), and immediately loaded onto non-denaturing 4% polyacrylamide gels, and electrophoresed in 1× Tris/glycine buffer (25 mM Tris/HCl, 192 mM glycine). The samples were run through the gel at 4°C for 2 h at 175 V, dried on filter paper at 80°C for 50 min, and exposed to HyBlot CL™ autoradiography film (Denville Scientific) at −80°C using an intensifying screen.

Zebrafish maintenance, breeding, and microinjection

Breeding, maintenance, and manipulation of zebrafish adults and embryos were performed as described (Westerfield 1995). Microinjection of zebrafish with morpholino modified antisense oligonucleotides tagged with 3′-carboxyfluorescein (MO) was performed as previously described (Evans et al. 2007). Morpholinos were designed to be complimentary to the EST-specific HSF4 mRNA sequence based on sequence data obtained from Genbank (accession # NM001013317; *hsf4*-MO (5′-TATAGAGCCTG GGTTCTCCTGCATG-3′)). Morpholinos with a five-base-pair mismatch were injected as a control for non-specific phenotypic effects; *hsf4* 5bpmm-MO (5′-TATtGAGg CTGGcTTCaCCTGgATG-3′; mismatched base pairs are indicated by lower case letters). Morpholino mismatch sequences were designed to be as similar as possible to the original morpholino sequence while still preventing hybridization, with an unaltered GC content. The mismatch sequence had no significant sequence identity to any known sequences within the zebrafish genome.

Morpholino stock solutions for *hsf4*-MO and *hsf4* 5bpmm-MO (Gene Tools, LLC, Oregon) were prepared in triple-distilled water to a final concentration of 8.333 μg/μL, aliquoted, and stored at −80°C until use. Stock MO were further diluted in Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM HEPES pH 7.6; Nasevicius and Ekker 2000) to concentrations of 1.73, 2.26, 3.46, and 5 μg/μL immediately prior to embryo injection. All MO were injected at a constant volume of 3.14 nL/injection using a Narashige IM-300 microinjector. Embryos were maintained in a 28°C incubator.

Treatment groups consisted of embryos injected with Danieau solution, *hsf4*-MO or *hsf4* 5bpmm-MO, and were compared with an uninjected treatment group. The diameter of the eye as well as the length of the body was measured in embryos showing strong fluorescein penetrance at 48 h post-fertilization (hpf). The effect of the MO on lens phenotype was determined by calculating the ratio of eye and lens diameter and eye surface area to total body length similar to that previously described (Evans et al. 2007).

RNA extraction and cDNA synthesis

RNA was extracted from zebrafish embryos collected at four different developmental time points—10, 30, 60 and 80 hpf. At least 30 embryos from each time point were collected into 1.5-mL microfuge tubes; water was removed, and samples were snap-frozen on dry ice. Frozen embryos were stored at −80°C until RNA extraction. RNA from embryos was extracted using the RNeasy RNA extraction kit (Qiagen). Briefly, embryos were homogenized using a pestle in Qiagen's RLT buffer. Samples were processed following the instructions from the RNeasy kit including the on-column DNase treatment and finally eluted from the column in 30 μL of RNase-free water and stored at −80°C until use.

Adult zebrafish tissues were collected as described by Gupta and Mullins (2010). Tissues from five adult fish were pooled into a tube containing RNAlater (Qiagen) solution and stored at −20°C until RNA extraction. Immediately prior to RNA extraction, adult tissues were removed from the RNAlater solution, frozen in liquid nitrogen, and ground using a pestle. Liver and spleen tissue were immediately resuspended in buffer and processed using an RNeasy extraction kit (Qiagen) as described above for embryos. Pulverized eye tissues were immediately resuspended and homogenized with a pestle in Tri Reagent® (Ambion) and subsequently extracted with chloroform. RNA was extracted from the resulting supernatant using the RNeasy extraction kit (Qiagen) as described above.

A sample of purified RNA was diluted tenfold with 10 mM Tris and dispensed into trUView cuvettes (Bio-Rad), and absorbance was measured at 260 nm using a SmartSpec Plus spectrophotometer (BioRad). The concentration of RNA in each sample was determined using the following equation—40 μg/mL × A_{260} × dilution factor, and RNA integrity was assessed using formaldehyde gel electrophoresis.

Synthesis of cDNA was performed using the RevertAid H minus first-strand cDNA synthesis kit (Fermentas). Reactions were performed using 2 μg of RNA for embryo samples and 1 μg of RNA for adult tissues. Reactions were performed as per the kit instructions using only oligo $(d)_T$ primers in the reaction. Samples were stored at −20°C until use.

Gene expression analysis by quantitative PCR

Quantitative (q)PCR was performed to determine the mRNA levels of the short EST-derived *hsf4* transcript using a forward primer specific to the coding region of the mRNA transcript (5′ GCCAAGGAAGTTCTGCCCAA 3′) and a reverse primer specific to the 3′ UTR of the transcript (5′ GCTAAAAGTGGTCTCGCCCC 3′). *Beta-actin* was used as a reference gene based on previous studies that examined a number of suitable reference genes during zebrafish development (Tang et al. 2007; McCurley and Callard 2008) and amplified in every reaction using forward primer 5′ CGAGCAGGAGATGGGAACC 3′ and reverse primer 5′ CAACGGAAACGCTCATTGC 3′. The cDNA was diluted fivefold in water, and 2 μL of the diluted cDNA was added to a 20-μL reaction mixture containing 10 μL of 2× SsoFast™ EvaGreen® Supermix (BioRad) and 300 μM final concentration of each primer. PCR reactions were performed in a BioRad MiniOpticon thermocycler.

PCR reactions were performed using triplicate technical samples and a single non-template control for each of three biological replicates tested. Reactions were performed using the following conditions: Denaturing reaction at 95°C for 10 min followed by 50 cycles of (95°C for 15 s, 62°C for 30 s, 72°C for 45 s). A melting curve was also generated for all samples after the completion of the PCR reactions to confirm the specificity of the primers. Critical threshold values (C_T) and melting curve analysis was performed using the BioRad CFX Manager software. A standard curve of cDNA dilutions was generated for each primer set to determine the efficiency of the reactions. Reaction efficiencies for *hsf4* and *beta-actin* were determined to be similar, and therefore relative gene expression was calculated using the delta-C_T method of normalizing gene expression (Anonymous 2010). Results are reported as the ratio of *beta-actin* to *hsf4* mRNA levels using the equation, Ratio (reference/target)=(Efficiency of reaction)$^{\text{Ct (Reference)}-\text{Ct(Target)}}$.

The PCR fragments from the above reaction as well as fragments generated from a second independent set of *hsf4*-specific primers (forward—5′ GAAGCCAGAGCG AGACGACA 3′; reverse—5′ AGCTGTGTTGAA AATTGTGTTTGG 3′) were each cloned into the pCR®4-TOPO vector using the TOPO TA Cloning® Kit following the Invitrogen product manual, and the identity of the PCR fragment was confirmed by sequencing using M13 primers at the Plant Biotechnology Institute (Saskatoon, SK).

Results

Identification of a novel zebrafish HSF4 protein sequence

A novel zebrafish HSF (zHSF4) was previously isolated from an EST screen of an embryonic cDNA library derived from zebrafish embryos spanning stages between fertilization to 72 hpf (Strausberg et al. 2002). The identified clone consists of a 1,650 bp full-length cDNA sequence (Genbank accession no. NM_001013317), corresponds to Mammalian Genome Collection (MGC)/ZGC:113344, and is referenced in the ZFIN library as ZDB-GENE-050306-18 (Sprague et al. 2006). Sequence analysis of the cloned cDNA reveals a significant homology to previously characterized zebrafish HSF1 and HSF2 nucleotide sequences (Råbergh et al. 2000; Yeh et al. 2006) and to mammalian HSF4. The identified mRNA sequence is encoded by a single *hsf4* gene that has been mapped to zebrafish chromosome 18, with the genomic sequence spanning 7,103 bp and consisting of eight exons and seven introns. Exons 1–3 encode the DBD, and exons 4–6 encode the HR-A/B domain (Sprague et al. 2006). Surprisingly, the predicted protein sequence for this novel zHSF4 is only 286 amino acid residues in length, which is much shorter than any other HSF protein sequences previously characterized. Very recently, a second, longer putative HSF4 transcript has also been predicted from the same gene (Ensemble Zv8, release 59), although it is not currently known if this represents a true gene product. The short predicted protein product of the EST-derived zebrafish HSF4 cDNA is very unique within the HSF family among all eukaryotic organisms, and therefore we focused our analysis on this novel vertebrate HSF4 sequence. Analysis of the predicted amino acid sequence using SMART and Pfam protein analysis tools (UniProtKB) indicates that amino acids 19–123 comprise a region with a high degree of identity to the DBD of proteins in the HSF superfamily. Sequence alignment (Fig. 1a) demonstrates the overall level of amino acid sequence conservation between this novel zebrafish HSF4 protein and zebrafish HSF1 and HSF2. The highly conserved HR-A/B trimerization domain spanning amino acid sequence 133–214 (Fig. 1a) can also be seen in the three zebrafish HSFs and is similar to that present in HSF protein sequences from several other species including human, mice, dogs, equine, *Xenopus laevis*, chicken, and rainbow trout (not shown). The HR-A/B domain identified by sequence alignment corresponds to a predicted coiled coil structure, characteristic of a leucine zipper domain, identified in the zHSF4 sequence by the UniProtKB software. However, the zHSF4 protein lacks an HR-C domain (Fig. 1b). Sequence analysis with UniProtKB software and sequence alignment could not identify a second coiled-coil structure or leucine zipper characteristic of an HR-C domain in the carboxy terminus of zHSF4. The

lack of an HR-C trimerization inhibition domain in the carboxy terminus of the protein is consistent with what has been reported for mammalian HSF4 protein sequences (Nakai et al. 1997). It is, however, important to note that the novel zHSF4 protein sequence is effectively 177–250 amino acids shorter than any other HSF identified in a vertebrate species. Thus, the lack of an HR-C domain in the carboxy terminus is not due to the presence of an amino acid sequence which does not contain a heptad repeat sequence as is the case for mammalian HSF4s, but instead to the fact that this region is completely absent in the zebrafish protein.

Phylogenetic analysis of the zebrafish HSF4 protein

Given the substantial difference in size of the zHSF4 protein when compared with known HSF sequences, we carried out an extensive comparison of HSF1, HSF2, HSF3, and HSF4 protein sequences from 11 species to identify to which HSF group the novel zHSF4 protein was most closely related. The zHSF4 protein clusters in a distinct group with the chicken and zebrafinch HSF4 proteins near the mammalian HSF4 sequence cluster (Fig. 2). However, the branch for the zHSF4 protein occurs sooner and is closer to the HSF1 group than the mammalian HSF4 cluster. Additionally, the distance between the zHSF4 protein and the mammalian HSF4 cluster is greater than that observed between the fish and mammalian proteins for either HSF1 or HSF2. Another interesting observation from this analysis is that the zHSF4 protein clusters with two avian HSF4 protein sequences, the chicken and zebrafinch. While the chicken and zebrafinch branches are close to each other, similar to what is observed for the HSF2 protein from these same species (Fig. 2), the close grouping of the avian proteins to zebrafish proteins appears to be unique to the HSF4 group. In contrast, the grouping of chicken and zebrafinch HSF2 proteins is much closer to that of the mammalian HSF2 while the zebrafish and trout HSF2 branch occurs more distant from the mammalian HSF2 proteins. Similarly, the chicken HSF1 protein is more closely grouped with the mammalian HSF1 proteins while the trout and zebrafish HSF1 proteins branch is closer to the single *Drosophila* HSF than to the mammalian HSF1.

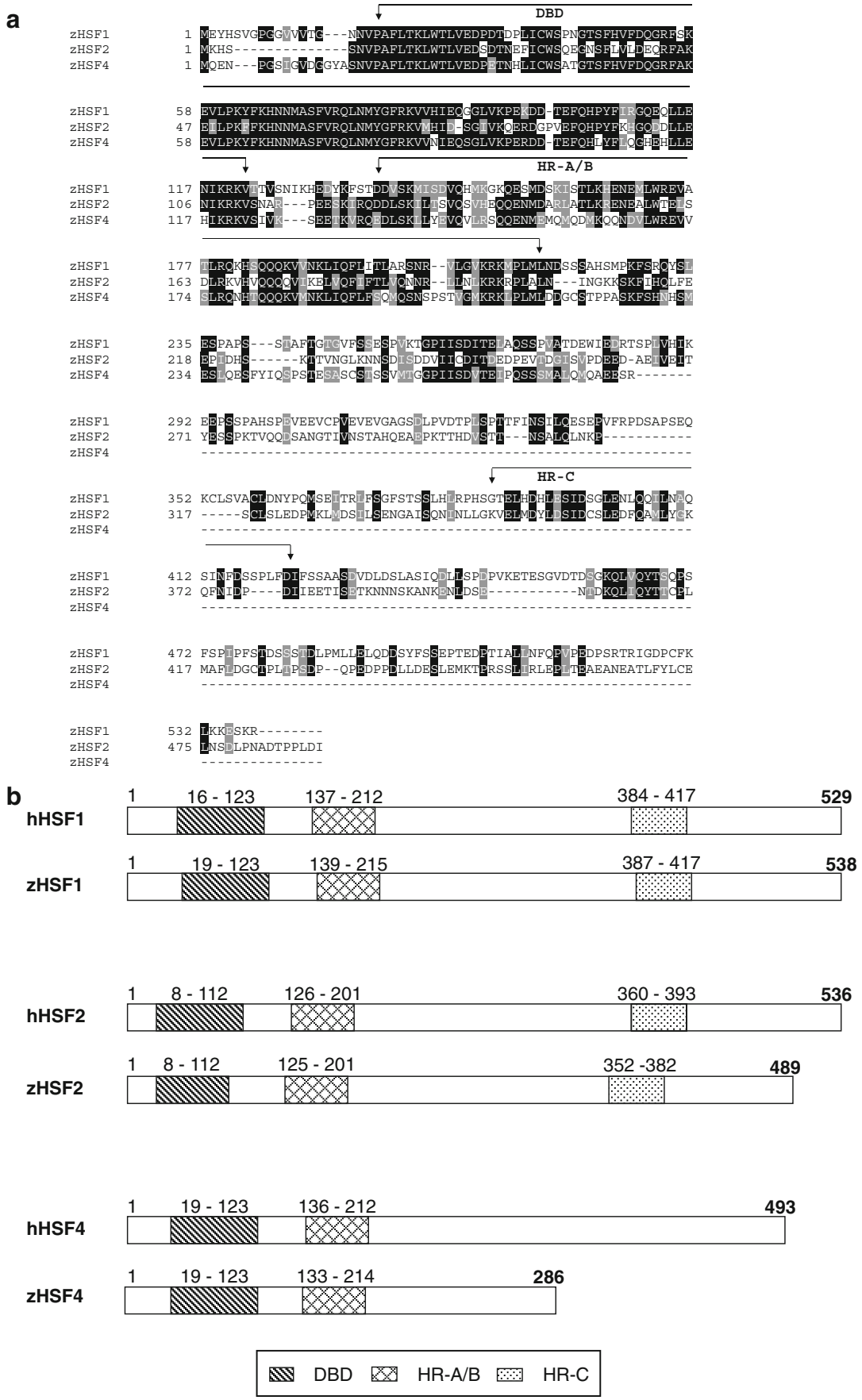
The early branching and distinct clustering of the zHSF4 protein observed in the phylogenetic analysis (Fig. 2) led us to perform a more thorough comparison of the amino acid sequence comprising the zHSF4 protein to other members of the HSF protein family (Tables 1, 2, 3, and 4). The putative amino acid sequences of the DBD, HR-A/B, and HR-C protein domains of the three zebrafish HSFs were compared between themselves and to HSF protein sequences from other species. This analysis revealed that the zHSF4 protein shares a high degree of identity in the DBD of zHSF1 (85%) and zHSF2 (73%) (Fig. 1 and Table 1).

Fig. 1 Comparison of the three identified zebrafish heat-shock factor protein sequences. **a** Zebrafish HSF1, HSF2, and HSF4 proteins are compared with each other by sequence alignment. Residues that are identical between the four sequences are shaded in *black* while amino acids that have some similarity are shaded in *grey*. The DBD, HR-A/B and HR-C protein domains are indicated by arrows above the sequence. **b** A diagrammatic representation of zebrafish HSF proteins and the corresponding human ortholog comparing the location of specific protein domains within each sequence. The range of amino acids comprising each domain is indicated *above* each domain and the total number of amino acids in each protein is shown at the *end* of each

Interestingly, the DBD of the predicted zHSF4 protein also shares 84–85% identity with the DBD of HSF1 proteins from seven other vertebrate species (Table 2) but, surprisingly, only a 72% amino acid identity with four mammalian HSF4 proteins (Table 1) and a maximum of 73% identity to HSF2 proteins from eight other species (Table 3). Of particular interest is the degree of the amino acid sequence identity observed between the zHSF4 protein and those predicted for two avian species, the chicken and the zebrafinch (Table 1). The zHSF4 protein had a total amino acid identity of 77% with the chicken and 79% with the zebrafinch, which is higher than what was calculated for zHSF4 in comparison to other zebrafish HSFs (30% for zHSF1 and 27% for zHSF2) as well as being higher than that observed for comparisons to any mammalian HSF4 sequence, (52–54%) (Table 1). When only the DBD and HR-A/B protein domains are compared between zebrafish and the two avian species, the similarity becomes even more striking. The DBD of the zHSF4 protein has a 94% amino acid identity with chicken and a 96% identity with the zebrafinch while the HR-A/B domain has an 82% and 83% identity, respectively. The similarities observed for these protein domains from both avian species and zHSF4 represent the highest level of amino acid conservation between the zHSF4 protein domains in all species compared (Tables 1, 2, 3, and 4). Analysis of the chicken and zebrafinch HSF4 protein sequences reveals that these proteins lack an HR-C domain similar to what has been observed for mammalian HSF4 proteins. However, like the zebrafish HSF4 protein, the amino acid composition of the avian HSF4 DBD domains shares a greater degree of identity with HSF1 DBDs than with mammalian HSF4 DBDs (data not shown).

Zebrafish HSF4 proteins bind to discontinuous HSE sequences with a pattern similar to HSF1

The data presented above clearly indicate that the zHSF4 DNA binding domain more closely resembles that of zebrafish and mammalian HSF1 proteins than it does mammalian HSF4. We thus examined the ability of the zHSF4 protein to bind to HSE DNA binding sites as determined by electrophoretic mobility shift assay using zHSF4 protein isolated and purified from a bacterial expression system. A DNA



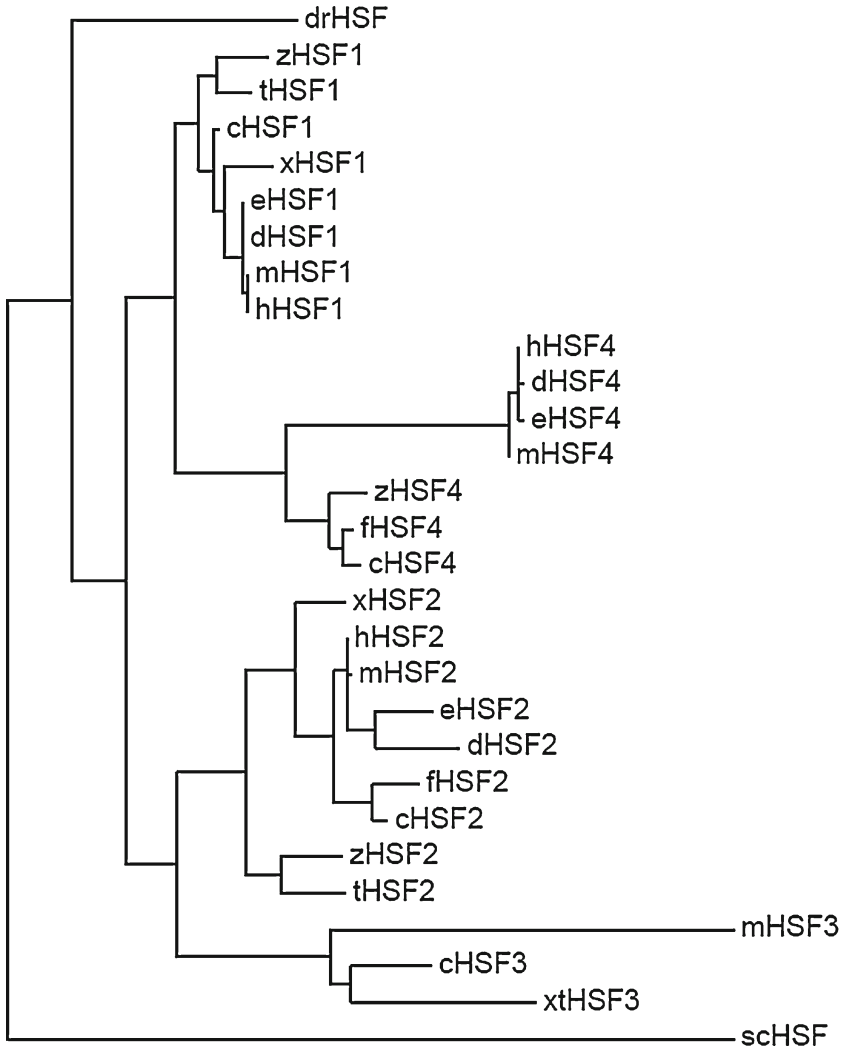


Fig. 2 Phylogenetic analysis of identified HSF protein sequences. An unanchored phylogenic tree of HSF protein sequences from 11 species deposited in Genbank was generated using the Phylogeny.fr software. These sequences include those identified for zebrafish (*z*), rainbow trout (*t*), human (*h*), mouse (*m*), dog (*d*), equine (*e*), *X. laevis* (*x*), *Xenopus tropicalis* (*xt*), *Saccharomyces cerevisiae* (*sc*), *Drosophila* (*dr*), zebrafinch (*f*), and chicken (*c*). Tree branch lengths depict relative evolutionary distances between proteins

oligo designed to be a perfect continuous inverted repeat sequence of nGAAn was used as the HSE target (Online Resource 2). We first demonstrated that the zHSF4 protein bound to HSE in a specific manner (Fig. 3). The addition of unlabelled HSE to the reaction at a concentration of 50 times the amount of labeled HSE was sufficient to completely compete the protein away from the ^{32}P-labeled HSE while addition of the same concentration of an oligo containing a

Table 1 Percentage amino acid identity of HSF4 protein functional domains between zebrafish and other species

Species	Amino acids (#)	zHSF1				zHSF2				zHSF4		
		Total	DBD	HR-A/B	HR-C	Total	DBD	HR-A/B	HR-C	Total	DBD	DBD HR-A/B
zHSF4	286	30	**85**	52	NA	27	73	45	NA			
hHSF4	492	30	75	45	NS	26	63	35	16	53	72	56
mHSF4	462	26	75	48	NS	20	63	36	NS	52	72	59
dHSF4	492	28	75	44	NS	27	63	35	NS	54	72	56
eHSF4	492	28	75	45	NS	27	63	35	NS	52	72	55
cHSF4	510	37	**84**	55	NS	31	70	45	32	77	**94**	**82**
fHSF4	504	37	**86**	53	NS	33	71	45	32	79	**96**	**83**

Amino acid sequences of the total protein, the DNA binding domain (DBD), heptad repeat A/B trimerization domain (HR-A/B), and the heptad repeat C (HR-C) domain of HSF4 sequences from human (h), mouse (m), dog (d), Equine (e), chicken (c), and zebrafinch (f) were compared with zebrafish (z) HSF sequences using NCBI BLAST program. Amino acid sequences that displayed higher than 80% identities are shown in bold

NS indicates no significant amino acid match was identified by BLAST

Table 2 Percentage amino acid identity of HSF1 protein functional domains between zebrafish and other species

Species	Amino acids (#)	zHSF1				zHSF2				zHSF4		
		Total	DBD	HR-A/B	HR-C	Total	DBD	HR-A/B	HR-C	Total	DBD	HR-A/B
zHSF1	538					38	73	49	35	57	**85**	49
hHSF1	529	58	**91**	75	42	38	73	51	NS	58	**84**	56
mHSF1	503	56	**91**	74	42	38	73	49	NS	56	**84**	55
dHSF1	554	61	**91**	77	48	33	73	51	NS	57	**84**	61
eHSF1	590	58	**91**	77	45	37	73	51	NS	58	**84**	60
xHSF1	530	59	**93**	74	48	37	75	49	32	59	**84**	55
cHSF1	491	55	**93**	75	45	36	72	49	35	58	**85**	56
tHSF1	513	66	**96**	75	77	37	74	53	35	57	**85**	55
drHSF	691	27	56	56	39	20	57	42	13	36	56	48
scHSF	833	12	47	21	NS	16	45	22	NS	23	44	20

Amino acid sequences of the total protein, the DNA binding domain (DBD), heptad repeat A/B trimerization domain (HR-A/B), and the heptad repeat C (HR-C) domain of HSF1 sequences from human (h), mouse (m), dog (d), Equine (e), *X. laevis* (x), chicken (c), rainbow trout (t), *Drosophila* (dr), and *S. cerevisiae* (sc) were compared with zebrafish (z) HSF sequences using NCBI BLAST program. Amino acid sequences that displayed higher than 80% identities are shown in bold

NS indicates no significant amino acid match was identified by BLAST

CAAT box (CBF) as a competitor was not able to displace the zHSF4 protein from the HSE oligo.

We next used EMSA to compare the ability of all three zebrafish HSF proteins to bind to four discontinuous HSE sequences (Online Resource 2). Recent studies have demonstrated that heat-shock factors can have different binding affinities for discontinuous HSE sequences. For example, it has been shown that mammalian HSF1 preferentially binds HSE sites with perfect nGAAn inverted sequences while mammalian HSF4 has similar binding affinities for perfect and discontinuous nGAAn sequences (Fujimoto et al. 2008; Yamamoto et al. 2009; Sakurai and Enoki 2010). Reactions were performed by adding 1 pmol of ^{32}P-labeled HSE oligo or modified (mod) HSE oligos to 750 ng of purified protein. As expected, zHSF4 bound specifically to the perfect HSE (Fig. 3), and little difference was observed between the abilities of the three zebrafish HSF proteins to bind to the perfect HSE (data not shown). All HSFs were able to bind HSEsn and a relatively small amount of each HSF bound to the HSEmod1 oligo while no protein binding was observed for three other modified HSE oligos (data not shown). Further analysis of the binding affinity of the three zebrafish HSFs was performed by competition assay with unlabelled HSEsn and HSEmod1. Addition of 50–100 pmol of HSEsn was able to effectively displace zHSF2 from the labeled HSE oligo while a concentration of 300 pmol was required

Table 3 Percentage amino acid identity of HSF2 protein functional domains between zebrafish and other species

Species	Amino acids (#)	zHSF1				zHSF2				zHSF4		
		Total	DBD	HR-A/B	HR-C	Total	DBD	HR-A/B	HR-C	Total	DBD	HR-A/B
zHSF2	489	35	73	49	42					47	73	45
hHSF2	536	36	72	49	39	60	**90**	61	77	44	73	41
mHSF2	517	37	72	49	39	59	**89**	61	77	44	73	41
dHSF2	578	33	59	49	39	56	74	61	77	36	61	41
eHSF2	533	33	58	49	39	56	74	61	77	38	58	41
xHSF2	515	36	73	45	35	58	**90**	65	65	41	73	39
cHSF2	563	36	69	52	39	59	**80**	61	77	41	72	41
fHSF2	630	32	66	48	39	57	77	60	77	42	70	45
tHSF2	511	36	72	55	35	68	**95**	66	87	47	70	43

Amino acid sequences of the total protein, the DNA binding domain (DBD), heptad repeat A/B trimerization domain (HR-A/B), and the heptad repeat C (HR-C) domain of HSF2 sequences from human (h), mouse (m), dog (d), Equine (e), *X. laevis* (x), chicken (c), zebrafinch (f), and rainbow trout (t) were compared with zebrafish (z) HSF sequences using NCBI BLAST program. Amino acid sequences that displayed higher than 80% identities are shown in bold

Table 4 Percentage amino acid identity of HSF3 protein functional domains between zebrafish and other species

Species	Amino acids (#)	zHSF1				zHSF2				zHSF4		
		Total	DBD	HR-A/B	HR-C	Total	DBD	HR-A/B	HR-C	Total	DBD	HR-A/B
mHSF3	492	16	53	29	NS	27	53	34	NS	28	50	28
xtHSF3	550	20	63	40	42	36	63	44	48	36	60	43
cHSF3	467	31	62	49	NS	34	63	51	45	46	63	48

Amino acid sequences of the total protein, the DNA binding domain (DBD), heptad repeat A/B trimerization domain (HR-A/B), and the heptad repeat C (HR-C) domain of HSF3 sequences from mouse (m), *X. tropicalis* (xt), and chicken (c) were compared with zebrafish (z) HSF sequences using NCBI BLAST program. Amino acid sequences that displayed higher than 80% identities are shown in bold

NS indicates no significant amino acid match was identified by BLAST

to effectively displace the zHSF1 and zHSF4 proteins (Fig. 4). Furthermore, zHSF2 was partially displaced from the labeled oligo when 1,000 pmol of HSEmod1 was added to the reaction; the same concentrations of this competamer did not displace any of the zHSF1 or zHSF4 proteins (Fig. 4). Thus, zHSF4 demonstrates a more constrained binding to discontinuous HSE sequences similar to that observed for HSF1 and in contrast to the less stringent HSE sequence requirements that have previously been reported for mammalian HSF4 proteins (Fujimoto et al. 2008; Yamamoto et al. 2009; Sakurai and Enoki 2010).

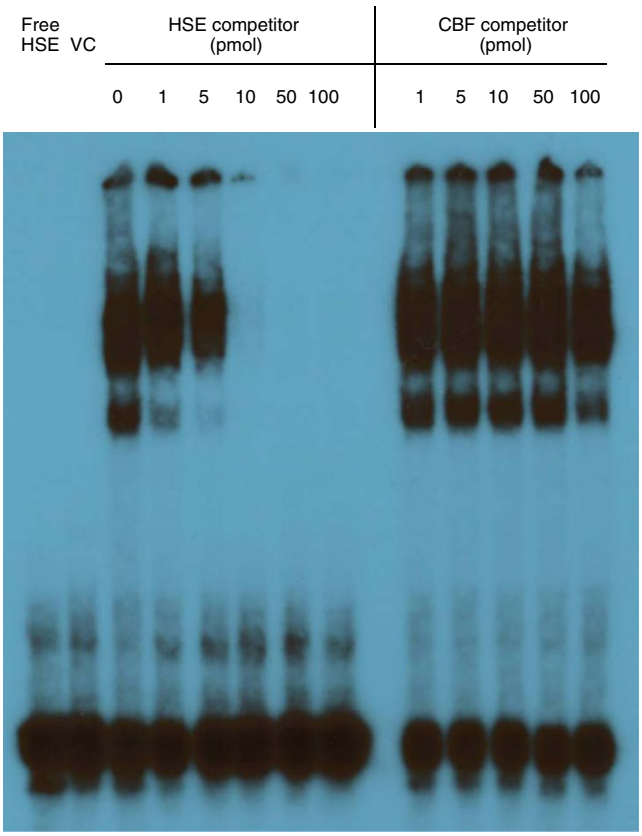


Fig. 3 Zebrafish HSF4 protein binds to an HSE sequence in a specific manner. Binding reactions were prepared containing 1 pmol of ^{32}P-labeled HSE oligo and 750 ng of either in vitro purified zHSF4 protein or protein purified from an *E. coli* control containing an empty pRSET vector (*VC*). An unlabeled HSE oligo or CBF oligo competitor was added (0–100 pmol of competitor per reaction) to binding reactions containing zHSF4-like protein. The binding specificity of the protein for each oligo sequence was determined by EMSA

Expression of *hsf4* mRNA occurs in a tissue- and developmental-specific manner

The observation that zHSF4 DNA binding characteristics more closely resemble HSF1 than mammalian HSF4 prompted us to investigate the expression patterns of *hsf4*. Quantitative PCR was used to detect *hsf4* mRNA levels in adult zebrafish tissues as well as in embryonic tissues from several developmental stages during the first 4 days of development. This represents time periods covering the early stages of lens formation between 10 and 30 h of development until a well-formed lens with secondary lens fibers is present by 80 h. This analysis demonstrated that *hsf4* transcripts were present in the adult eye but were undetectable in the liver and spleen (Fig. 5a). Expression analysis of embryos collected at 10, 30, 60, and 80 hpf demonstrated that *hsf4* mRNA is undetectable during development until 60 hpf (Fig. 5b). Levels of *hsf4* mRNA were similar at both the 60 and 80 hpf developmental stages. PCR products from analysis of the embryonic tissue at 80 hpf were sequenced to confirm that the DNA product matched that of the intended short EST-derived *hsf4* target sequence (data not shown).

We have previously demonstrated the effectiveness of morpholino modified antisense oligonucleotide (MO) microinjection in fertilized zebrafish eggs to knockdown HSP and HSF expression (Evans et al. 2005, 2007). Thus, we used a similar approach to examine HSF4 in zebrafish embryos. Embryos were injected with several concentrations of *hsf4*-MO as well as *hsf4* 5bpmm-MO and compared with Danieau injected and uninjected controls (Online Resource 3) to determine the maximum *hsf4*-MO concentration for use in subsequent

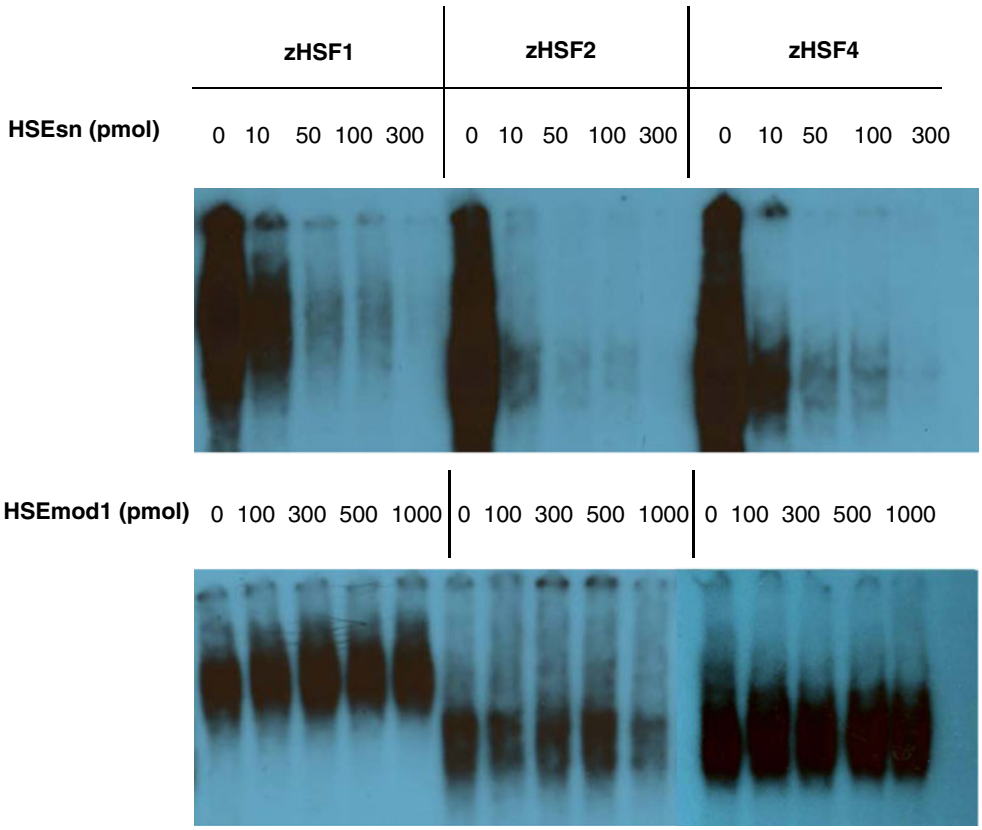


Fig. 4 Zebrafish HSF proteins can bind to certain discontinuous HSEs. Purified in vitro generated zHSF1, zHSF2, and zHSF4 protein (750 ng per reaction) were added to binding reactions containing 1 pmol of ^{32}P-labeled HSE oligo. An unlabeled modified HSE oligo was added to the binding reactions as a competitor. The binding specificity of the protein for each HSE oligo sequence was determined by EMSA. HSEsn oligo was added to each reaction in quantities ranging from 0 to 300 pmol of competitor per reaction. HSEmod1 was added to each reaction in quantities ranging from 0 to 1,000 pmol

experiments without causing significant non-specific phenotypic abnormalities. No significant change in ratios between body to eye length, body length to lens diameter, or eye surface area to body length were observed at 48 hpf (Table 5), indicating that morpholino microinjection had no impact on lens and eye development. In addition, no cataract development was observed in injected embryos after 96 hpf as has previously been observed for *zhsf1*MO-injected embryos (Evans et al. 2007). These data are not surprising given the lack of HSF4 mRNA expression prior to 60 h of development and the known titration of morpholinos below effective levels that is known to occur beginning at 50–60 h of development (Evans et al. 2005).

Discussion

Two HSFs have previously been characterized in zebrafish. In 2000, Råbergh et al. identified two isoforms of HSF1 in adult zebrafish tissues, and in 2006, Yeh et al. identified HSF2 in zebrafish embryos. In this study, we report a unique zebrafish HSF4 isoform that was originally identified in an EST screen of a cDNA library (Strausberg et al. 2002) and subsequently through annotation of zebrafish genomic sequence data. The predicted amino acid sequence of zHSF4 shows strong identity to other HSFs which is in agreement with the high levels of amino acid conservation that is characteristic of this protein family (Fig. 1; Tables 1, 2, 3, and 4). The lack of an HR-C domain suggests that, similar to mammalian HSF4, zHSF4 may also be constitutively trimerized in the cell (Nakai et al. 1997; Björk and Sistonen 2010). However, unlike the mammalian proteins, the lack of an HR-C domain is due to the severe C-terminal truncation of zHSF4 compared with the mammalian and avian forms of HSF4. Indeed, this truncation makes zHSF4 the smallest HSF of any class identified to date. Function of the C-terminal region of the mammalian HSF4 proteins is at present unknown, but the relatively high degree of sequence conservation across this region (73–88%) of the mammalian proteins suggests that it may be of functional or structural importance. Clearly, any such potential function in this region would not be present within the truncated zebrafish form of HSF4.

The phylogenetic analysis of complete HSF protein sequences from 11 species demonstrates that the zebrafish and avian proteins form a separate subgroup of the HSF4 cluster, where zHSF4 groups, very closely with chicken and zebrafinch, predicted HSF4 protein sequences. The close relationship of zHSF4 to avian HSF4 appears to be unique to HSF4 proteins as the branching distances between these species is much greater for HSF1 and HSF2. While this group of zebrafish and avian HSF4 proteins does cluster closer to the mammalian HSF4 proteins than to any of the

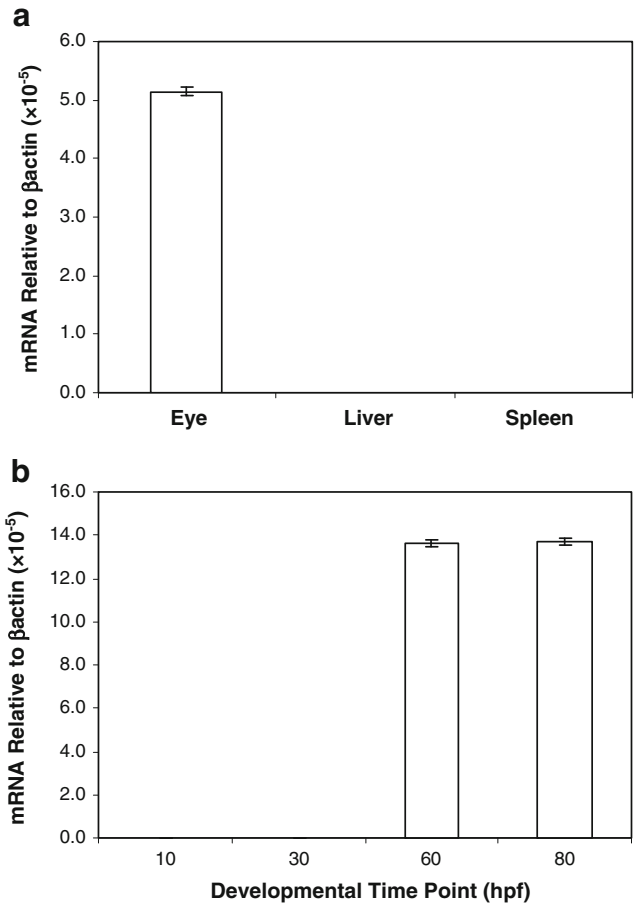


Fig. 5 Zebrafish *hsf4* mRNA levels are tissue- and development-stage-specific. Quantitative PCR was performed on cDNA prepared from three adult tissues (**a**) and embryonic tissue from four developmental stages (**b**) to determine relative mRNA expression levels for *hsf4*. The data represent a single experiment where a similar pattern was seen in three independent biological replicates. The *graph* represents *hsf4* Ct levels normalized to the *beta-actin* (βactin) reference gene Ct levels. *Error bars* represent the coefficient of variation (*CV*) from three technical replicates calculated from the square root of the sum of the squares for the CV(target) plus the CV(reference)

HSF1, HSF2, or HSF3 groups, the zebrafish/avian group branches from HSF1 much sooner in the tree. However, the avian proteins do not exhibit the C-terminal truncation observed for this zHSF4 isoform and are similar in amino acid length to the mammalian proteins. In combination, these data suggest that the HSF4 protein family arose from a duplication event of an ancestral HSF1 gene and that zHSF4 may have subsequently lost a portion of the C-terminal.

Variations in the amino acid sequence of different domains within different HSFs provide these proteins with the ability to have variable functions. Takemori et al. 2009 demonstrated that specific amino acid regions of the hHSF1 DBD and HR-A/B domain regulate the ability of HSF1 to bind to discontinuous HSE sequences. Enoki and Sakurai (2011) demonstrated that sequence differences in the HR-C domain of zebrafish HSF1a and HSF1c isoform affected oligomerization,which resulted in different binding affinities for discontinuous HSEs between the two isoforms. Recent studies of human and mice HSFs have demonstrated that different HSFs will recognize variations of HSE sequences, with HSF1 preferring a continuous inverted 5′nGAAn3′ repeat sequence while HSF2 and HSF4 are capable of binding to disordered versions of this sequence (Fujimoto et al. 2008; Takemori et al. 2009; Yamamoto et al. 2009; Sakurai and Enoki 2010). In mouse lens tissue, 222 binding sites for HSF4 were identified, and of those binding sites, only six had perfect nGAAn repeats in the HSE sequences (Fujimoto et al. 2008). Some binding sites had one or no GAA sequences. Mutant HSEs with an ambiguous HSE sequence (nGnnn) could partially compete mHSF4 from a perfect HSE in an EMSA, but the same ambiguous sequence could not compete away mHSF1 or mHSF2 (Fujimoto et al. 2008). Our results from similar EMSA experiments using zebrafish HSF proteins in HSE binding assays demonstrate that the zHSF4 protein does bind to a perfect HSE in a specific manner. However, competition assays demonstrated that zHSF4 binds to discontinuous HSEs in a more restricted manner very similar to the binding pattern observed for zHSF1.

It was unexpected when our analysis revealed that the DBD of zHSF4 was more similar to mammalian HSF1 than to mammalian HSF4 but shared an extremely high degree of identity with the DBD of avian HSF4. The high percentage

Table 5 Knockdown of *hsf4* expression does not result in an altered eye phenotype

3.46 μg/μL MO	Embryos (#)	Body/eye length		Body length/lens diameter		Eye length/eye diameter		Eye surface area/body length	
		Average	SD	Average	SD	Average	SD	Average	SD
Uninjected	144	12.4	0.73	37.9	3.1	3.07	0.18	4.95	0.74
Danieau Injected	95	12.6	1.1	38.8	2.9	3.09	0.26	4.92	0.77
hsf4-MO	50	12.4	0.94	38.9	3.8	3.17	0.32	5.41	0.57
hsf4 5bpmm-MO	17	12.3	0.65	38.1	3.0	3.10	0.25	5.06	0.38

Embryos were injected with 3.14 nL of Danieau solution or MO (3.46 μg/μL), and several measurements were performed on the eye and body of embryos 48 h after injection. Ratios of body length to eye length, eye surface area, and lens diameter were calculated for each of the treatment groups

of amino acid identity between zHSF1 and zHSF4 DBD (85%) does, however, support our observations from binding assays that these two proteins would bind to discontinuous HSEs in a similar manner unlike the case previously reported in mammalian systems (Fujimoto et al. 2008). The high level of identity observed between the fish and avian HSF4 proteins with HSF1, coupled with the relatively low affinity of zHSF4 for discontinuous HSE sequences when compared with mammalian HSF4, suggests that target genes for the zebrafish and avian HSF4 transcription factors may not be fully shared with those regulated by mammalian HSF4. At present, target genes for HSF4 in fish and avian species have not been identified.

Analysis of *hsf4* gene expression in zebrafish adult tissues identified the eye as the primary tissue expressing *hsf4* (Fig. 5). This is consistent with other studies that have identified an important role for mammalian HSF4 in lens development and differentiation of lens fibers. Mutations in HSF4 genes have been linked to several human families with hereditary congenital cataracts as well as several dog breeds that also frequently exhibit juvenile cataracts (Bu et al. 2002; Smaoui et al. 2004; Ke et al. 2006; Mellersh et al. 2006; Engelhardt et al. 2007). Several of these mutations occur in the DNA binding domain of the protein and affect the ability of HSF4 to bind to HSE sequences (Enoki et al. 2010). Cataracts are also observed in mouse models carrying mutations introduced into the HSF4 gene (Fujimoto et al. 2004). The onset of cataract formation in the lenses of mammals with HSF4 mutations is due in part to improper regulation of the crystallin genes, especially gamma crystallin genes, by HSF4 (Somasundaram and Bhat 2004; Shi et al. 2009). HSF4 has also been shown to act cooperatively with HSF1 in lens epithelial cells to regulate cell proliferation as well as having a role in the regulation of Hsp27 during lens development (Fujimoto et al. 2004; Min et al. 2004). These studies suggested that zHSF4 would represent a good candidate to be involved in zebrafish lens development and in cooperative regulation of lens-specific *hsp70* gene expression together with zHSF1 (Evans et al. 2007). However, MO knockdown of HSF4 had no impact on eye phenotype in zebrafish embryos at 48 hpf, the point in development when both Hsp70 and HSF1 knockdown results in a clear small lens phenotype (Evans et al. 2005, 2007). The lack of any detectable *hsf4* gene expression in developing zebrafish embryos prior to 60 hpf (Fig. 5) may provide an explanation for the lack of phenotypic effect observed in the MO knockdown studies. While MO are a very effective tool to knockdown gene expression, they become diluted in the cells as the embryos develop, and typically MO-induced phenotypes are severely reduced by 70 hpf and are absent by 90 hpf. Development of the zebrafish eye lens begins prior to 24 hpf, with hsp70 expression beginning by 30 hpf (Blechinger et al. 2002). Lens development and growth continue into later embryogenesis and larval/juvenile stages. Indeed, lens growth in fish continues into adulthood. The results of the gene expression analysis together with the lack of an observable phenotype in the MO knockdown embryos would suggest that if *hsf4* plays a role in the zebrafish eye, it would occur in later stages of development. As indicated above, lens formation in mouse-carrying deletions of HSF4 are normal until later embryogenesis, with defects only becoming apparent during late gestation and postnatally (Fujimoto et al. 2004; Shi et al. 2009). In mouse, lens vesicles are completely separated from the overlying epithelium by E11, suggesting that HSF4 also plays no role in earlier events of lens formation in this species. In any event, these data strongly suggest that zHSF4 and zHSF1 do not work cooperatively to regulate early aspects of zebrafish lens formation, such as *hsp70* induction. This does not rule out the possibility of such cooperative function later in zebrafish lens development beyond the time period during which microinjected antisense morpholino oligonucleotides are effective (Evans et al. 2005). However, the distinct differences in sequence identity of the DBD and of the HSE-binding properties for zHSF4 as compared with what has been previously reported for mammalian HSF4s suggests that lens-specific gene targets and/or mechanism of regulation will likely differ in part from that observed in mammals.

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