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TISSUE-SPECIFIC EXPRESSION OF ZEBRAFISH (*DANIO RERIO*) HEAT SHOCK FACTOR 1 mRNAS IN RESPONSE TO HEAT STRESS

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Summary

All organisms respond to environmental, chemical and physiological stresses by enhanced synthesis of an evolutionarily conserved family of proteins known as heat shock proteins (HSPs) or stress proteins. Certain HSPs are also expressed constitutively during cell growth and development, and they function as molecular chaperones. The transcriptional regulation of *hsp* genes is mediated by the heat shock transcription factor (HSF). The stress response has been studied mostly in mammalian cell lines or organisms normally maintained under constant laboratory conditions. There is much less information on the regulation of the stress response of animals, such as fish, that have to tolerate large fluctuations in environmental and internal conditions. To characterize the regulation of the heat shock response in fish, we have cloned the first heat shock transcription factor from fish, zebrafish *Danio rerio*. Phylogenetic analysis confirms that the isolated zebrafish HSF belongs to the HSF1 family and is therefore designated zHSF1. Analysis by reverse transcriptase polymerase chain reaction (RT-PCR) shows the presence of two zHSF1 mRNA forms that are expressed in a tissue-specific fashion upon exposure to heat stress. Both forms are expressed in gonads under all conditions; in liver and to a lesser extent in the gills, the longer splice form of zHSF1 disappears upon heat shock. We present evidence for a unique tissue-specific regulation of HSF1 upon exposure to elevated temperature.

Key words: heat shock, heat shock factor, zebrafish, *Danio rerio*, heat shock protein.

Introduction

The expression of heat shock proteins (HSPs) is regulated at both transcriptional and post-transcriptional levels. The transcriptional regulation of *hsp* genes is mediated by the heat shock transcription factor (HSF), which is present as an inactive form in unstressed cells and is activated by stress stimuli (for a review, see Morimoto, 1998). Cloning of multiple HSFs has revealed the complexity of the regulatory mechanisms of the heat shock response among eukaryotes (for a review, see Morimoto, 1998). In yeast (Sorger and Pelham, 1988; Wiederrecht et al., 1988) and *Drosophila* (Clos et al., 1990), only one HSF involved in the regulation of the heat shock response has been identified. However, in higher eukaryotes, several HSFs with distinct roles have been cloned, three in human (Rabindran et al., 1991; Schuetz et al., 1991; Nakai et al., 1997), chicken (Nakai and Morimoto, 1993) and tomato (Scharf et al., 1990) and two in mouse (Sarge et al., 1991). Heat shock factor 1 (HSF1) is activated by classical stresses such as heat stress (Sarge et al., 1993) and metal toxicity (Baler et al., 1993). In all the cell types and organisms studied, HSF1 is constitutively expressed and its activity is regulated at the post-translational level, through oligomerization, nuclear translocation and hyperphosphorylation (Baler et al., 1993; Cotto et al., 1996). Unlike HSF1, HSF2 is not responsive to stress stimuli, but is abundantly expressed and activated at certain stages of mouse embryogenesis (Rallu et al., 1997), spermatogenesis (Sarge et al., 1994; Alastalo et al., 1998) and haemin-induced erythroid differentiation of K562 cells (Sistonen et al., 1992; Pirkkala et al., 1999).

Most studies on stress responses have been conducted with organisms that are seldom exposed to large fluctuations in their natural environment. Among the more than 20 000 species of fish, however, some are able to tolerate large fluctuations in environmental conditions, while others live within a very narrow range of, for example, temperatures and oxygen tensions.

The stress response in fish has largely been studied in cell lines and in primary cells and to a lesser extent in whole fish, and most of these studies describe the effects of various stressors on HSP synthesis (for a review, see Iwama et al., 1998). Stressor-induced HSP synthesis has been studied with respect to heat shock (Heikkila et al., 1982; Dietz and Somero, 1992, 1993), heavy metals (Heikkila et al., 1982; Misra et al.,

1989; Ryan and Hightower, 1994; Sanders et al., 1995; Grosvik and Goksoyr, 1996), bacterial infections (Forsyth et al., 1997), viral infections (Cho et al., 1997) and environmental pollutants (Grosvik and Goksoyr, 1996; Vijayan et al., 1997, 1998). Little is known about the regulatory mechanisms of the heat shock response in fish. Using transcription blockers such as actinomycin D, it has been proposed that HSP synthesis is regulated at the level of transcription (Currie and Tufts, 1997). Indeed, we have previously reported that an HSF1-like factor is involved in the induction of *hsp*70 mRNA in rainbow trout (*Oncorhynchus mykiss*) (Airaksinen et al., 1998).

In this study, we characterized the molecular mechanism of the heat shock response in fish by identifying the transcriptional regulator involved in HSP expression. We have cloned the first heat shock factor from a fish, the zebrafish *Danio rerio*, and named it zHSF1 on the basis of phylogenetic analysis. Our results reveal the presence of two zHSF1 isoforms with a unique difference in structure that are expressed in a tissue-specific manner upon heat shock. These findings provide new insights into the heat shock response and may also provide an explanation for the cell-type- and stressor-specific differences that have been observed in *hsp70* gene expression in zebrafish in previous studies.

Materials and methods

Animals and cell culture

Zebrafish *Danio rerio* were obtained from a local aquarium store and allowed to acclimatize for 1 week prior to exposure to stress. They were maintained at 28 °C on a 12 h:12 h light:dark cycle and were fed daily. Established fish cell lines used in the present study were as follows: BF-2 (ATCC CCL 91) from muscle of fry bluegill sunfish *Lepomis machrochirus*; CHSE-214 (ATCC CRL 1681) from embryo chinook salmon *Oncorhynchus tshawytscha*; EPC from epithelial papilloma of common carp *Cyprinus carpio* (Fijan et al., 1983) and RTG-2 (ATCC CCL 55) from gonad of rainbow trout *Oncorhynchus mykiss*. The cells were cultured to confluence at 20 °C under atmospheric air in Eagle's minimum essential medium (with Earle's salts) supplemented with 10 % foetal bovine serum, penicillin (100 i.u. ml^{-1}), streptomycin (100 μg ml^{-1}), L-glutamine (2 mmol l^{-1}) and 1.5 % sodium bicarbonate, pH 7.4. All cell culture medium components were from Gibco BRL Life Technologies Ltd.

cDNA library screening

Synthesis of cDNA was performed with 6 μg of total RNA isolated from BF-2, EPC, CHSE-214 or RTG-2 cells or rainbow trout liver at 42 °C with oligo dT primers (Invitrogen, Carlsbad, CA, USA) and AMV reverse transcriptase (Promega, Madison, WI, USA). The probe for screening cDNA libraries was prepared by polymerase chain reaction (PCR) (*Taq* DNA polymerase, Promega) with degenerate oligonucleotide primers based on sequence comparisons of known HSF DNA-binding domains (DBDs) and part of the hydrophobic heptad repeat (HR-A/B) (forward, 5′-GTC/GCCGGCCTTCCTGA-CCAAGCTGTGG-3′; reverse, 5′-CACCTCCCT/GCCACAG-C/GGCTCTGTTCTC-3′). An expected PCR product of 474 base pairs (bp) was obtained only from BF-2 cell cDNA. The PCR product was gel-purified (Qiagen, The Netherlands) and subcloned into a pGEM T vector (Promega), and JM109 cells (Promega) were transformed with the plasmid. The 474 bp PCR product obtained, corresponding to the BF-2 HSF DBD and part of HR-A/B, was random-primer-labelled using a Redi Prime kit (Amersham Life Sciences, Buckinghamshire, UK) and used as a probe to screen a zebrafish embryo or heart cDNA library (Stratagene, La Jolla, CA, USA) and a juvenile rainbow trout cDNA library (kind gift of Dr Thomas T. Chen, University of Connecticut, USA). Approximately 10^7 plaques of each library were screened, and plaques were lifted according to standard protocols (Stratagene). The membranes (Hybond N, Amersham) were hybridized (6× standard saline citrate, SSC, 40 % formamide, 5× Denhardt's solution, 0.1 % sodium dodecyl sulphate, SDS, 100 μg ml^{-1} yeast tRNA) with the labelled BF-2 DBD probe overnight at 42 °C, washed to final conditions of 0.1× SSC, 0.1 % SDS and visualized by autoradiography. After tertiary screening, one possible positive clone containing the full-length cDNA was identified from the zebrafish heart cDNA library. The cDNA was excised from the phage, subcloned into pBluescript SK vector according to the manufacturer's (Stratagene) instructions and sequenced using ABI Prism 377 (Perkin Elmer, UK). The sequence was analyzed using the BESTFIT and GAP options in the GCG Wisconsin program package, version 8.1-Unix (Genetics Computer Group Inc., Madison, WI, USA).

Phylogenetic analyses

Phylogenetic analyses were performed using the zebrafish HSF nucleotide sequence and 10 HSF nucleotide sequences obtained from GenBank. Sequence alignments were conducted using the PILEUP programs in the GCG Wisconsin package, version 8.1-Unix. Phylogenetic analyses, including 1000 bootstrap replicates, were conducted using the PHYLIP software package, version 3.5c (Felsenstein, 1989).

Southern blot analysis

Genomic DNA was isolated from gills, gonads and liver of zebrafish or whole fish, and 10–20 μg of DNA was digested with *Eco*RI or *Sca*I, separated on a 0.8 % agarose gel and transferred to a nylon membrane (Hybond N, Amersham). The blot was hybridized as described above with a 235 bp cDNA probe, corresponding to a poorly conserved region between nucleotides 1958 and 2192, and obtained from the zHSF1 full-length clone by digestion with *Hind*III and *Eco*RV. The blot was washed to final conditions of 42 °C, 0.1× SSC, 0.1 % SDS.

Northern blot analysis and RT-PCR

RNA was isolated from fish cells and tissues using the RNAzolB method according to the manufacturer's (Tel-Test Inc. Friendswood, TX, USA) instructions. To isolate RNA from tissues, liver samples were pooled from 4–5 fish, gill

samples from three fish and gonads from 1–2 fish. RNA samples (15 µg per lane) were separated on a 1.2 % agarose gel containing formaldehyde and blotted onto nylon filters. Blots were hybridized with a [α-^{32}P]dCTP-labelled plasmid specific for human *hsp70* (Wu et al., 1985) or human β-actin, pHFβA1 (Gunning et al., 1983), as a probe overnight at 42 °C. Radiolabelling of the probes was performed using a nick translation kit (Promega). Blots were washed to final conditions of 42 °C, 0.1× SSC, 0.1 % SDS and visualized by autoradiography.

HSF expression levels in zebrafish tissues were analyzed using RT-PCR with primers (forward, 5′-TCACTGGAACTG-GCGTCTTC-3′; reverse, 5′-CGATGGCTGCGACGTGTA-CT-3′) amplifying a 605 bp fragment (base pairs 996–1600) of the zHSF1.

The *hsp70* expression levels were analyzed using RT-PCR with primers (forward, 5′-ATCCTGACCATTGAAGACGG-3′; reverse, 5′-TGTTCAGTTCTCTGCCGTTG-3′) amplifying a 457 bp fragment of the partial zebrafish *hsp70* sequence available in the GenBank (Lele et al., 1997).

The *hsc70* expression levels were analyzed using RT-PCR with primers (forward, 5′-TGAAGCACTGGCCTTTTAAT-3′; reverse, 5′-CCAGCAGTAGATTTGACCTC-3′) amplifying a 412 bp fragment of the zebrafish *hsc70* sequence available in the GenBank (Graser et al., 1996). Synthesis of cDNA was performed as described above with 6 µg of RNA. To test for DNA contamination of RNA samples, cDNA synthesis and subsequent amplification reactions were also performed in the absence of reverse transcriptase. The optimum annealing temperature for HSF was first determined using a temperature gradient program (Eppendorf Mastercycler Gradient) and was found to be 63 °C. The samples were subjected to 30 cycles of PCR amplification. The same conditions were used for amplification of *hsp70* and *hsc70*.

The ratios were quantified using a computerized image analysis scanner (Microcomputer Imaging Device version M4, Imaging Research, Inc.) or a fluoroscanner (Molecular Dynamics).

Western blot analysis

Whole cell extracts were prepared from gills, gonads and liver of zebrafish as described previously (Airaksinen et al., 1998). A 15 µg sample of whole cell extract was separated on an 8 % SDS–polyacrylamide gel and transferred to nitrocellulose membranes (Schleicher & Schuell) using semi-dry transfer (Bio-Rad). Filters were blocked for 2 h in 3 % nonfat dry milk in phosphate-buffered saline (PBS) with 0.3 % Tween 20. Western blot analysis was performed using a monoclonal mouse anti-HSP70 antibody (dilution 1:10 000) (clone 3a3, Affinity Bioreagents, Golden, CO, USA), which detects both the constitutive and inducible forms of HSP70. Horseradish-peroxidase-conjugated rabbit anti-mouse immunoglobulin (Amersham) was used as a secondary antibody, and the signal was developed using an enhanced chemiluminescence method according to the manufacturer's (Amersham) instructions.

Results and discussion

Isolation of the first HSF from fish – zebrafish HSF belongs to the HSF1 family

In fish, no regulatory factors involved in the heat shock response have been isolated. To examine the regulation of the heat shock response in zebrafish, we cloned the zebrafish HSF. Using PCR with degenerate primers corresponding to known HSF DNA-binding domains (DBDs) and hydrophobic heptad repeats (HR-A/B) of other organisms, we were able to clone the corresponding domain of 474 bp from the bluegill sunfish cell line BF-2. Nucleotide sequence analysis of the BF-2 DBD showed a high homology of 80 % to other HSF1 DBDs (human and mouse 83 %, chicken 84 %), of 70 % to HSF2 DBDs (human 69 %, mouse 71 % and chicken 69 %) and of 73 % to chicken HSF3 DBD. The 474 bp PCR product obtained was then used as a probe to screen cDNA libraries from zebrafish and rainbow trout. A positive clone was obtained from a zebrafish heart cDNA library corresponding to a full-length HSF of 2299 bp with an open reading frame (ORF) of 512 amino acid residues (Fig. 1). The predicted amino acid sequence showed an overall homology (amino acid identity) of 60 % to all HSF1s and 40 % to HSF2s. The high homology of the well-conserved regions, i.e. the DBD and the HR-A/B and HR-C in the fish HSF1 and other HSFs, supports the concept of conservation throughout evolution (Table 1). Interestingly, the predicted amino acid sequence of the DBD of BF-2 showed 91 % homology to the zebrafish DBD, which is within the same range as the homology between zHSF1 and other HSF1 family members. When nucleotide sequences were compared, the

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1    MEYHSVGPGGVVVTGNNVPAFLTKLWTLVEDPDTDPLICWSPNGTSFHVF
51   DQGRFSKEVLPKYFKHNNMASFVRQLNMYGFRKVVHIEQGGLVKPEKDDT
                                          •  ‡   •  ‡
101  EFQHPYFIRGQEQLLENIKRKVTTVSNIKHEDYKFSTDDVSKMISDVQHM
      •  ‡   •  ‡   •  ‡   •  ‡          ‡‡  •• ‡‡  ••
151  KGKQESMDSKISTLKHENEMLWREVATLRQKHSQQQKVVNKLIQFLITLA
         •
201  RSNRVLGVKRKMPLMLNDSSSAHSMPKFSRQYSLESPAPSSTAFTGTGVF
251  SSESPVKTGPIISDITELAQSSPVATDEWIEDRTSPLVHIKEEPSSPAHS
301  PEVEEVCPVEVEVGAGSDLPVDTPLSPTTFINSILQESEPVFRPDSAPSE
                ‡   •  ‡   •  ‡   •      •
351  QKCLSVACLDKTELHDHLESIDSGLENLQQILNAQSINFDSSPLFDIFSS
401  AASDVDLDSLASIQDLLSPDPVKETESGVDTDSGKQLVQYTSQPSFSPIP
451  FSTDSSSTDLPMLLELQDDSYFSSEPTEDPTIALLNFQPVPEDPSRTRIG
501  DPCFKLKKESKR
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Fig. 1. Predicted amino acid sequence of zebrafish heat shock transcription factor (zHSF1a) cDNA. The DNA-binding domain (DBD) is boxed in red. The hydrophobic heptad repeats (HR) are underlined, HR-A/B in blue and HR-C in green, and the hydrophobic amino acid residues within these are indicated with a dot or double dagger. The sequence has been deposited in the GenBank database, accession no. AF159134.

Table 1. *Sequence comparison of zebrafish heat shock factor 1 with other heat shock factors*

	Percentage identity			
	ORF	DBD	HR-A/B	HR-C
hHSF1	64	91	76	43
hHSF2	41	74	49	40
mHSF1	64	91	75	43
mHSF2	41	74	49	40
cHSF1	62	93	76	47
cHSF2	39	64	51	40
cHSF3	38	55	51	40
dHSF	35	57	57	41
xHSF	60	94	75	47
bHSF	ND	91	ND	ND

The percentage amino acid identity of zebrafish ORF (open reading frame), DBD (DNA-binding domain), HR-A/B (heptad repeat A/B) and HR-C (heptad repeat C) to heat shock factor (HSF) genes from human (h), mouse (m), chicken (c), *Drosophila* (d), *Xenopus* (x) and bluegill sunfish (b) compared using the University of Wisconsin Genetics Computer Group program.

ND, not determined.

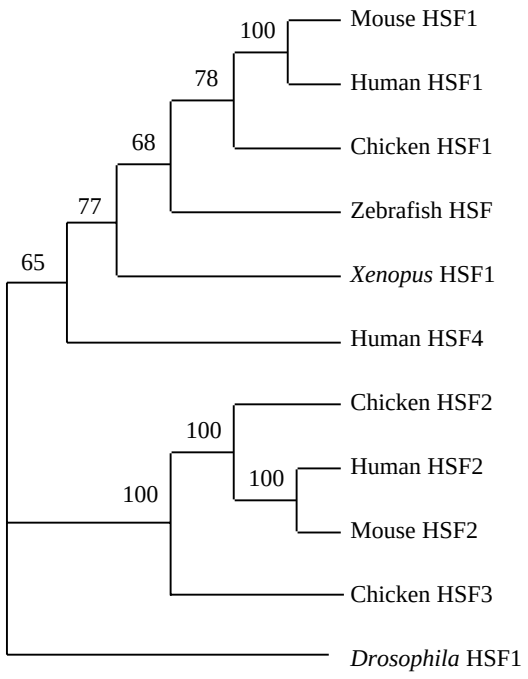


Fig. 2. Phylogenetic relationship of 11 HSF genes. Full-length nucleotide sequences were aligned, and phylogenetic analysis was carried out using the PHYLIP phylogeny inference package. The SEQBOOT program was used to create 1000 bootstrap replicates of the sequence alignment data. DNADIST (employing the Kimura two-parameter distance matrix) was used to create nucleic acid sequence distance matrices, which were used as input data for the phylogenetic-tree-fitting program FITCH (Fitch–Margoliash method) with settings for analysis of 1000 multiple data sets. The CONSENSE program was used to calculate the occurrence ratios of particular branchings among 1000 trees and to show the results as an unrooted consensus tree. The values next to the branches in the unrooted consensus tree indicate bootstrap confidence limits from 1000 replicates.

BF-2 DBD was more homologous to human and mouse HSF1 (83 %) and to chicken HSF1 (84 %) than it was to zebrafish HSF1 (81 %). This may indicate either that several distinct HSFs are present in fish or that different fish species have surprisingly divergent HSFs. The divergence in itself would not be surprising since the evolutionary distances among fishes can be as much as 500 million years.

We used phylogenetic analysis to verify that the zebrafish HSF obtained belonged to the HSF1 family (Fig. 2). The *Drosophila* HSF1 created an outgroup in the unrooted phylogenetic tree. Human, mouse, chicken and *Xenopus* HSF1 sequences formed one clade, which also included the zebrafish HSF. Human HSF4 created an outgroup for this clade. Human, mouse and chicken HSF2 sequences grouped together, separately from chicken HSF3. Bootstrap analysis indicated that the branchpoints on the tree are well-supported. The phylogenetic analysis confirmed that the isolated zebrafish HSF belongs to the HSF1 family (Fig. 2), and it is therefore called zHSF1.

zHSF1 is present in the zebrafish genome

Because the genomic sequence of the zHSF1 gene is not yet known, we detected the presence of the zHSF1 gene by Southern blot hybridization. For this purpose, we used a 235 bp probe corresponding to a poorly conserved region in the 3′ end of the zHSF1 mRNA sequence. Genomic DNA isolated from gills, gonads, liver and whole fish was cleaved using *Eco*RI and *Sca*I and analyzed by Southern blotting. Cleavage with *Eco*RI

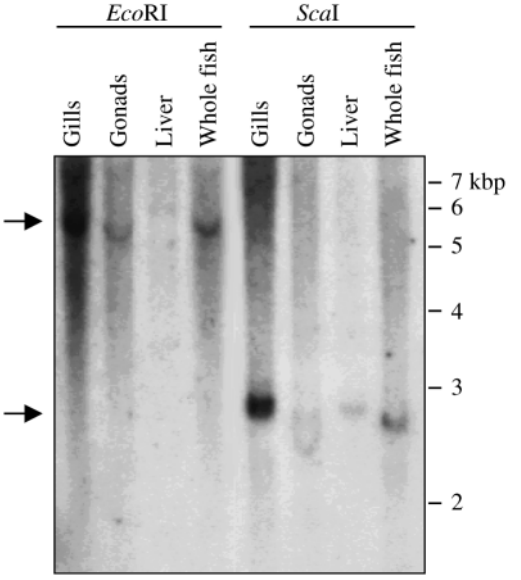


Fig. 3. The presence of zHSF1 in the zebrafish genome demonstrated by Southern blot hybridization. Genomic DNA (15 µg per lane) isolated from gills, gonads, liver and whole fish was digested using *Eco*RI and *Sca*I and probed with a 235 bp cDNA probe corresponding to a poorly conserved region of the zHSF1 domain, between nucleotides 1958 and 2192. Southern blotting shows a single band of approximately 5.5 kbp after digestion with *Eco*RI and a single band of approximately 2.8 kbp after digestion with *Sca*I. The positions of DNA size markers are shown on the right.

gave a single band of approximately 5.5 kbp and cleavage with *Sca*I gave a single band of approximately 2.8 kbp (Fig. 3), indicating that the zHSF1 is present in the zebrafish genome.

Two isoforms of zHSF1 are expressed in a tissue- and temperature-dependent manner

We next examined the expression of zHSF1 in zebrafish tissues using RT-PCR with zHSF1-specific primers to study the expression levels in gills, gonads and liver. The gills were chosen since they are immediately exposed to any changes in environmental conditions, the gonads because of their importance in reproduction, and the liver because of its role as the central metabolizing and detoxifying organ. The expected product of 605 bp was observed in all tissues in both untreated and heat-shocked fish (Fig. 4A). In addition, a band of approximately 700 bp was observed in all control tissues. In reactions performed without reverse transcriptase, no bands were observed, thus eliminating the possibility of DNA contamination of RNA samples (data not shown).

Cloning and sequence analysis of the longer band revealed that this new 683 bp product corresponded to the previously cloned zHSF1, but had an extra 78 bp sequence (26 amino acid residues) inserted immediately adjacent to and continuous with the N terminus of the HR-C domain (Fig. 4B). The originally cloned zHSF1 was called zHSF1a and the form containing the extra 78 bp was called zHSF1b (total length 2377 bp). The gonads of fish kept at 28 °C and of fish exposed to temperatures of 33, 35 and 37 °C all contained zHSF1b. In liver, however, zHSF1b was observed in untreated fish, but it started to disappear upon heat stress and was totally absent at 35 and 37 °C (Fig. 4A). In gills, a modest decrease in zHSF1b level was observed upon heat stress; however, this was not evident in all samples studied. Interestingly, in zHSF1b, there was a one nucleotide change that caused a substitution of lysine to asparagine immediately before the inserted new sequence (Fig. 4B). The inserted 26 amino acid sequence in zHSF1b does not show any sequence homology to the hydrophobic heptad repeats in the splice forms of mouse and *Schistosoma* HSFs (Goodson and Sarge, 1995; Levy Holtzman and Schechter, 1996), although some potential hydrophobic HR motifs are present (Fig. 4B).

Sequence homology analysis search revealed 54 % amino acid identity and 74 % similarity to a consensus zinc finger motif in the zinc finger protein OZF (only zinc finger) (Le Chalony et al., 1994). In zHSF1, there are two cysteine residues located prior to the insertion site (Figs 1, 4B) that, together with the two histidines in the inserted sequence, could form a putative zinc finger motif. Zinc finger proteins are usually

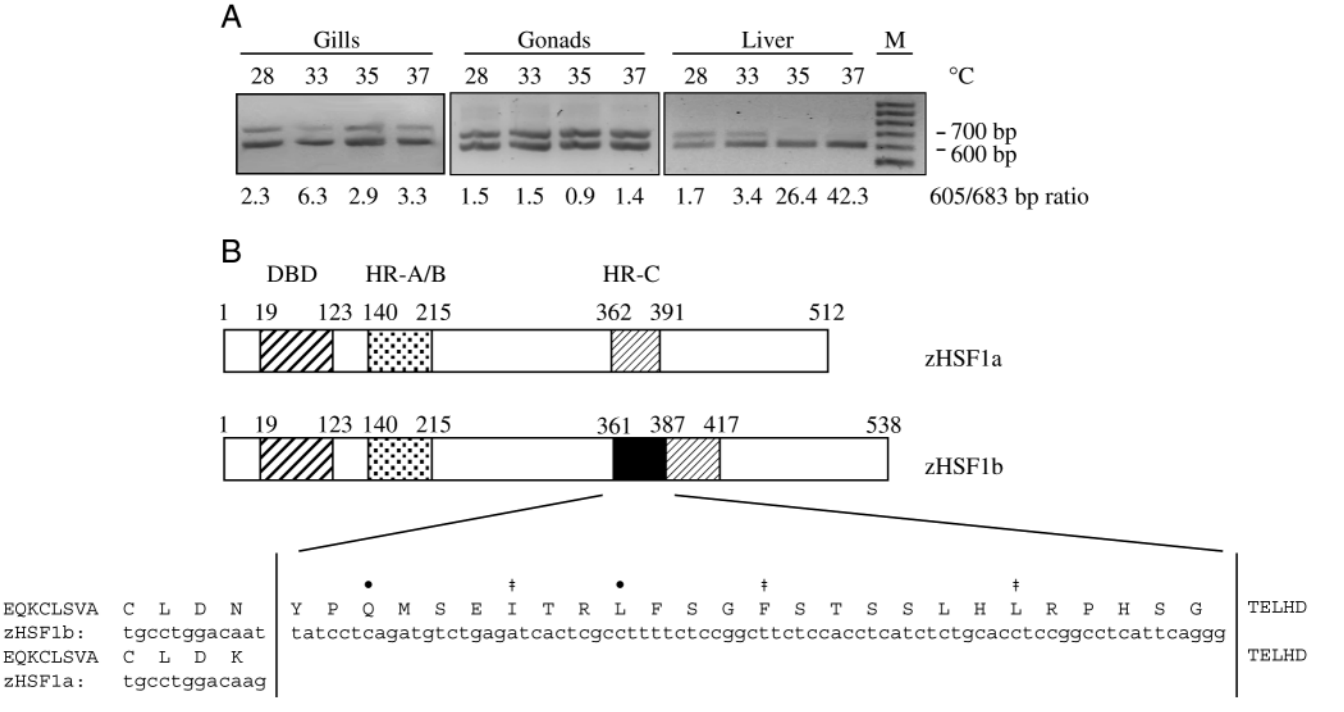
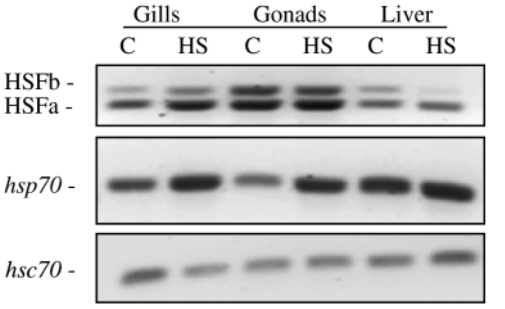


Fig. 4. Two isoforms of zHSF1 are expressed in a tissue- and temperature-dependent manner in zebrafish. (A) Reverse transcription polymerase chain reaction (RT-PCR) of zHSF1 forms in gills, gonads and liver in untreated (28 °C) and heat-shocked (33, 35, 37 °C) fish. The intensities of zHSF1a and zHSF1b were determined using fluoroimaging, and the calculated zHSF1a/zHSF1b ratios are indicated below each panel. M indicates the DNA size marker. (B) Schematic presentation of the two zHSF1 forms. The DNA-binding domain (DBD) and hydrophobic heptad repeats (HR-A/B and HR-C) are shown. The nucleotide sequence (lowercase letters) and predicted amino acid sequence (uppercase letters) of zHSF1b are shown at the bottom of the figure. The borders of zHSF1a are indicated by a vertical line. Note the substitution of lysine in the zHSF1a form with asparagine in the zHSF1b form immediately before the inserted sequence. The hydrophobic amino acids are indicated with a dot or double-dagger. The sequences have been deposited in the GenBank data base, zHSF1a with accession number AF159134 and zHSF1b with accession number AF159135.

involved in DNA/RNA binding and may act as tissue-specific modulators of gene expression (Le Chalony et al., 1994). Interestingly, the suppressor of the hairy-wing locus in *Drosophila*, which encodes a protein containing 12 zinc fingers, has been shown to repress heat-shock-induced transcription when positioned between sites that bind proteins required for heat-shock-induced transcription (Holdridge and Dorsett, 1991). With regard to the tissue-specific expression, it is plausible that the inserted sequence in zHSF1b could, by some protein/protein interactions, repress the heat shock response.

Quantitative analysis comparing *hsp70* and the two zHSF1 isoforms, as well as *hsp70* and *hsc70* from the same tissue samples and amplified using the same PCR conditions, was performed next. The results show that *hsp70* is induced by heat shock in all tissues (Fig. 5). A comparison of the ratios of the zHSF1a and zHSF1b isoforms, as well as the zHSF1 isoforms and *hsp70*, shows that the gills, and particularly the liver, have a higher *hsp70*/zHSF1b ratio than the gonads, indicating that they contain a lower level of zHSF1b compared with the gonads, in both control and heat-shocked fish (Fig. 5).



HSFa/HSFb	4.7	5.2	1.9	2.0	2.5	15.5
hsp70/HSFa	2.5	2.8	0.7	1.9	4.7	6.1
hsp70/HSFb	4.3	5.1	0.9	2.4	7.0	14.2
hsp70/*hsc70*	1.5	3.5	1.4	2.7	2.7	3.7

Fig. 5. Comparison of the levels of the genes for the two zHSF1 isoforms, *hsc70* and *hsp70*, in different zebrafish tissues. Reverse transcription polymerase chain reaction (RT-PCR) was performed with the same samples for all primer pairs and with the same amplification conditions. The intensities of zHSF1b (683 bp), zHSF1a (605 bp), *hsc70* (412 bp) and *hsp70* (457 bp) were determined using fluoroimaging, and ratios were calculated and are indicated below the panels. C indicates control fish kept at 28 °C, and HS indicates heat-shocked fish kept at 37 °C for 1 h.

hsp70 – the target gene for HSF1

Because *hsp70* is one of the target genes for HSF1 in other organisms, we studied the effect of heat shock on *hsp70* mRNA levels in zebrafish tissues. Steady-state levels of *hsp70* mRNA in the gills, gonads and liver of zebrafish exposed to 28, 33, 35 and 37 °C were analyzed by northern blot analysis. A 1 h heat shock at 37 °C markedly increased *hsp70* mRNA levels in gills and liver, but only slightly in gonads compared with controls (28 °C), which contained low levels of *hsp70* mRNA (Fig. 6A). A slight increase in *hsp70* mRNA was also observed after 1 h at 35 °C in all tissues and after 1 h at 33 °C in liver (Fig. 6A). A comparison of the results from the RT-PCR analysis of *hsp70* induction (Fig. 5) and those obtained from northern blot analysis (Fig. 6A), reveals a good correlation in the level of *hsp70* induction, i.e. stronger induction in gills and liver than in gonads. The very low *hsp70* induction in the gonads in this analysis may be because, in northern analysis, the *hsp70* levels were normalized to levels of human β-actin, while in RT-PCR they were normalized to levels of zebrafish *hsc70*.

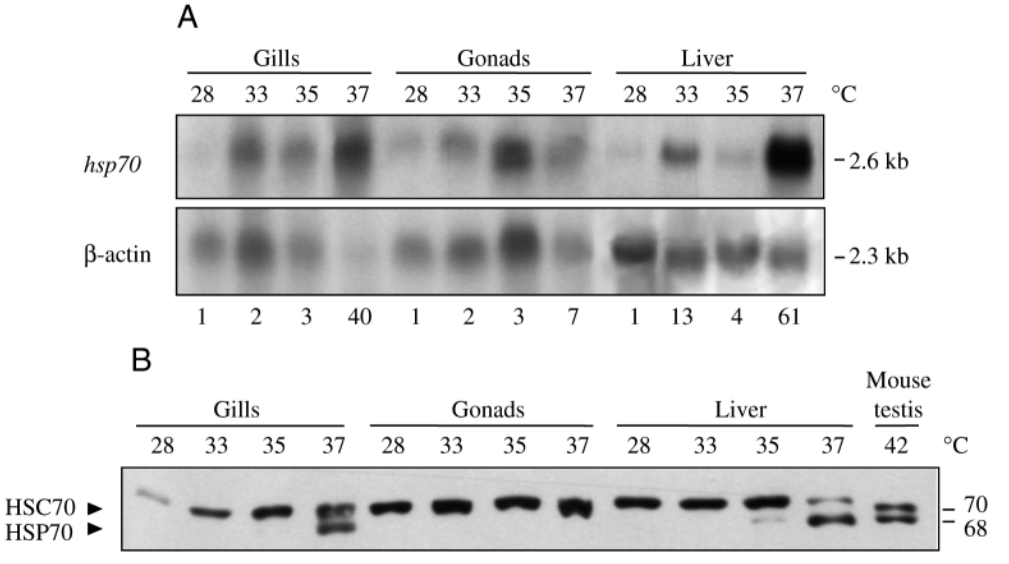


Fig. 6. Northern and western blot analyses of zebrafish tissues exposed to heat shock for 1 h. (A) Northern blot analysis of total RNA isolated from gills, gonads and liver of untreated (28 °C) and heat-shocked (33, 35, 37 °C) fish. Quantification of *hsp70* mRNA performed using phosphoimaging is indicated below each lane. A value of 1 indicates the level of *hsp70* mRNA accumulation in control samples, and values greater than 1 indicate the fold induction upon heat stress compared with controls. β-Actin was used for normalization of the data. In liver and gills, a marked increase in *hsp70* mRNA is observed upon heat shock treatment, while only a small increase is observed in gonads. (B) Western blot analysis of zebrafish tissues as indicated in A and analyzed using an HSP70 antibody that recognizes both the constitutive (HSC70) and inducible (HSP70), forms. In gonads, HSC70 is observed at all temperatures, with only a slight induction of HSP70 after 1 h at 37 °C. In gills, HSP70 is seen upon heat shock, and in liver HSP70 is increased with a corresponding reduction in the amount of the constitutive HSC70. Whole-cell extract of mouse testis was used as a positive control for the antibody, showing HSP68 and HSP70.

Several inducible forms of HSP70 protein were observed upon heat shock in liver and gills

We next performed western blot analysis to investigate whether the observed increase in *hsp70* mRNA level was reflected in a corresponding increase in HSP70 protein level (Fig. 6B). In gonads, the constitutive HSC70 was observed at all temperatures, with a slight induction of the inducible HSP70 after 1 h at 37 °C. In gills, the inducible HSP70 was observed upon heat shock at 37 °C (Fig. 6B). A third intermediate band was observed after longer exposure, possibly indicating the presence of additional HSP70 proteins (results not shown). In liver, the inducible HSP70 was observed after a 1 h heat shock at 35 °C and more pronouncedly at 37 °C, with a corresponding reduction in the amount of the constitutive HSC70 (Fig. 6B). The presence of several different isoforms of HSP70 protein has been reported previously in fathead minnow (*Pimephales promelas*) in response to heat stress (Dyer et al., 1991) and exposure to copper (Sanders et al., 1995) and in coho salmon (*Oncorhynchus kisutch*) with bacterial kidney disease (Forsyth et al., 1997). In zebrafish embryos, several different *hsp70* genes have been isolated (Krone et al., 1997; Lele et al., 1997). One of these, the *hsp70-4* gene, is strongly heat-inducible but shows no expression at control temperatures (Lele et al., 1997). The expression of *hsp70* has also been shown to be stressor-specific, since ethanol treatment of zebrafish embryos, in contrast to heat shock, induces only a slight increase in *hsp70* mRNA levels (Lele et al., 1997). It has previously been suggested that cell-type- and stressor-specific differences in gene expression in zebrafish embryos may be due to a differential activity of HSF or to the presence of various HSF isoforms (Krone et al., 1997).

In conclusion, cloning and expression analysis of the first heat shock factor from fish, zHSF1, reveal several new and intriguing aspects of the heat shock response. Sequence homology analysis of the DBD of the cloned bluegill sunfish HSF suggests that fish may either have several distinct HSFs or that different fish species have surprisingly divergent HSFs. It has been suggested that multiple HSFs have developed in eukaryotes as a consequence of the need to be able to respond to different kinds of stress (Morimoto, 1998). The existence of multiple HSFs may also be a consequence of the need for co-regulation of the heat shock genes in response to specific signals (Morimoto, 1998). Given the environmental variability in the aquatic environment, it is conceivable that fishes have several HSFs with distinct functions. However, further studies are required to investigate the possible presence and the function of multiple HSFs in fish. The most striking result in our study was the finding of a novel form of zHSF1, zHSF1b, which is expressed in a tissue-dependent manner in response to heat shock. Although tissue-dependent constitutive expression patterns of HSF1 isoforms have been described in mouse (Goodson and Sarge, 1995), their functional roles have not been studied in detail. The relevance of our finding that zHSF1b is regulated differently in gills and liver compared with gonads, and its possible consequence for the heat shock response of zebrafish, remains to be clarified. Tissue-specific patterns of synthesis of heat shock proteins have been observed in various fish species in previous studies (Dyer et al., 1991; Dietz and Somero, 1993; Airaksinen et al., 1998). It has been suggested that the variation in the heat shock response among tissues could support the hypothesis that the thermal limits of an organism may be governed by certain tissues more than by others (Dyer et al., 1991). Our finding of two distinct forms of zHSF1 indicates that alternative splicing may provide a mechanism for generating cell- and stressor-specific gene expression in zebrafish.

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