Studies on the Regulatory Mechanisms Involved in the Expression of Heat-Shock Protein Genes from Rainbow Trout

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CONTENTS

INTRODUCTION

Poikilotherms like fish are directly affected by ambient temperature, which is among the most pervasive of environmental factors (Hawkins, 1996). Temperature influences their metabolism, activity levels, spawning, development and growth; and because of selective pressures associated with these processes, temperature is an ecological resource, influencing the proportion of potential habitat that is suitable for a species (Hawkins, 1996). In this manner, adaptive responses to ambient water temperature are essential for the survival of aquatic ectotherms. In particular, adaptation to heat stress is a critical factor for the viability of cold-adapted fish such as salmonids because they receive heat stress at moderately low temperatures. For instance, the ultimate incipient lethal temperature for rainbow trout, at which half of the fish tested die within 1 week, is about 26.2 °C (Kaya, 1978).

At a cellular level, all organisms respond to heat stress by inducing the synthesis of a group of proteins called the heat-shock proteins (HSPs) (Lindquist and Craig, 1988). The response is the most highly conserved genetic system known, existing in every organism in which it has been sought, from archaebacteria to eubacteria, from plants to animals (Lindquist and Craig, 1988). HSPs are traditionally classified by molecular weights into several families (Sonna *et al.*, 2002). For example, a 70-kDa heat-shock protein belongs to the HSP70 family. HSP family members possess three principal biochemical functions: molecular chaperone activity, regulation of cellular redox state, and regulation of protein turnover (Sonna *et al.*, 2002).

The inducible HSP expression is regulated by the heat-shock transcription factors (HSFs) (Pirkkala *et al.*, 2001). In response to various inducers such as heat stress, most HSFs acquire DNA binding activity to the heat-shock element (HSE), thereby mediating transcription of the heat-shock genes, which results in accumulation of HSPs (Pirkkala *et al.*, 2001) as shown in Fig. 0-1. In vertebrates, several members of the HSF family, HSF1–HSF4, have been found (Pirkkala *et al.*, 2001). Among the family members, HSF1 is functionally analogous to yeast and *Drosophila* HSF as the principal stress-induced transcription factor (Morimoto, 1998).
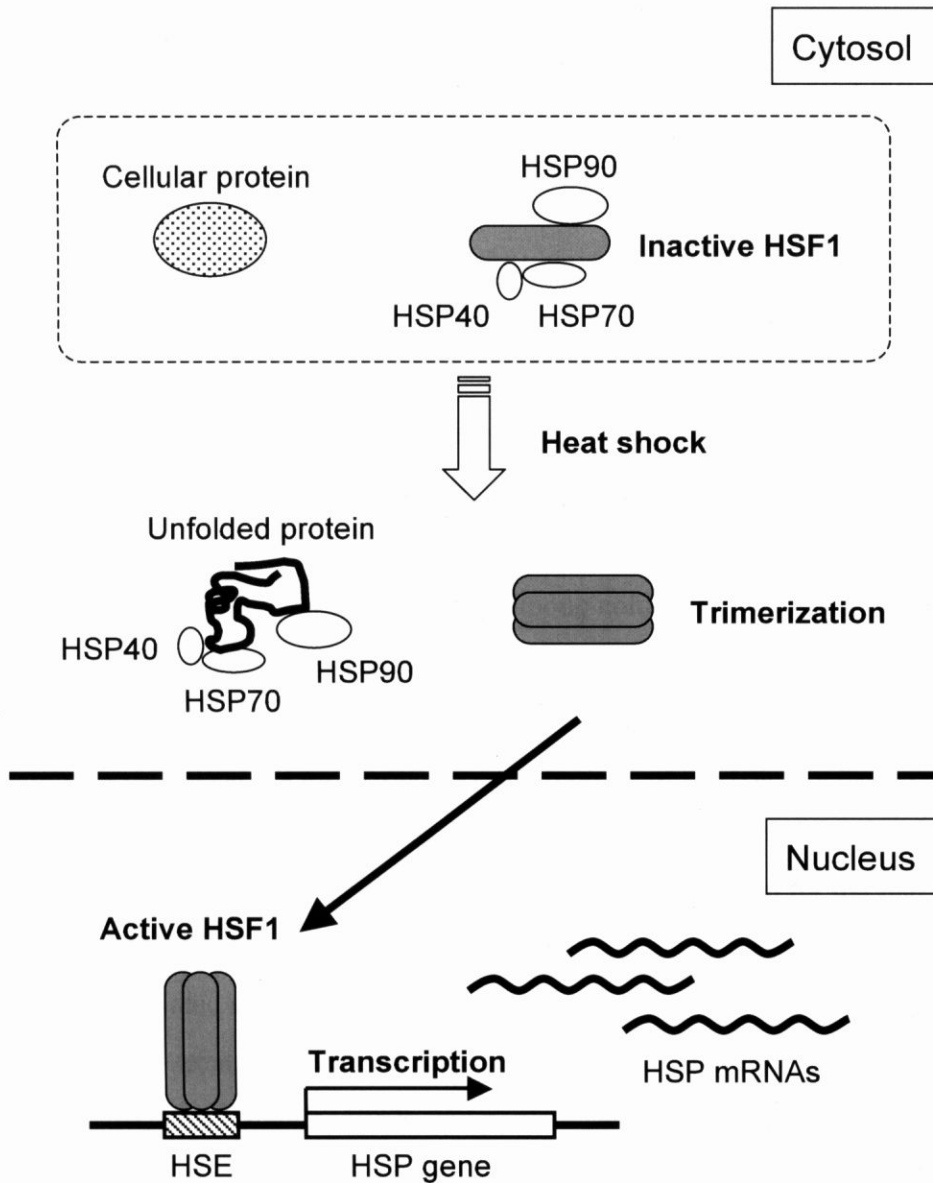
Fig. 0-1. Model of the regulatory mechanisms underlying the expression of heat-shock protein genes in mammalian cells. HSF1 is present in the cytosol as inactive monomers under normal conditions. The inactivation of HSF1 is associated with the binding of HSPs to the protein. When unfolded proteins accumulate in the cytosol by heat shock, HSPs dissociate from HSF1 to function as molecular chaperones. Subsequently, HSF1 acquires DNA binding ability through trimerization and translocates to the nucleus. HSF1 binds to HSEs in the upstream region of HSP genes and activates their transcription.

Published studies using teleost cultured cells clearly demonstrate that teleosts can mount a stress response very similar to that observed with other vertebrates (Hightower and Renfro, 1988). In regard to HSPs, most studies in fish have been performed at the protein level, and these results suggest that the cellular stress response is likely to be playing some role in enhancing the survival and health of the stressed fish (Basu *et al.*, 2002). However, HSP genes have only been cloned from a modest number of different fish species, and molecular studies of the fish HSPs are still in their early descriptive phase (Basu *et al.*, 2002).

Thus, the present study was undertaken to examine the gene structure, the mRNA expression profiles and the mechanism of transcriptional regulation of fish HSPs. For this purpose, rainbow trout was chosen as an experimental model fish because of the following reasons. First, temperature tolerance is an important trait from both an economic and an evolutionary perspective in fishes, particularly among cold-water salmonids such as rainbow trout (Somorjai *et al.*, 2003). Second, since rainbow trout is one of the most intensively studied fishes in a wide range of research areas (Thorgaard *et al.*, 2002), considerable information is available about the physiology and biology of the fish as a background for molecular studies. Third, since rainbow trout have evolved by tetraploidization from a diploid ancestor (Ohno *et al.*, 1968), wider-scale DNA sequence information for the fish will provide an excellent and distinctive system for studying the aftermath of a genome-wide duplication event and the associated structural and regulatory gene changes (Thorgaard *et al.*, 2002).

In rainbow trout, the major stress-inducible protein in the cells has been reported to be Hsp70 (Kothary and Candido, 1982), and partial cDNAs encoding Hsp70 have been isolated (Kothary *et al.*, 1984b). Based on these data, the present study began with isolation of a full-length cDNA encoding rainbow trout Hsp70. Chapter I describes the isolation of *Hsp70* and its expression profile in rainbow trout RTG-2 cells exposed to heat stress. Next, to comprehensively explore the expression profile of HSP genes, isolation of genes encoding the other HSP family members was undertaken. Chapter II describes the isolation of HSP genes and their expression profiles in RTG-2 cells in response to heat stress. Finally, to investigate the molecular mechanism of transcriptional regulation of the HSP genes, isolation of a gene encoding HSF was undertaken. Chapter III describes the isolation and mRNA expression of *HSF1* and the activation of the transcription factor by heat stress.

According to the nomenclature used in the previous publication (Gething, 1997a), fully capitalized names were used to denote the protein family (e.g. HSP90, HSP70, HSP60 etc.) and initial capital letter was used for specific family members (e.g. Hsp90, Hsc70, Cct8 etc.) in this thesis.

ABBREVIATIONS

ANOVA, analysis of variance

CCT, chaperonin containing TCP1

cDNA, complementary DNA

DBD, DNA-binding domain

DDBJ, DNA Data Bank of Japan

DIG, digoxigenin

DNA, deoxyribonucleic acid

EDTA, ethylenediaminetetraacetic acid

EGS, ethylene glycol bis(succinimidylsuccinate)

EMSA, electrophoretic mobility shift assay

ER, endoplasmic reticulum

EST, expressed sequence tag

GRP, glucose-regulated protein

HA, hemagglutinin

HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid

HR, hydrophobic repeat

HSC, heat-shock cognate

HSE, heat-shock element

HSF, heat-shock transcription factor

HSP, heat-shock protein

Ig, immunoglobulin

IOD, integrated optical density

mRNA, messenger RNA

NCBI, National Center for Biotechnology Information

NLS, nuclear localization signal

ORF, open reading frame

PCR, polymerase chain reaction

PVDF, poly(vinylidene difluoride)

RACE, rapid amplification of cDNA ends

RNA, ribonucleic acid

RT-PCR, reverse transcription polymerase chain reaction

SD, standard deviation

SDS, sodium dodecyl sulfate

SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SSC, standard saline citrate

TCP1, t-complex polypeptide 1

TPR, tetratricopeptide repeat

UTR, untranslated region

CHAPTER I   CLONING AND mRNA EXPRESSION PROFILE OF Hsp70

Heat-shock protein 70 (Hsp70) is the major stress-inducible protein in vertebrates and highly conserved throughout evolution. The transcripts of Hsp70 are induced under various physiological stresses and the resulting translation products act as the molecular chaperone that mediates the correct assembly and localization of intracellular proteins (Miao *et al.*, 1997). In fish, *Hsp70*s have been cloned from a number of species; however, most studies of Hsp70 have been performed at the protein level (Basu *et al.*, 2002). Hence, relatively little is known about the expression profiles of Hsp70 transcripts in fish cells exposed to a variety of stresses.

Until now, two partial cDNA sequences encoding Hsp70 have been isolated from rainbow trout (Kothary *et al.*, 1984b). Their sequences have been used as probes to detect Hsp70 mRNA in rainbow trout cells exposed to stresses such as heat shock (Currie *et al.*, 1999; Kothary *et al.*, 1984a; Lund *et al.*, 2000; Samples *et al.*, 1999), sodium arsenite (Kothary *et al.*, 1984a), and hypoxia (Currie *et al.*, 1999). However, rainbow trout have evolved by tetraploidization from a diploid ancestor (Ohno *et al.*, 1968), raising the possibility that multiple *Hsp70*s exist in the genome, as inferred from Southern blot analysis (Kothary *et al.*, 1984b). Furthermore, a heat-shock cognate 70 (Hsc70) gene has been identified in rainbow trout (Zafarullah *et al.*, 1992). Hsc70 is a member of the HSP70 family that is constitutively expressed in unstressed cells (Hightower and Leung, 1997). Nevertheless, only the partial, not full-length, cDNA sequences mentioned above have been used to detect rainbow trout Hsp70 transcripts. Therefore, an unambiguous interpretation on mRNA expression profiles of *Hsp70* and following evaluation of heat stress on rainbow trout were likely impossible. To this end, much more detailed information is required on the possible existence of the multiple *Hsp70*s and their sequence variations along with differences from *Hsc70*. The resulting information is prerequisite to utilization of *Hsp70* expression profiles as a molecular biomarker for stressed state of fish. Furthermore, it is interesting to explore different functional properties of the possible multiple Hsp70s of fish in terms of evolutionary interpretation.

Thus, the purpose of the study was to isolate full-length cDNA clones encoding rainbow trout Hsp70 and to investigate their mRNA expression profiles during heat stress. This chapter describes the identification of two distinct heat-inducible *Hsp70*s in rainbow trout and their different mRNA accumulation levels in cultured cells exposed to heat stress.

Section 1   Isolation and sequence analysis of cDNAs encoding HSP70

Rainbow trout is likely to have duplicate *Hsp70*s attributed to ancestral tetraploidization. Furthermore, since Hsp70 is a member of the highly conserved HSP70 family, homologous genes such as *Hsc70* are generally present in vertebrate genomes. Thus, to specifically analyze the mRNA expression profile of rainbow trout *Hsp70*, it is essential to identify the number of *Hsp70*s in rainbow trout genome and to determine their full-length DNA sequences. This section describes the isolation of full-length cDNAs encoding Hsp70 and their gene structures.

Materials and Methods

Construction of cDNA library

Rainbow trout *Oncorhynchus mykiss* were obtained from the Nikko Station of the National Research Institute of Fisheries Science (Tochigi Prefecture, Japan) and reared on a commercial diet at 10 °C. One individual of rainbow trout (3 months old) was heat-shocked for 30 min in an aquarium at 25 °C, and the whole body was homogenized in TRIzOL Reagent (Invitrogen, Carlsbad, CA, USA) using a power homogenizer (Polytron; Kinematica, Lucerne, Switzerland). The total RNA was isolated according to the manufacturer's instructions. Poly(A) RNA was purified from the total RNA using an Oligotex-MAG mRNA Purification kit (Takara Bio, Otsu, Japan). A directional cDNA library was constructed from poly(A) RNA using a SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning kit (Invitrogen) according to the manufacturer's instructions.

cDNA cloning

Screening of the cDNA library was performed using a GENETRAPPER cDNA Positive Selection System (Invitrogen). An oligonucleotide probe for a cDNA capture hybridization was designed based on the sequence of a rainbow trout *Hsp70* genomic clone isolated in a preliminary study (GenBank accession no. AB062281). The nucleotide sequence of the probe was 5'-TGGTCCTGGTGAAGATGAGG-3'. Biotinylation of the probe and cDNA capture hybridization were performed according to the manufacturer's instructions. The captured single-stranded DNA was repaired and transfected into ELECTROMAX DH10B cells (Invitrogen) by using an electroporation apparatus (MicroPulser; Bio-Rad Laboratories, Hercules, CA, USA). The electroporation was carried out at 1.8 kV using 20-μl cells in a 0.1-cm-gap cuvette. White colonies were selected after transformation, and whether they were truly positive were analyzed by PCR using the above-mentioned probe sequence as a sense primer and a SP6 promoter primer (5'-GTGACACTATAGAAGAGCTATGACGTC-3') as an antisense primer. Subsequently, the positive clones were sequenced with an automated DNA sequencer (Gene Rapid; Amersham Biosciences, Piscataway, NJ, USA) after labeling with a Thermo Sequenase Cy5.5 Dye Terminator Sequencing kit (Amersham Biosciences).

Phylogenetic analysis

Amino acid sequences of vertebrate Hsp70s and Hsc70s were retrieved from the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/Welcome-e.html). A phylogenetic tree was constructed from the multiple sequence alignment by using the neighbor-joining method (Saitou and Nei, 1987), as implemented in CLUSTAL W (Thompson *et al.*, 1994). A graphical output of the tree was visualized by the program TREEVIEW (Page, 1996).

Southern blot analysis

Genomic DNA was isolated from RTG-2 cells by using a DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each 10-µg DNA was digested with restriction enzymes *Bam*HI, *Eco*RI, and *Hin*dIII, electrophoresed on a 1% agarose gel, and capillary-transferred to a nylon membrane (Hybond N+; Amersham Biosciences) with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). DNA was UV-crosslinked to the membranes, which were subsequently hybridized for 1 h in PerfectHyb hybridization solution (Toyobo, Osaka, Japan) with digoxigenin (DIG)-labeled DNA probes. Probes specific for *Hsp70a* and *Hsp70b* were designed in their 3'-untranslated regions (UTRs), namely, nucleotides 2561–2773 and 2029–2191, respectively. The GenBank accession numbers of the two *Hsp70*s are as follows: *Hsp70a*, AB176854 and *Hsp70b*, AB176855. The probes were labeled with a PCR DIG Probes Synthesis kit (Roche Diagnostics, Mannheim, Germany). The hybridized membranes were washed twice with 2× SSC plus 0.1% SDS at room temperature for 5 min, and then twice with 2× SSC plus 0.1% SDS at 68 °C for 15 min. The chemiluminescent detection of the probes was performed with a DIG Luminescent Detection kit (Roche Diagnostics) according to the manufacturer's instructions. The positive signals were detected by exposure on Hyperfilm-ECL (Amersham Biosciences).

Results

To obtain full-length cDNA clones encoding Hsp70, a plasmid cDNA library containing 1.1×10^6 independent clones was constructed from a heat-shocked juvenile of rainbow trout and screened. After cDNA capture hybridization and *Escherichia coli* transformation, 14 out of 93 colonies were PCR-positive. Each positive clone was partially sequenced (approximately 400 bases) from the 5' end and, consequently, 12 out of 14 clones showed sequences highly homologous to those of Hsp70 cDNAs previously reported for other eukaryotic species. Moreover, the clones were divided into two groups based on the

nucleotide sequences, and each group was comprised of six identical clones. The clones having the longest size from each group were selected, named C1 and C11, and their complete cDNA sequences were determined. Consequently, clones C1 and C11 were 2804 and 2229 bp in length, respectively. Hereafter, these are referred to as *Hsp70a* and *Hsp70b*, respectively.

The deduced amino acid sequences of *Hsp70a* and *Hsp70b* both contain 644 residues with calculated molecular weights of 71 015 and 70 988, respectively (Fig. 1-1). The two proteins shared 98.1% identity with variations in 12 amino acid residues. Furthermore, rainbow trout Hsp70s shared many identical amino acid residues with those of other vertebrates (Fig. 1-1). For example, Hsp70a and Hsp70b showed 84.5% and 84.2% identity with human Hsp70.1, respectively.

Fig. 1-2 shows a phylogenetic tree constructed with cytosolic HSP70 family members in vertebrates. The tree was composed of the three major groups: mammalian Hsp70, fish Hsp70, and vertebrate Hsc70. Among the three groups in the tree, rainbow trout Hsp70s belonged to the fish Hsp70 group, confirming the isolated cDNA clones to be those of *Hsp70*s.

Fig. 1-3 shows the Southern blots of rainbow trout *Hsp70a* and *Hsp70b*. To accurately distinguish between the two genes, the DNA probes were designed to hybridize to 3'-UTR specific to respective genes. Consequently, the band patterns differed between *Hsp70a* and *Hsp70b*, suggesting that the two rainbow trout Hsp70s were encoded by distinct genes in the genome.

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1    MSSAKGPSIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPNNTVF    rainbow trout Hsp70a
1    .....................................................................    rainbow trout Hsp70b
1    ...P..IA.............S..........................NA..........L.........    zebrafish Hsp70
1    .--..KTA...........................................................L..Q....    rat Hsp70.1/2
1    .--..AAA..........................................................L..Q....    human Hsp70.1

71   DAKRLIGRKFNDQVVQADMKHWPFKVVSDGGKPKVQVDYKGENKSFNPEEISS|MVLVKMR|EIAEAYLGQK    rainbow trout Hsp70a
71   .............................................C........|.......|.........    rainbow trout Hsp70b
71   ........R.D.H...S.....S....R.......A.EHS..D.T..............K........    zebrafish Hsp70
69   ..........G.P...S.......Q..N..D......N.....R..Y.........T..K........HP    rat Hsp70.1/2
69   ..........G.P...S.......Q.IN..D......S....T.A.Y.........T..K........YP    human Hsp70.1

141  VSNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAAIAYGMDKGKSRERNVLIFDLGGGTFDVS    rainbow trout Hsp70a
141  .............................................................M.......    rainbow trout Hsp70b
141  .T...........E.H.........V.....A......RP......L.....S...........E......    zebrafish Hsp70
139  .T...................................................L.RTGKG................    rat Hsp70.1/2
139  .T...................................................L.RTGKG................    human Hsp70.1

211  ILTIEDGIFEVKATAGDTHLGGEDFDNRLVSHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTLSSSS    rainbow trout Hsp70a
211  .....................................................................    rainbow trout Hsp70b
211  ..................V......T.N.........................................    zebrafish Hsp70
209  ....D.............D.............................V.................T    rat Hsp70.1/2
209  ....D...................N......................V.................T    human Hsp70.1

281  QASIEIDSLFEGIDFYTSITRARFEEMCSDLFRGTLEPVEKALRDAKMDKAQIHDVVLVGGSTRIPKVQK    rainbow trout Hsp70a
281  ...............................R.........G......................    rainbow trout Hsp70b
281  .........Y..............L.........D.................I...........I..    zebrafish Hsp70
279  ...L......................L...........L.......L.............    rat Hsp70.1/2
279  ...L......................L......S............L.......L.............    human Hsp70.1

351  LLQDFFNGRELNKSINPDEAVAYGAAIQAAILSGDKSENVQDLLLLDVAPLSLGIETAGGVMTALIKRNT    rainbow trout Hsp70a
351  .....................................................................    rainbow trout Hsp70b
351  ..................V.....M..T.G....M...........................    zebrafish Hsp70
349  .........D..............V.....M...................L...............S    rat Hsp70.1/2
349  .........D..............V.....M...................L...............S    human Hsp70.1

421  TIPSKQTQIFTTYSDNQPGVMIQVYEGERAMTKDNNLLGKFELSGIPPSPRGVPQIEVTFDIDANGILNV    rainbow trout Hsp70a
421  ........T...................................A.....................    rainbow trout Hsp70b
421  ...T....T.A.........L...F...G......D.T....A.....................    zebrafish Hsp70
419  ...T....T........L...........R......R.......A.................    rat Hsp70.1/2
419  ...T.............L...................R........A.................    human Hsp70.1

491  AAVDKSTGKENKITITNDKGRLSKEDIERMVQDADKYKAEDDAQREKMAAKNSLESYAFNMKSSVEDDNM    rainbow trout Hsp70a
491  S...............................I.........................    rainbow trout Hsp70b
491  S.A......Q................E......E.......L....IS...........N.......L    zebrafish Hsp70
489  T.T......A................E......E.ER.....EV...RV....A..........A...EGL    rat Hsp70.1/2
489  T.T......A................E......E.E......EV...RVS...A..........A...EGL    human Hsp70.1

561  KGKISEEDKKKVMDRCNQTISWLENNQLGDKEEYEHQLKELEKVCQPIITKLYQQGGMPTGCCGDQARTS    rainbow trout Hsp70a
561  .....Q......V...D.............................................    rainbow trout Hsp70b
561  ......R.VEK..EAV.R......A..........-....A.G..A....AA    zebrafish Hsp70
559  ......A.....L.K.QEV....DS.T.AE...FV.KRE...R..N...SG...GA.A.-.AG.FG.QAP    rat Hsp70.1/2
559  ......A.....L.K.QEV....DA.T.AE.D.F..KR....Q..N...SG...GA.G.-.PG.FG.QGP    human Hsp70.1

631  SGVSSQGPTIEEID                   rainbow trout Hsp70a
631  ..D...........                   rainbow trout Hsp70b
630  ..A.A.......V.                   zebrafish Hsp70
628  K.G.GS......V.                   rat Hsp70.1/2
628  K.G.GS......V.                   human Hsp70.1
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Fig. 1-1. Comparison of the amino acid sequences of rainbow trout Hsp70a and
Hsp70b with those of other vertebrate Hsp70s. Dots represent amino acid residues
that are identical to those of the rainbow trout Hsp70a sequence, and dashes indicate
gaps set to maximize the alignment. The box represents the location of the sequence
used for designing a probe to screen rainbow trout *Hsp70*s. GenBank accession
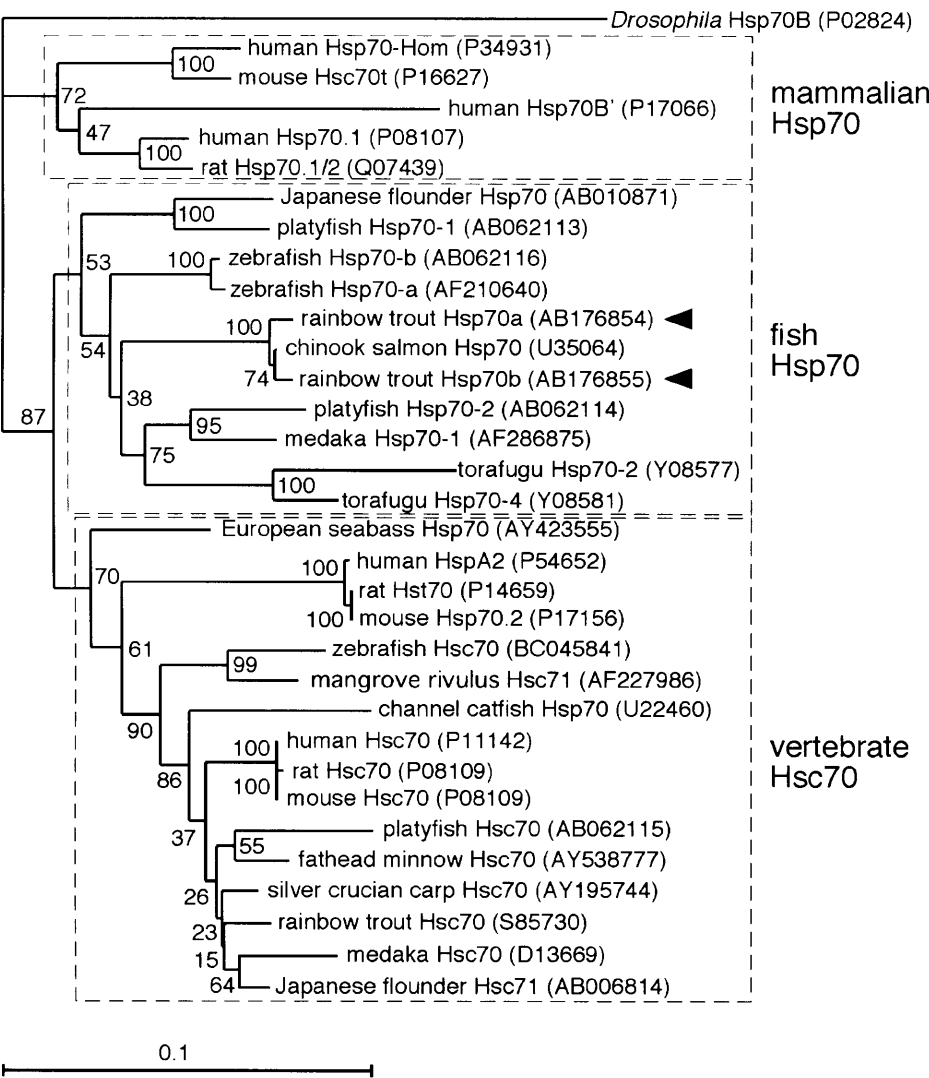numbers for cited genes are presented in Fig. 1-2.

Fig. 1-2. Phylogenetic analysis of cytosolic HSP70 family members in vertebrates. Arrowheads indicate the position of rainbow trout Hsp70a and Hsp70b. The three groups of HSP70 family members are boxed. *Drosophila* Hsp70B was used as an outgroup. Numbers at the nodes represent the percentage of bootstrap values (1000 replicates). The scale bar represents a phylogenetic distance of 0.1 amino acid substitutions per site. GenBank or Swiss-Prot accession numbers for cited proteins are indicated in parentheses.
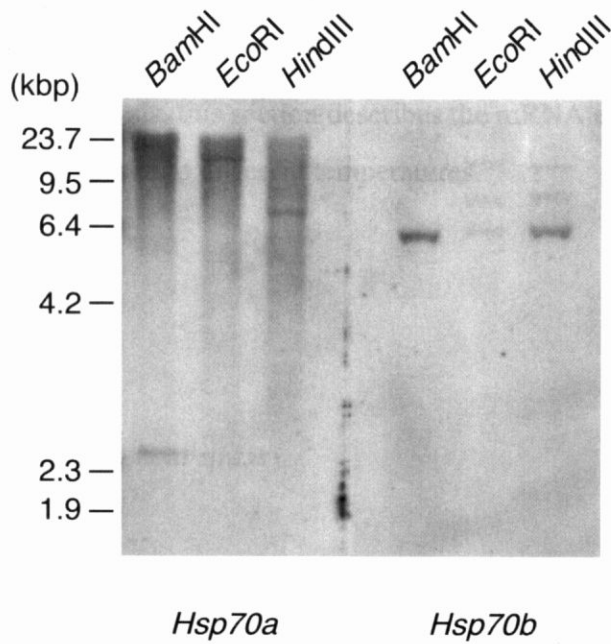
Fig. 1-3. Genomic Southern blot analysis of rainbow trout *Hsp70a* (left panel) and *Hsp70b* (right panel). RTG-2 genomic DNA digested with *Bam*HI, *Eco*RI, and *Hin*dIII was separated by agarose gel electrophoresis, blotted onto a nylon membrane, and hybridized with DIG-labeled probes. The probes are complementary to sequences in 3'-UTR of the two genes. λ DNAs digested with *Hin*dIII were used as size markers and are indicated on the left.

Section 2   mRNA expression profiling of *Hsp70*—Temperature shift experiment

In the preceding section, two distinct genes encoding Hsp70 were identified in rainbow trout, and the nucleotide sequences of their 3'-UTR were found to have low homology with each other. Indeed, each *Hsp70* was specifically detected in Southern blot analysis by using the part of their 3'-UTR sequences as DNA probes. By applying the same probes to Northern blot analysis, this section describes the mRNA expression profiling of *Hsp70*s in RTG-2 cells exposed to different temperatures.

Materials and Methods

Cell cultures and exposure to heat stress

RTG-2 cells, immortalized rainbow trout gonadal fibroblasts (Wolf and Quimby, 1962), were cultured at 20 °C in Leibovitz's L-15 medium (Invitrogen) supplemented with 5% fetal bovine serum and antibiotic–antimycotic mixture (100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Invitrogen). The culture dishes were sealed with Parafilm and immersed into a water bath at 24, 28, and 32 °C for 1 h each.

Northern blot analysis

Total RNA was isolated from RTG-2 cells with TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions. For Northern blotting, 5 μg of the total RNA was separated on a 1.2% agarose-formaldehyde gel and capillary transferred to a Hybond N+ nylon membrane with 10× SSC. Hybridization and detection of target genes were performed through Southern blot analysis as described in Section 1 of this chapter. A probe specific for *Hsc70* was designed in its 3'-UTR, namely, nucleotides 6188–6408, based on the genomic

DNA sequence (GenBank accession no. S85730) previously reported (Zafarullah *et al.*, 1992).

Quantification and statistical analysis of mRNA accumulation levels

The fluorograms of Northern blots were scanned in 8-bit grayscale into Adobe
Photoshop (Adobe Systems, San Jose, CA, USA) at 300 dpi using a flatbed scanner
(GT-8700F; Seiko Epson, Tokyo, Japan) and accompanying software. The integrated optical
density (IOD) of positive bands was quantified using Gel-Pro Analyzer software (Media
Cybernetics, Silver Spring, MD, USA). The relative amounts of Hsp70 mRNAs were
calculated by dividing IOD of *Hsp70* by that of *Hsc70*. To confirm the validity of using
Hsc70 as an internal standard, possible changes in the amounts of Hsc70 mRNAs themselves
during heat stress were tested with one-way analysis of variance (ANOVA) at the significance
level of $P<0.05$.

Results

Fig. 1-4 shows the expression profiles of rainbow trout Hsp70 mRNAs in RTG-2
cells exposed to different heat shock temperatures for 1 h. Neither Hsp70a nor Hsp70b
mRNAs were detected at the control temperature (20 °C), whereas both mRNAs were
apparently induced at 24 °C and above (Fig. 1-4A). In contrast, Hsc70 mRNA was
constitutively expressed at any temperatures examined (Fig. 1-4A). A single mRNA species
was detected at 24 °C for both *Hsp70*s, where the length of Hsp70a mRNA differed from that
of Hsp70b mRNA. The apparent sizes of the bands were consistent with the calculated ones
based on the cloned cDNAs described in the preceding section. Besides the single mRNA
species at 24 °C, longer mRNA species were detected at 28 and 32 °C for each *Hsp70* (Fig.
1-4A). Hereafter, for convenience, the shorter and longer mRNA species are referred to as
Hsp70-short and Hsp70-long mRNAs, respectively.

A

Temperature (°C)

20 24 28 32

28S —

18S —

Hsp70a

28S —

18S —

Hsp70b
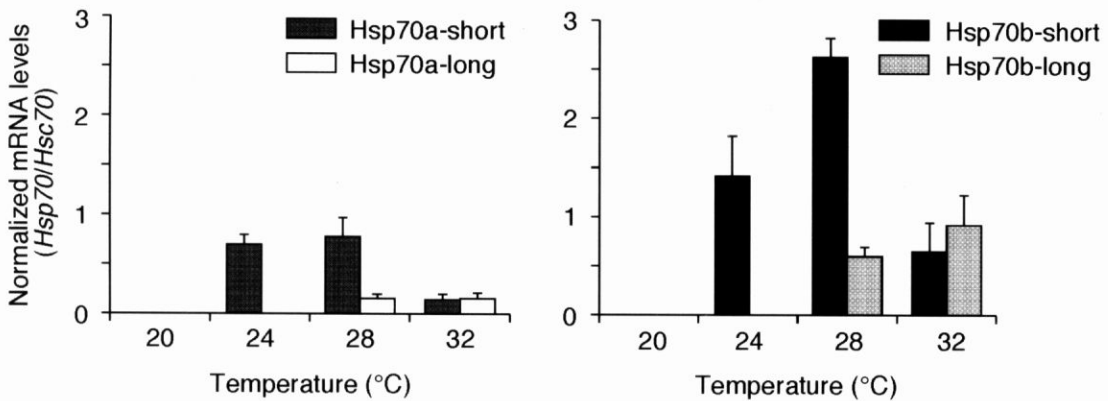
28S —

18S —
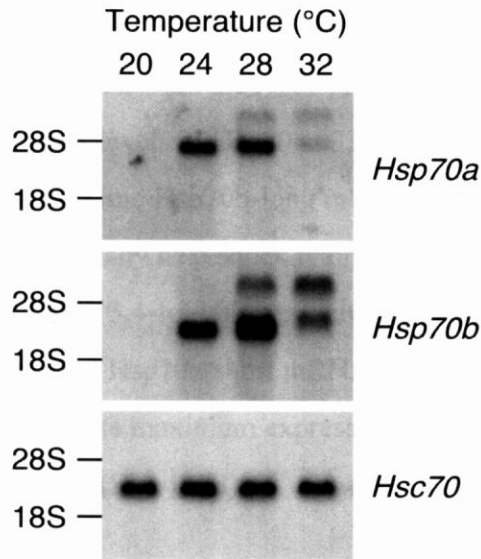
Hsc70

B

Fig. 1-4. Expression profiles of Hsp70 mRNAs in RTG-2 cells exposed to different temperatures. (A) Northern blot analysis of *Hsp70a*, *Hsp70b*, and *Hsc70*. The probes are complementary to sequences in 3'-UTR of respective genes. The positions of 28S and 18S rRNAs are indicated on the left as size markers. The blots are representatives of three independent experiments. (B) Quantification of Northern blot analysis. The amounts of Hsp70 mRNAs normalized to those of Hsc70 mRNAs are shown. The values were calculated by dividing IOD of Hsp70 mRNA by that of Hsc70 mRNA, and are presented as means ± SD of three independent experiments.

To compare the expression levels of different mRNA species, the amounts of the *Hsp70* transcripts were determined by measuring IOD of the bands. IOD of Hsc70 mRNA was used as an internal standard because there was no statistically significant difference in the level of Hsc70 mRNA at different temperatures ($P>0.05$, one-way ANOVA). In this regard, however, IOD at 32 °C was excluded from the ANOVA because of its large variance. Therefore, the normalized mRNA levels of Hsp70 at 32 °C were calculated for reference. As shown in Fig. 1-4B, Hsp70b-short and Hsp70b-long mRNAs displayed higher expression levels than Hsp70a counterparts at any heat shock temperatures tested. For example, the mean value of Hsp70b-short mRNA was 3.4-fold higher than that of Hsp70a-short mRNA at 28 °C. In addition, the expression level of Hsp70a-short mRNA was almost as high as that of Hsc70 mRNA at 24 and 28 °C, whereas the maximum expression level of Hsp70b-short mRNA was 2.6-fold higher than that of *Hsc70* (Fig. 1-4B).

Section 3   mRNA expression profiling of *Hsp70*—Time course experiment

In the preceding section, a 1 h heat shock at 28 °C gave rise to the highest accumulation levels of Hsp70 mRNAs. When heat shock is prolonged, how do the accumulation levels change in RTG-2 cells? To answer this question, this section describes the mRNA expression profiling of *Hsp70*s during prolonged heat stress.

Materials and Methods

Cell cultures and exposure to heat stress

RTG-2 cells were cultured at 20 °C as described in Section 2 of this chapter. The culture dishes were sealed with Parafilm and immersed into a water bath at 28 °C for 1.5, 3, 6, 12, and 24 h.

Northern blot analysis

Northern blot analysis was performed as described in Section 2 of this chapter.

Quantification and statistical analysis of mRNA accumulation levels

Quantification and statistical analysis of mRNA expression levels were performed as described in Section 2 of this chapter.

Results

Fig. 1-5 shows the expression profiles of Hsp70 mRNAs during prolonged heat stress at 28 °C. Since there was no statistically significant changes in the level of Hsc70 mRNA during heat stress ($P>0.05$, one-way ANOVA), IOD of Hsc70 mRNA was again used as an internal standard. In this experiment, Hsp70 mRNA levels reached the maximum after 3 h at 28 °C and then decreased (Fig. 1-5B). Hsp70a-long and Hsp70b-long mRNAs disappeared by 12 h, whereas Hsp70a-short and Hsp70b-short mRNAs remained detectable after 24 h. Although *Hsp70a* and *Hsp70b* had similar expression profiles, the levels of Hsp70b-short and Hsp70b-long mRNAs were consistently higher than Hsp70a counterparts during heat stress (Fig. 1-5B).
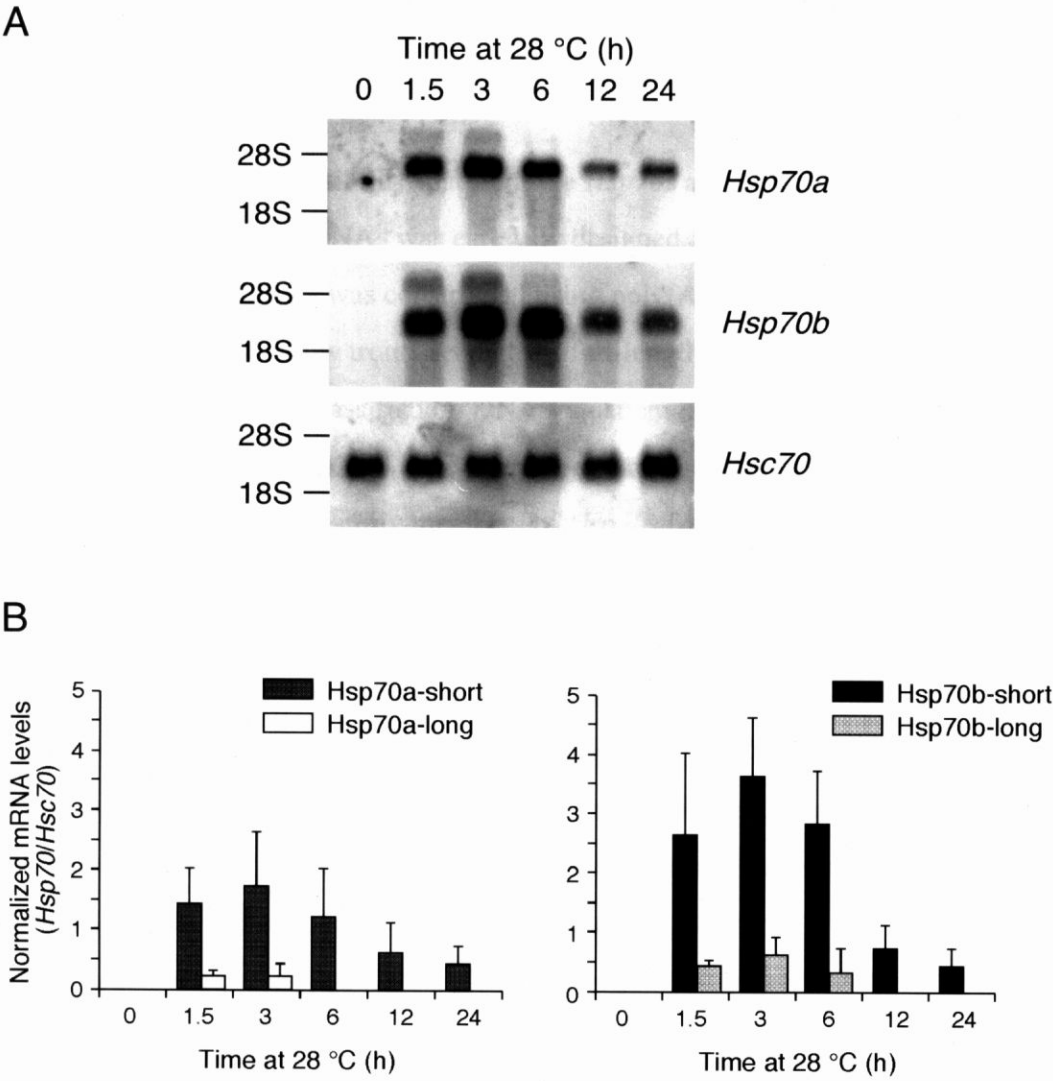
A

B

Fig. 1-5. Expression profiles of Hsp70 mRNAs in RTG-2 cells exposed to prolonged heat stress at 28 °C. (A) Northern blot analysis of *Hsp70a*, *Hsp70b*, and *Hsc70*. The probes are complementary to sequences in 3'-UTR of respective genes. The positions of 28S and 18S rRNAs are indicated on the left as size markers. The blots are representatives of three independent experiments. (B) Quantification of Northern blot analysis. The amounts of Hsp70 mRNAs normalized to those of Hsc70 mRNAs are shown. The values were calculated by dividing IOD of Hsp70 mRNA by that of Hsc70 mRNA, and are presented as means ± SD of three independent experiments.

Section 4   Discussion

In this chapter, two distinct *Hsp70*s were identified in rainbow trout, and their mRNA expression profiles were separately investigated using RTG-2 cells exposed to heat stress.

Considering the possibility that multiple *Hsp70*s are expressed in rainbow trout cells, a screening to identify the cDNAs was carefully designed. First, to eliminate individual variations, the cDNA library was constructed using poly(A) RNA prepared from one individual of juvenile rainbow trout. Second, 12 full-length cDNA clones encoding Hsp70 were isolated and correctly classified by DNA sequencing. Consequently, it was found that two *Hsp70*s, *Hsp70a* and *Hsp70b*, were expressed in rainbow trout. Until now, two cDNA clones have been isolated as family members of *Hsp70*s from rainbow trout: *THS70.7* and *THS70.14* (Kothary *et al.*, 1984b); however, both clones were only partially sequenced. Comparing the partial cDNAs with the complete cDNA sequences isolated in the present study, *THS70.14* was found to correspond to *Hsp70a*. On the other hand, it could not be identified with certainty that *THS70.7* is either *Hsp70a* or *Hsp70b* because *THS70.7* was the fragment of the coding region of *Hsp70* that is highly conserved. In addition, a genomic clone of rainbow trout *Hsp70* was previously isolated (GenBank accession no. AB062281), which corresponds to *Hsp70a* and has an intronless open reading frame.

Rainbow trout Hsp70s were highly homologous to those from other vertebrates (Fig. 1-1). Phylogenetic analysis of cytosolic Hsp70 family members indicates that there is a fish Hsp70 group containing rainbow trout Hsp70s (Fig. 1-2). Moreover, the phylogenetic tree showed another two groups: mammalian Hsp70 and vertebrate Hsc70. Even if other Hsp70 family members, glucose-regulated protein 75 (Grp75) and 78 (Grp78), were included in the analysis, the clustering of the three groups was not altered (data not shown). Some *Hsp70*s belonging to the fish Hsp70 group are heat-inducible; e.g., Japanese flounder *Paralichtys olivaceus Hsp70* (Yokoyama *et al.*, 1998), platyfish *Xiphophorus maculatus* and zebrafish *Danio rerio Hsp70*s (Yamashita *et al.*, 2004). Therefore, rainbow trout Hsp70s are suggested to be heat-inducible forms, and indeed, their transcription was induced by heat stress (Figs. 1-4 and 1-5).

Southern blot analysis indicated that rainbow trout Hsp70a and Hsp70b are encoded by distinct genes in the genome (Fig. 1-3), which would be common among salmonid fishes because, as suggested by Ohno *et al.* (1968), most of them have evolved by tetraploidization from a diploid ancestor. In fact, Rise *et al.* (2004) have reported the presence of distinct forms of several genes in Atlantic salmon *Salmo salar* by preliminary comparisons of its expressed sequence tag (EST) clusters. Thus, rainbow trout *Hsp70a* and *Hsp70b* are suggested to be paralogous genes.

Northern blot analysis showed that both *Hsp70a* and *Hsp70b* were induced by heat stress in RTG-2 cells (Figs. 1-4 and 1-5). In this analysis, Hsp70a and Hsp70b mRNAs could be clearly distinguished by using probes specific to respective genes. As shown in Fig. 1-4, the mRNA levels of *Hsp70a* and *Hsp70b* decreased at 32 °C and the variance of data at this temperature was large, suggesting that a temperature of 32 °C was too severe for rainbow trout cells. For instance, the synthesis of Hsps was not observed at 32 °C in RTG-2 cells, and the temperature range in which RTG-2 cells could survive for at least 7 days was 0–28 °C (Mosser *et al.*, 1986). Furthermore, Mosser *et al.* (1986) reported that RTG-2 cells became shriveled and took on a threadlike appearance after 1 h at 32 °C. The observation in the present study was consistent with these results, and there was no observable change in the cell morphology after 1h at 24 and 28 °C (data not shown). Thus, temperatures exceeding 28 °C would be too stressful for RTG-2 cells.

A new finding is that longer mRNA species were induced in rainbow trout cells at above 28 °C for both *Hsp70a* and *Hsp70b* (Fig. 1-4). Although the expression of multiple mRNA species has been reported for several heat-shock genes (Dellavalle *et al.*, 1994; Takechi *et al.*, 1994), two different mRNA species from a single *Hsp70* have not yet been detected. Since the longer mRNA species were detected only under severe heat stress conditions, they may be useful as a biomarker for the high degrees of heat stress. However, whether such mRNAs are induced at the individual level remains to be clarified. Now, what mechanisms underlie the generation of different Hsp70 mRNA species under severe heat stress condition? There are three possible mechanisms: (i) polyadenylation and deadenylation [e.g., *Drosophila Hsp70* (Dellavalle *et al.*, 1994)], (ii) defective splicing [e.g., *Drosophila Hsp83* (Dellavalle *et al.*, 1994)], and (iii) alternative splicing [e.g., mouse *Hsp47* (Takechi *et*

al., 1994)]. Among them, it is most likely that the mRNA species are generated by alternative splicing. One reason is that the lengths of the longer mRNA species appear to be too long if they are polyadenylated forms of the shorter mRNA species (Figs. 1-4A and 1-5A). For example, a polyadenylated form of *Drosophila* Hsp70 mRNAs has poly(A) tails of about 120 to 160 nucleotides (Dellavalle *et al.*, 1994). Another reason is that rainbow trout Hsc70 mRNA, which contains introns in the genomic DNA (Zafarullah *et al.*, 1992), showed no change in length at above 28 °C (Fig 1-4A). This indicates that splicing is not defective after severe heat stress. Taken together, the longer mRNA species of the rainbow trout *Hsp70*s are thought to be alternatively spliced products. However, the possibility that polyadenylation and deadenylation may be involved in the event cannot be excluded because the sequences of poly(A) tails from the longer mRNA species have not yet been determined.

Another finding is that the accumulated mRNA levels of *Hsp70b* were higher than that of *Hsp70a* during heat stress (Figs. 1-4 and 1-5). This difference seems not due to the difference of labeling efficiency between the two DIG-labeled probes. The efficiency for both probes were evaluated by gel electrophoresis, confirming that there was no significant difference between the signal intensities of the two bands (data not shown). However, quantitative PCR analysis will be needed to discuss more accurately the difference of the mRNA levels. On the other hand, the mRNA expression profiles of the two genes were similar to each other in both temperature shift (Fig. 1-4) and time course experiments (Fig. 1-5). In the time course experiment, prolonged heat stress at 28 °C for longer than 3 h caused a decrease in Hsp70 mRNA levels (Fig. 1-5B). These results generally agreed with the induction pattern of Hsp70 mRNA previously reported (Kothary *et al.*, 1984a), in which, however, multiple Hsp70 mRNA species were examined together without separation.

At the protein level, the synthesis of Hsp70 has been reported to attain a peak at 2 h and maintain high levels between 2 and 6 h after treatment of RTG-2 cells at 28 °C (Mosser *et al.*, 1986). These results are roughly consistent with the mRNA expression profiles of Hsp70 shown in Fig. 1-5. In addition, Mosser *et al.* (1987) reported that the induction of HSP synthesis at 28 °C paralleled the development of thermotolerance in RTG-2 cells. On the other hand, rainbow trout Hsp70a and Hsp70b have not been clearly distinguished at the protein level as well as at the mRNA level. Nevertheless, two proteins supposed to be Hsp70

have been detected in heat-stressed RTG-2 cells (Airaksinen *et al.*, 1998; Mosser *et al.*, 1986). Their apparent molecular sizes were 70 and 68 kDa (Mosser *et al.*, 1986), and 69 and 67 kDa (Airaksinen *et al.*, 1998). Considering the predicted molecular weights described in Results of Section 1, Hsp70a and Hsp70b seem to be their equivalents.

CHAPTER II   CLONING AND mRNA EXPRESSION PROFILES OF HSP FAMILY MEMBERS

To resolve the regulation and functional significance of HSPs, fish are ideal organisms because they are ectothermic vertebrates and naturally exposed to thermal stress in their environment (Basu *et al.*, 2002). However, most studies on HSPs in fish have been performed at the protein level, and HSP genes have only been cloned from a modest number of different species (Basu *et al.*, 2002). Furthermore, since the structure and the mRNA expression of HSP genes have been analyzed individually and separately, a comprehensive mRNA expression profile of HSP genes has not been established in cells from a single fish species. Additionally, HSPs are good examples of molecular level biomarkers to perceive sublethal cellular damages as the result of an environmental stress (Ryan and Hightower, 1996). However, the use of HSPs as indicators for stress states of fish in general is premature (Iwama *et al.*, 2004), in part because little information is available about a comprehensive stress response profile of HSP genes in fish cells.

Thus, this chapter describes the isolation of multiple HSP genes from rainbow trout and analysis of their expression profiles at the mRNA levels. In this regard, the mRNA levels between unstressed and heat-shocked cells were quantitatively compared using real-time RT-PCR analysis.

Section 1   Cloning of HSP cDNAs expressed in heat-shocked RTG-2 cells

To comprehensively analyze the mRNA expression profile of *HSP*s, DNA sequence information for each gene is prerequisite to the specific detection. However, *HSP*s have not been cloned from rainbow trout except for *Hsc70* reported by Zafarullah *et al.* (1992) and *Hsp70*s described in the preceding chapter. Thus, isolation of genes encoding HSP family members is required, and it is desirable that *HSP*s would be isolated as many as possible at the same time. This section describes an efficient isolation of cDNAs encoding HSPs from RTG-2 cells and the features of their gene structures.

Materials and Methods

Cell Culture

RTG-2 cells were cultured at 15 °C as described in Section 2 of Chapter I.

Construction of directional cDNA library

Five 100-mm culture dishes with confluent RTG-2 cells were heat-shocked at 25 °C for 24 h in an incubator. Cells were lysed with TRIZOL Reagent (Invitrogen), and total RNA was isolated according to the manufacture's instructions. Poly(A) RNA was isolated from the total RNA using an Oligotex-MAG mRNA Purification kit (Takara Bio). A directional cDNA library was constructed using a SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning kit (Invitrogen). cDNAs were constructed from 5 µg of poly(A) RNAs using a *Not*I primer-adapter. After addition of a *Sal*I adapter, cDNAs were digested with *Not*I and size-fractionated by column chromatography according to the manufacturer's instructions. The digests were ligated into plasmid vector pSPORT1, and the plasmids were introduced into ELECTROMAX DH10B competent cells (Invitrogen) using an electroporation apparatus

(MicroPulser; Bio-Rad Laboratories). The electroporation was performed using a 0.1-cm-gap cuvette containing 1 µl of the plasmid DNA and 20 µl of the competent cells at 1.8 kV and one pulse. The cells were plated onto LB agar plates containing 100-µg/ml ampicillin.

Sequencing and bioinformatic analysis

Two hundred white colonies were arbitrarily selected for isolation of plasmid DNA. The plasmid DNA was purified using a conventional alkaline/SDS lysis method. The 5' end each of cDNA inserts was sequenced with a T7 promoter primer (5'-TAATACGACTCACTATAGG-3') using a Thermo Sequenase II dye terminator cycle sequencing kit (Amersham Biosciences) and an automated DNA sequencer (373A; Applied Biosystems, Foster City, CA, USA). After removing the vector sequence, the deduced amino acid sequences were compared with sequences in the nr protein database using the blastx program in the NCBI BLAST homepage (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

To clone multiple HSP genes at the same time, 200 cDNAs were arbitrarily isolated from heat-shocked RTG-2 cells and their 5' ends were sequenced. The readable sequence length was approximately 340 bp. The data are summarized in Table 2-1, where the BLAST hits with an E value $\geq 10^{-5}$ were defined as unknown. Out of 200 clones, 121 clones (60.5%) showed significant similarities (E value $<10^{-5}$) with proteins in the NCBI database, and 11 clones (5.5%) were putatively identified as HSP family members. Additionally, four clones (2.0%) were classified as "chaperone-related proteins", which was designated in the present study, including proteins with chaperone or catalytic activity for protein folding.

Table 2-1. List of cDNAs isolated from heat-shocked RTG-2 cells

Putative genes	No. of clone	% of total clones
HSP family members		
heat-shock cognate protein 70	3	
heat-shock protein 90β	2	
heat-shock protein 70	2	
glucose-regulated protein 78	1	
chaperonin containing TCP1, subunit 8 (Cct8)	1	
heat-shock protein 47	1	
DnaJ (Hsp40) homolog, subfamily A, member 4	1	
Subtotal	**11**	**5.5**
Chaperone-related proteins		
progesterone receptor-related protein p23	2	
14-3-3B2 protein	1	
FK506 binding protein 2	1	
Subtotal	**4**	**2.0**
Others		
cytochrome *c* oxidase subunit I	6	
elongation factor 1α	5	
cytoskeletal β-actin	4	
ferritin H-2	4	
K18, simple type I keratin	4	
CD81	2	
cyclin G1	2	
sorting nexin	2	
thymosin β	2	
trafficking protein particle complex 5	2	
type I collagen α2 chain	2	
singletons	71	
Subtotal	**106**	**53.0**
Unknown	**79**	**39.5**
Total	**200**	**100.0**

Next, the cDNA clones encoding HSP family members were completely sequenced, resulting in identification of nine genes (Table 2-2). These clones were designated as in Table 2-2, based on their deduced protein homology with HSP family members of other vertebrates. Among them, two types having different sequences were found each in *Hsp90* and *Hsc70*. They were designated as *Hsp90βa* and *Hsp90βb*, and *Hsc70a* and *Hsc70b*, respectively. Both Hsp90βa and Hsp90βb cDNAs contained complete open reading frame (ORF), and their deduced amino acid sequences shared 97.7% identity with each other. The two proteins contained the conserved C-terminal tetrapeptide motif, EEVD (data not shown), which plays an important role in cofactor binding mediated by a tetratricopeptide repeat (TPR) domain (Scheufler *et al.*, 2000). A BLAST search indicated that both proteins had the highest amino acid identity with Atlantic salmon *Salmo salar* Hsp90β (Table 2-2). Meanwhile, Hsc70b cDNA contained an incomplete ORF, and its deduced protein lacked 121 amino acid residues from the N-terminus compared with that of Hsc70a cDNA. The cDNA sequence of Hsc70a was identical to that of the genomic clone reported previously as rainbow trout *Hsc71* (Zafarullah *et al.*, 1992). For *Hsp70*, one clone was isolated and identified as *Hsp70a*, which was cloned from a juvenile rainbow trout in the preceding chapter. The two Hsp70a proteins

Table 2-2. Summary of cloned cDNAs encoding HSP family members

HSP family	Rainbow trout RTG-2 cDNA			The best matching sequence in the blastp program			
	Clone no.	Designation	Accession no.	Protein name	Species	Accession no.	Amino acid identity (%)
HSP90	Q120	*Hsp90βa*	AB196457	Hsp90β	Atlantic salmon	AF135117	98.9
	R046	*Hsp90βb*	AB196458	Hsp90β	Atlantic salmon	AF135117	97.8
HSP70	R032[a]	*Grp78*	AB196459	Grp78	zebrafish	BC052971	90.1[b]
	R058	*Hsp70a*	AB176854[c]	Hsp70	rainbow trout	AB062281[d]	99.4
	Q126	*Hsc70a*	AB196460	Hsc71	rainbow trout	S85730[d]	100.0
	Q110[a]	*Hsc70b*	AB196461	Hsc71	rainbow trout	S85730[d]	79.2[b]
HSP60	Q123	*Cct8*	AB196462	Cct8	zebrafish	BC050492	90.7
HSP47	R100	*Hsp47*	AB196463	Hsp47	zebrafish	BC071301	83.5
HSP40	Q086[a,e]	*DnaJ homolog*	AB196464	DnaJ homolog	*Xenopus*	BC042291	78.9[b]

[a] cDNA clone with an incomplete 5' end.
[b] partial sequence identity.
[c] cDNA clone isolated in Chapter I.
[d] genomic clone.
[e] cDNA clone containing putative intron(s).

derived from different sources showed only one amino acid variation, namely, T429I (data not shown). For *BiP/Grp78*, one incomplete cDNA clone, which lacked the 5' end, was isolated. The deduced protein possessed a C-terminal tetrapeptide KDEL (data not shown), which is conserved in BiP/Grp78 and required for retrieval of BiP/Grp78 molecules that leave the endoplasmic reticulum (ER) (Munro and Pelham, 1987). Besides those described above, cDNAs encoding chaperonin containing TCP1 subunit 8 (Cct8), Hsp47 and DnaJ homolog were identified. Cct8 and Hsp47 cDNAs contained complete ORFs, whereas DnaJ homolog cDNA was a partial fragment containing putative intron(s). The deduced Hsp47 protein contained an RDEL tetrapeptide motif at the C terminus (data not shown) that acts as an ER-retention signal (Satoh *et al.*, 1996).

Section 2   Accumulation of HSP mRNAs in unstressed and heat-shocked RTG-2 cells

As a result of the preceding section, DNA sequence information for several *HSP*s became available. Based on this information, it is possible to design probes and primers specific to respective genes. By using these probes and primers, this section describes the mRNA expression profiles of *HSP*s and the quantitative analysis of their mRNA accumulation levels before and after heat stress.

Materials and Methods

RNA preparation

Control RTG-2 cells were cultured at 20 °C as described in Section 2 of Chapter I. The culture dishes were sealed with Parafilm and immersed into a water bath at 28 °C for 3 h. This heat-shock condition gave rise to the highest accumulation levels of Hsp70 mRNAs in Chapter I. Total RNA was isolated from the control and heat-shocked RTG-2 cells with TRIZOL Reagent (Invitrogen) according to the manufacturer's instruction. For real-time RT-PCR, 20 µg of each RNA was incubated at 37 °C for 20 min with 10 U RNase-free DNase I (Takara Bio) in a 50-µl reaction volume containing 20 U RNase inhibitor (Toyobo). RNAs were extracted with phenol:chloroform:isoamyl alcohol (25:24:1 mixed, pH5.2; Nacalai Tesque, Kyoto, Japan) followed by ethanol precipitation.

Northern blot analysis

Five micrograms of total RNA were separated on a 1% agarose–formaldehyde gel and capillary transferred to Hybond N+ nylon membranes (Amersham Biosciences) with 10× SSC. RNAs were UV-crosslinked to the membranes, which were subsequently hybridized at

68 °C for 1 h in a PerfectHyb hybridization solution (Toyobo) containing DIG-labeled DNA probes. The probes were labeled with a PCR DIG Probes Synthesis kit (Roche Diagnostics) using primers specific to 3'-UTR of each gene (Table 2-3). The hybridized membranes were washed twice with 2× SSC plus 0.1% SDS at 68 °C for 5 min, and then twice with 2× SSC plus 0.1% SDS at 68 °C for 15 min. The chemiluminescent detection of the probes was performed with a DIG Luminescent Detection kit (Roche Diagnostics) according to the manufacturer's instruction. Positive signals were detected by using a luminescent image analyzer (LAS-1000 mini; Fuji Photo Film, Tokyo, Japan).

Table 2-3. Primers used for synthesis of DIG-labeled probes

Gene	Accession no.	Primer sequence [a]	Nucleotide positions	Product length (bp)
Hsp90βa	AB196457	F: 5'-AATGGGTAACCTGGTCAGTG-3' R: 5'-CTGAATACAGACAGGTCTGA-3'	2301–2600	300
Hsp90βb	AB196458	F: 5'-GTCTCAAACCTACACACCTG-3' R: 5'-CTACATAGCTACCGGTCCAA-3'	2231–2510	280
Grp78	AB196459	F: 5'-TCTGGAGTGGCACAGATGTA-3' R: 5'-CATGTTACCCTTCACCCAGA-3'	1911–2170	260
Hsp70a	AB176854	F: 5'-ACCACGAAATTGGGGAGAAA-3' R: 5'-TGCAATGTCCAACAATGAAA-3'	2544–2756	213
Hsp70b	AB176855	F: 5'-AGAGATTGACTAAAGTGAGGGAT-3' R: 5'-ACATTTTATTTGCAATGTCC-3'	2012–2174	163
Hsc70a	AB196460	F: 5'-ACCTCCCCCTAACAAGCAAA-3' R: 5'-AGGCATTGTGACAAAGGCAG-3'	2091–2310	220
Hsc70b	AB196461	F: 5'-CCACCATTGAGGAAGTCGAT-3' R: 5'-CAGGACTCAAAATGTAGACA-3'	1601–1810	210
Cct8	AB196462	F: 5'-AAACACTGGGGCATCAAACT-3' R: 5'-ATGTCATCGCTCCTTCCATC-3'	1559–1856	298
Hsp47	AB196463	F: 5'-CCAGGAAATGGCACATGTAT-3' R: 5'-TATAAGCATGCTGTCGGGTC-3'	1381–1670	290
β-actin	AB196465	F: 5'-TGTACCCATCCCAAACGACC-3' R: 5'-TCCTCAGCTGCATGATAGAA-3'	1201–1490	290

[a] F, forward primer; R, reverse primer.

Quantitative RT-PCR analysis

Quantitative RT-PCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen) and ABI PRISM 7000 Sequence Detection System (Applied Biosystems). One-step RT-PCR was performed in a 25-μl total reaction volume including 12.5 μl of 2× QuantiTect SYBR Green RT-PCR master mix, 0.25 μl QuantiTect RT mix, 50 ng RNA template, and 0.2 μM each of target specific primers designed to amplify a part of 3'-UTR of each gene (Table 2-4). To quantify each target transcript, a standard curve was constructed with serial dilutions of total RNA extracted from heat-shocked (28 °C, 3 h) RTG-2 cells for every set of primers. Reverse transcription was performed at 50 °C for 30 min, and thermal cycling conditions were as follows: 95 °C for 15 min, and 40 cycles of 95 °C for 5 s and 60 °C for 31 s. After PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product, as displayed by a single peak (data not shown). The control, containing all the reaction components except for the template, was included in all experiments. The amount of

Table 2-4. Primers used for real-time RT-PCR

Gene [a]	Primer sequence [b]	Nucleotide positions	Product length (bp)
Hsp90βa	F: 5'-AATGGGTAACCTGGTCAGTG-3' R: 5'-CTGAATACAGACAGGTCTGA-3'	2301–2600	300
Hsp90βb	F: 5'-AATGCTGTCTCAAACCTACACACC-3' R: 5'-ATACCATAAAAGGACACCGACCA-3'	2225–2335	111
Grp78	F: 5'-TCTGGAGTGGCACAGATGTA-3' R: 5'-CATGTTACCCTTCACCCAGA-3'	1911–2170	260
Hsp70a	F: 5'-CGGGAGTTGTAGCGATGAGA-3' R: 5'-CTTCCTAAATAGCACTGAGCCATAA-3'	2495–2634	140
Hsp70b	F: 5'-AGGCCCAACCATTGAAGAGA-3' R: 5'-GCAATGTCCAGCAATGCAATA-3'	1997–2163	167
Hsc70a	F: 5'-ACCTCCCCCTAACAAGCAAA-3' R: 5'-AGGCATTGTGACAAAGGCAG-3'	2091–2310	220
Hsc70b	F: 5'-GCCCAATCTGTAGTAAAGCCAAG-3' R: 5'-CTCCAGGACTCAAAATGTAGACAAA-3'	1669–1813	145
Cct8	F: 5'-CTCTACCCTTTTGACCACCTAACC-3' R: 5'-CTCCTTCCATCACACAGTAACCAC-3'	1698–1847	150
Hsp47	F: 5'-CCAGGAAATGGCACATGTAT-3' R: 5'-TATAAGCATGCTGTCGGGTC-3'	1381–1670	290
β-actin	F: 5'-TGGGGCAGTATGGCTTGTATG-3' R: 5'-CTCTGGCACCCTAATCACCTCT-3'	1624–1788	165

[a] Accession numbers for genes are in Table 2-3.
[b] F, forward primer; R, reverse primer.

each HSP mRNA was then normalized to the abundance of a housekeeping gene, *β-actin*. To evaluate the validity of using *β-actin* as an internal standard, possible changes in the amounts of β-actin mRNA were tested before and after heat shock by using λ poly(A)$^+$ RNA-A (Takara Bio) as an external standard. Subsequently, the normalized values of each HSP mRNA in heat-shocked cells were divided by those in controls. Student's unpaired *t* test was used for group comparisons.

Results

To investigate whether the cloned HSP genes are actually transcribed in rainbow trout cells, Northern blot analysis was performed using DNA probes specific to 3'-UTR of respective genes. In this regard, the DnaJ homolog gene was excluded from this analysis because its cDNA was incomplete where 3'-UTR was uncertain. Meanwhile, *Hsp70b* isolated in Chapter I was included. Consequently, single bands were detected for all genes examined except *Hsp70*s where two mRNA species having different sizes were detected in heat-shocked cells irrespective of *Hsp70a* and *Hsp70b* as observed in Chapter I (Fig. 2-1). Therefore, it was concluded that the cloned genes are actually transcribed in RTG-2 cells.

Although Hsp70a and Hsp70b transcripts were apparently induced by heat shock, changes in mRNA accumulation levels were ambiguous for the other HSP family members
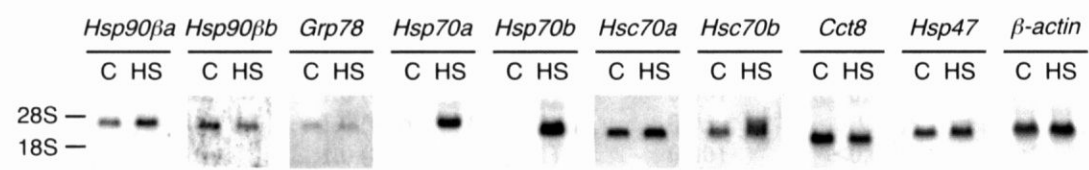

Fig. 2-1. mRNA accumulation levels of *HSP*s in control (C) and heat-shocked (HS) RTG-2 cells as revealed by Northern blot analyses. The probes are complementary to sequences in 3'-UTR of respective genes. The positions of 28S and 18S rRNAs are indicated on the left as size markers. The blots are representatives of three independent experiments.

(Fig. 2-1). Thus, to accurately determine the changes, quantitative RT-PCR analysis was performed. In this regard, the β-actin gene was used as an internal standard for normalizing the mRNA accumulation levels of HSP genes, because no significant differences were found in the β-actin mRNA levels between control and heat-shocked cells ($P = 0.36$, Student's t test, two-sided), at least under the heat-shock conditions applied here (data not shown). Consequently, significantly increased after heat shock were the mRNA accumulation levels of five HSP genes, namely, *Hsp70a*, *Hsp70b*, *Hsc70a*, *Hsc70b*, and *Hsp47* (Table 2-5). In particular, the accumulation levels of Hsp70a and Hsp70b mRNAs were dramatically increased after heat shock, and the mean values in heat-shocked cells were 480- and 510-fold of those in controls, respectively (Table 2-5). The increased levels of Hsc70a, Hsc70b and Hsp47 mRNAs were 1.3- to 2.8-fold on average after heat shock and considerably lower than those of Hsp70 mRNAs (Table 2-5). Further, when *Hsc70a* and *Hsc70b* were compared, the increased mRNA level of *Hsc70b* was more remarkable than that of *Hsc70a*, as suggested by a statistical analysis (Table 2-5). On the other hand, there were no differences in mRNA levels of four other HSP genes between control and heat-shocked cells with a significance level of 0.05 (Table 2-5).

Table 2-5. mRNA accumulation levels of *HSP*s relative to those in control

Gene	Control (20 °C)[a]	Heat shock (28 °C, 3 h)[a]	Significance level[b]	P value[b]
Hsp90βa	1.0 (0.5)	1.0 (0.6)	0.05	0.99[c]
Hsp90βb	1.0 (0.4)	1.1 (0.4)	0.05	0.77[c]
Grp78	1.0 (0.4)	1.1 (0.5)	0.05	0.78[c]
Hsp70a	1.0 (0.3)	480 (170)	0.001	<0.001[d]
Hsp70b	1.0 (0.4)	510 (150)	0.001	<0.001[d]
Hsc70a	1.0 (0.3)	1.3 (0.4)	0.05	0.0083[d]
Hsc70b	1.0 (0.3)	2.8 (0.8)	0.001	<0.001[d]
Cct8	1.0 (0.6)	0.7 (0.2)	0.05	0.12[c]
Hsp47	1.0 (0.3)	1.6 (0.1)	0.001	<0.001[d]

[a] Values are means (SD); n = 6.
[b] The Student's unpaired t test was used for group comparisons.
[c] Two-sided test.
[d] One-sided test.

Section 3   Discussion

In this chapter, multiple genes encoding HSP family members were isolated, and their mRNA accumulation levels were comprehensively compared in RTG-2 cells before and after heat shock. To accurately compare the mRNA levels of these HSP genes, real-time RT-PCR was used.

In preliminary experiments, it was found that the mRNA accumulation level of the β-actin gene was reduced in RTG-2 cells after 24-h exposure to heat shock at 25 °C (data not shown). Accordingly, it was considered that the ratios of mRNAs of the HSP genes to those of the house-keeping gene would be relatively high in the heat-shocked cells, and utilization of the cells as a source of a cDNA library was attempted to isolate HSP genes as many as possible. As expected, multiple HSP genes were identified by arbitrarily isolating cDNAs from the RTG-2 cells (Table 2-1). Among the cloned genes, two cDNAs having different sequences were found in each of *Hsp90* and *Hsc70*. These were suggested to be paralogous genes as in the case of rainbow trout *Hsp70*s in Chapter I. Although only one clone could be identified each for the other HSP family genes in the present study, each may have a possible paralog because it has been inferred that most of the rainbow trout genes are duplicated (Ohno *et al.*, 1968; Palti *et al.*, 2004).

Sequence analyses of the two Hsp90 cDNAs showed that both deduced proteins were highly homologous to Atlantic salmon Hsp90β. In vertebrates, two major Hsp90 isoforms are present in the cytosol, namely, Hsp90α and Hsp90β (Sreedhar *et al.*, 2004). Hsp90α is highly heat-inducible, whereas Hsp90β is expressed constitutively at a high level at normal temperatures and its expression is weakly dependent on heat shock (Scheibel and Buchner, 1997). The two Hsp90 genes in the present study, designated *Hsp90βa* and *Hsp90βb*, were constitutively expressed in RTG-2 cells irrespective of heat-shock treatment (Fig. 2-1 and Table 2-5), suggesting that both genes actually encode a β isoform of Hsp90. On the other hand, Sathiyaa *et al.* (2001) isolated a partial fragment of rainbow trout Hsp90 cDNA, showing that its transcripts were induced by heat shock in hepatocytes as revealed by Northern blot analysis. The reported partial sequence is not identical to the Hsp90s in the present study, raising the possibility that there exists at least one α isoform of Hsp90 besides

the two β isoforms in rainbow trout. This possibility is also supported by the result that an α isoform of Hsp90 has been isolated from other salmonid fish, chinook salmon *Oncorhynchus tshawytscha* (Palmisano *et al.*, 1999).

In contrast to other HSP family members examined, the accumulation levels of Hsp70 mRNAs were dramatically increased after heat shock (Fig. 2-1 and Table 2-5). In Northern blot analysis (Fig. 2-1), two mRNA species having different sizes were detected in heat-shocked cells irrespective of *Hsp70a* and *Hsp70b* as described in Chapter I. Since nearly the same regions were detected for the two genes in Northern blot and real-time RT-PCR analyses, it is inferred that the accumulation levels of Hsp70 mRNAs in the latter analysis were those of integrated values of the two mRNA species detected in the former analysis. In any case, given the magnitude of the changes in the mRNA levels, it is strongly suggested that *Hsp70*s are the most useful biomarkers of heat stress among HSP family members in fish, at least in rainbow trout.

The study in this chapter demonstrated that there exist two *Hsc70*s in rainbow trout cells like *Hsp70*s in Chapter I. Zafarullah *et al.* (1992) isolated a genomic clone encoding Hsc70 from rainbow trout and designated it as *Hsc71*, which corresponds to the *Hsc70a* in this chapter (Table 2-2). Hsc70 is a member of the HSP70 family and exhibits constitutive expression (Hightower and Leung, 1997). It has been reported that Hsc71 mRNA levels did not significantly increase after heat shock in RTG-2 cells (Zafarullah *et al.*, 1992), and similar results were obtained by Northern blot analysis in Chapter I. However, by using quantitative RT-PCR analysis, it was demonstrated that the accumulation levels of both Hsc70a and Hsc70b mRNAs were significantly increased after heat shock in the present study (Table 2-5). Considering that several putative HSEs have been found in the promoter region of *Hsc71* (Zafarullah *et al.*, 1992), it is likely that there exist functional HSEs for the rainbow trout *Hsc70*s. Furthermore, since the mRNA accumulation levels in heat-shocked cells differed between *Hsc70a* and *Hsc70b*, the two genes may have promoters with different activities.

Cct8, one of the subunits of the CCT complex abundant in eukaryotic cytosol, is a member of the chaperonin/HSP60 family (Kubota and Willison, 1997). Until now, the heat shock inducibility of CCT subunits has not been investigated in fish. In rainbow trout RTG-2 cells, quantitative RT-PCR analysis showed that there was no significant increase in the Cct8

mRNA levels after heat shock (Table 2-5). Likewise, it has been reported that no significant increase of CCT subunits was detected in response to heat shock at protein levels in both HeLa and mouse BALB/3T3 cells (Kubota *et al.*, 1999). However, as discussed by Kubota *et al.* (1999), CCT may be induced depending on the type of stress, cells, organisms and other environmental conditions.

In RTG-2 cells, the mRNA accumulation levels of *Hsp47* were significantly increased after heat shock (Table 2-5). It is known that *Hsp47* expression is induced by heat shock at the transcriptional level in zebrafish embryos (Pearson *et al.*, 1996) and chicken embryonic fibroblasts (Hirayoshi *et al.*, 1991). In vertebrate cells, Hsp47 is located in the lumen of ER (Nagata, 1997), as is BiP/Grp78 (Gething, 1997b). In rainbow trout, however, the cellular localizations of Hsp47 and Grp78 have not been determined. Considering that rainbow trout Hsp47 and Grp78 had ER retention signals (data not shown), it is suggested that both proteins are located in the ER lumen like their counterparts from other vertebrates. In addition, consistent with mammalian cells (Gething, 1997b), the results in this chapter indicate that rainbow trout Grp78 mRNA was constitutively expressed in unstressed cells (Fig. 2-1 and Table 2-5).

The enhanced synthesis has been observed with polypeptides having 100, 87, 70, 68, 60, 39, and 27 kDa in RTG-2 cells exposed to heat shock at 28 °C (Mosser *et al.*, 1986). Under the same heat-shock conditions, the enhanced syntheses of a similar set of HSPs have also been found by the other research group (Kothary and Candido, 1982), although there were slight differences in the polypeptide size together with an additional polypeptide having 32 kDa. Judging from the molecular sizes, it is thought that the above-mentioned polypeptides belong to either of the HSP100, HSP90, HSP70, HSP60, HSP40, and HSP27 families. Among these, the genes encoding the HSP90, HSP70, HSP60, and HSP40 families were cloned in this chapter. Unfortunately, however, cDNAs encoding HSP100 and HSP27 families could not be found in 200 cDNAs arbitrarily isolated in the present study.

CHAPTER III   CLONING AND CHARACTERIZATION OF HSF1

The expression of HSP genes is regulated by heat-shock transcription factors (HSFs) that bind to a specific *cis*-acting element, namely, HSE (Morimoto, 1998; Pirkkala *et al.*, 2001; Wu, 1995). In vertebrates, genes encoding four types of HSFs, HSF1–HSF4, have been cloned. Among the HSF family members, HSF1 is the principal transcriptional factor activated by exposure to stresses such as heat shock, and this protein is known to form homotrimers that bind DNA (Morimoto, 1998; Pirkkala *et al.*, 2001; Wu, 1995).

During evolution, fish have adapted to live in various ambient temperatures. Reflecting such adaptations, the threshold temperature for HSP induction differs between cold- and warm-adapted fish. For example, HSPs are induced in the 26–30 °C range in rainbow trout RTG-2 cells (Mosser *et al.*, 1986), whereas HSP70 is induced in the 35–37 °C range in zebrafish tissues (Råbergh *et al.*, 2000). However, little is known about the molecular mechanisms underlying the difference in HSP induction temperatures among fish species. To date, Råbergh *et al.* (2000) reported the isolation of one *HSF1* cDNA from zebrafish, which is a warm-adapted fish. Although a cDNA fragment encoding an HSF has also been cloned from bluegill sunfish *Lepomis machrochirus* (Råbergh *et al.*, 2000), a full-length HSF1 cDNA clone has not been isolated from any fish other than zebrafish. Some authors (Airaksinen *et al.*, 1998; Le Goff and Michel, 1999) have reported the presence of a protein that possesses HSF1-like activity in rainbow trout; however, a gene encoding HSF1 itself has not been identified in this cold-adapted fish.

Thus, the purpose of the study was to isolate a cDNA clone encoding rainbow trout HSF and to characterize its translation products. This chapter describes the existence of two distinct HSF1 isoforms in rainbow trout and the evidence for heterotrimer formation of these isoforms *in vitro*.

Section 1   cDNA cloning and genomic organization of HSF1

As a result of the preceding chapters, it was shown that some rainbow trout *HSP*s, especially *Hsp70*s, are induced at the transcriptional level by heat stress. This suggests that cells of rainbow trout have an HSF as do other vertebrates. Thus, to demonstrate the existence of the transcription factor, this section describes the cDNA cloning of rainbow trout HSF and its gene structure.

Materials and Methods

Cell culture and animals

RTG-2 cells were cultured at 15 °C as described in Section 2 of Chapter I. Rainbow trout (3 months old), which were used to extract total RNA for RT-PCR, were obtained from the Nikko Station of the National Research Institute of Fisheries Science (Tochigi Prefecture, Japan) and reared on a commercial diet at 15 °C.

cDNA cloning

A random primed λZAPII cDNA library was constructed by using a λZapII predigested *Eco*RI/CIAP-treated vector kit (Stratagene, La Jolla, CA, USA) with RNA isolated from RTG-2 cells as described below. Approximately 1.2×10^6 plaques were screened at 2×10^5 plaques per 140×100-mm plate by hybridization of duplicate nitrocellulose membranes with a 2.7-kb fragment of chicken HSF1 cDNA (Nakai and Morimoto, 1993) as a probe. The membranes were soaked in 2× SSC for 5 min, prehybridized at 42 °C for 2 h with hybridization buffer [6× SSC, 1× Denhardt's solution, 0.15% SDS, and 100 μg/ml denatured calf thymus DNA (Invitrogen)], and hybridized with a ^{32}P-labeled DNA probe in the same buffer at 42 °C for 16 h. The membranes were then rinsed twice with 2× SSC plus 0.1% SDS

at room temperature for 5 min per rinse, washed twice in 2× SSC plus 0.1% SDS at 50 °C for 20 min per wash, dried, and exposed to X-ray film for 2 days. Positive clones were isolated through three rounds of screening. Phagemid pBluescript SK (–) was excised from purified plaques with helper phage according to the manufacturer's instructions.

The 5'- and 3'- termini of rainbow trout HSF cDNAs were isolated by rapid amplification of cDNA ends (RACE). A directional cDNA library constructed in Section 1 of Chapter II was used as the PCR template. For 5'-RACE, the first PCR was performed with the M13 reverse primer (5'-AGCGGATAACAATTTCACACAGG-3') as a sense primer and a rainbow trout *HSF1*-specific primer (5'-ATCTTTCTCTTCATCCCCAGGACT-3') as an antisense primer. The nested PCR was performed with the T7 promoter primer (5'-TAATACGACTCACTATAGGG-3') as a sense primer and *HSF1*-specific primers (for *HSF1a*, 5'-TGCCTTTTATGTTCTGCACGA-3'; for *HSF1b*, 5'-CCTCCCTCCACAGAGCTTCA-3') as antisense primers. For 3'-RACE, the first PCR was performed with the M13 forward primer (5'-CCCAGTCACGACGTTGTAAAACG-3') as a sense primer and *HSF1*-specific primers (for *HSF1a*, 5'-GAAGCAGCTTGTCCAGTACACTAA-3'; for *HSF1b*, 5'-GAAGCAGCTGGTCCAGTACACCTC-3') as antisense primers. The nested PCR was performed with the SP6 promoter primer (5'-ATTTAGGTGACACTATA-3') as a sense primer and *HSF1*-specific primers (for *HSF1a*, 5'-CGGACCTCCCCACTCTGCTGGAGA-3'; for *HSF1b*, 5'-TCCCCACTCTGCTGGAGCTGGAGG-3') as antisense primers. The amplified products were subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA).

Sequence determination

Nucleotide sequences were determined from both strands by a 373A DNA sequencer (Applied Biosystems) and a Thermo Sequenase II Dye Terminator Cycle Sequencing kit (Amersham Biosciences).

Phylogenetic analysis

A phylogenetic tree was constructed as described in Section 1 of Chapter I. The setting parameters were as follows: MATRIX, BLOSUM; GAPOPEN, 10.0; GAPEXT, 0.05; GAPDIST, 8; MAXDIV, 40; ENDGAPS, off; NOPGAPS, off; NOHGAPS, off.

Southern blot analysis

Genomic DNA was isolated from RTG-2 cells by a GenomicPrep Cells and Tissue DNA Isolation kit (Amersham Biosciences) according to the manufacturer's instructions. Ten micrograms of genomic DNA was digested with *Bam*HI, *Eco*RI, or *Hin*dIII, resolved by electrophoresis on a 1% agarose gel, and transferred to a nylon membrane (Hybond N+; Amersham Biosciences). The membranes were hybridized for 1 h in PerfectHyb hybridization solution (Toyobo) with DIG-labeled DNA probes. As the probes, the 3'-UTRs of *HSF1a* and *HSF1b* were labeled by a PCR DIG Probes Synthesis kit (Roche Diagnostics). The probed regions of *HSF1a* and *HSF1b* correspond to nucleotides 1651–2027 and 1691–2052, respectively. The hybridized membranes were washed twice with 2× SSC plus 0.1% SDS at room temperature for 5 min, and then twice with 0.1× SSC plus 0.1% SDS at 68 °C for 15 min. The chemiluminescent detection of the probes was performed with a DIG luminescent detection kit (Roche Diagnostics) according to the manufacturer's instructions. The positive signals were detected by exposure on Hyperfilm-MP (Amersham Biosciences).

Results

By screening an RTG-2 cDNA library using a chicken HSF1 cDNA probe, two positive clones, named C1 and C2, were isolated. Sequence analysis revealed that these two clones encoded distinct isoforms of HSF. Clone C1 was a partial cDNA containing an insert of 983 nucleotides encoding the DNA-binding domain of HSF, whereas clone C2 contained an insert of 2771 nucleotides including introns and an ORF encoding 513 amino acids.

By using 5'- and 3'-RACE, the full-length cDNAs of clones C1 and C2 without introns were determined to be 2083 bp and 2142 bp, respectively. Clones C1 and C2 were predicted to encode proteins of 501 and 513 amino acids, respectively (Fig. 3-1). Phylogenetic analysis indicated that the two proteins belong to the HSF1 cluster (Fig. 3-2). Accordingly, clones C1 and C2 are referred to hereafter as rainbow trout HSF1a and HSF1b, respectively.

The sequence identity between the two predicted proteins was 86.4% (Table 3-1). By contrast, the whole ORF of the rainbow trout HSF1s showed low homology to those of other vertebrate HSF1s. For example, rainbow trout HSF1a and HSF1b showed 55.3% and 56.4% identity to human HSF1, respectively (Table 3-1).

Next, the structural features of HSF1a and HSF1b were examined in comparison to those of other vertebrate HSF1s. HSF1 has been reported to contain conserved regions referred to as the DNA-binding domain (DBD), and the amino-terminal and carboxyl-terminal hydrophobic heptad repeats (HR-A/B and HR-C, respectively) (Morimoto, 1998; Pirkkala *et al.*, 2001; Wu, 1995). Multiple sequence alignment demonstrated that both of the rainbow trout HSF1s contained these conserved domain structures (Fig. 3-1), as shown schematically in Fig. 3-3.

Region I (DBD) of the rainbow trout HSF1s showed high similarity to the corresponding region of zebrafish, chicken, and human HSF1 (Table 3-1); for instance, DBD of rainbow trout HSF1b shared 90.7% identity with that of human HSF1. By contrast, regions II (HR-A/B) and IV (HR-C) of the rainbow trout HSF1s showed less similarity to the corresponding regions of other vertebrate HSF1s (Table 3-1). However, the actual heptad repeats of hydrophobic amino acids are conserved across the whole HSF1 family (Fig. 3-1). In addition, two KRK tripeptides, which are conserved among characterized HSF1 family

members, were identified in both of the rainbow trout HSF1s (Fig. 3-1). In contrast to the highly conserved regions described above, regions III and V of rainbow trout HSF1s showed low similarity to the counterparts of other vertebrate HSF1s (Table 3-1). Notably, region V showed low similarity across the HSF1 family, even between rainbow trout HSF1a and HSF1b (78.8% identity; Table 3-1).

```
                                      DBD
1  MFFHGGGSGGVVVS  GSNVPAFLVKLWTLIEDPDTDPLICWSPNGNSFHVFDQGQFSKDVIPRYFKHSNMTSFVRQLNMYGFRKVVHIE  rtHSF1a
1  ....V...V.....  .N..I....I.....V....................CR...E...K....N..A...................  rtHSF1b
1  ..Y.SV.P.....T-.N..I....I.....V................T.......R...E...K....N..A...................  zHSF1
1  ..GP.AAAAA.GAGPG....S...T....V...E.........S...........A.E...K....N..A...................  cHSF1
1  .DLAV.PGAA-----GP...I....I.....VS.....A.......S...........A.E...K....N..A...................  mHSF1
1  .DLPV.PGAA-----GP...I....VS.....A.......S...........A.E...K....N..A...................  hHSF1

                                    HR-A/B
89  QGGLVKPEKDDMEFQHPYFIRGQEPLLENIKRKVTNVSNAKHEDLKMSSDDVSKILTNVQNIKGKQETIDSQIIAMKHENETLWREVASL  rtHSF1a
89  ...........T......I....H.............V..DE.........H.................A........  rtHSF1b
89  ...........T............Q...........T..I....Y.F.T....MISD..HM.....SM..K.STL.....M.......T.  zHSF1
91  ...........I...........H...........S..SI.N..I.VRQ.N.T.L..DI.VM.....SM..KL.......A........  cHSF1
86  ........R..T.....C.L....Q..........S..TL.S..I.IRQ.S.TRL..D..LM.....CM..KLL.......A........  mHSF1
86  ........R..T.....C.L....Q..........S..TL.S..I.IRQ.S.T.L..D..LM.....CM..KLL.......A........  hHSF1

179  RQKHVQQQKVVNKLIQFLVTLVQSNRVLGVKRKIPLMLNDSSSAHSMPKYSRQYSLEHLQAS----SSISPSGSSFTSTGLFTAESSLNN  rtHSF1a
179  ....A.........................................L..F.........LQGSPA..A..AP..N.......P....  rtHSF1b
179  ....S..............I..AR......V...M..................F.......----------.PA..STA..G..V.SP.PPVKT  zHSF1
181  ....A.............IS.......I..V...................VH----GS.PYAA.SPAYSGSNIYSPD..I.S  cHSF1
176  ....A.............IS.......I..V.......S..N....V...G.......VH--GPGPY.APSPAYS.SS.YSSDAVTSS  mHSF1
176  ....A.............IS.......I..V.......G...........F....VH--GSGPY.APSPAYS.SS.YAPDAVASS  hHSF1

265  GPIISDVTDLAQASHVEVTNEWMEES  TSPLVHIKEEPSSHV  LEEVCPAEVVIG GAGVQVDTPLSPINFINSILQESNEPNAA  rtHSF1a
269  ...........P.......I.......P.-.........A.............I.F.........S  rtHSF1b
259  ......I.E...S.P.A .D..I.DR  .........PAHSPEV.....V..EV.A.SDLP.......TT.........EPVS.L  zHSF1
267  ........E...S.PSASPSGSLD.R---S..V.R.....P.PSRSPK-------------------------------.NEPS---  cHSF1
262  ......I.F..PT.PLASPGRSID.RPISS.T..RV.Q..P.PPHSPRVI.AS.--------.RPSSM.......TA..D...R..FPTP..  mHSF1
262  ......I.E..P..PMASPGGSID.RPLSS....RV....P.PPQSPRV..AS.--------.RPSS...L...TAI.D...R..EPAP.S  hHSF1

                                     HR-C
347  ............STPTTPTEQKCLSLACLDK-SEISDHLDSIDNGLENLQTILNAQSINFESSPFFEFFSSSLPGSEFDL--  rtHSF1a
351  ...........PA.................CR................................t..I.........  rtHSF1b
345  .........TP..S.......V......T..H...E...S......Q.........D...L.DI...A--A.DVG.--  zHSF1
317  --------TTTAAAGN..EQPQPQE....V......-N..N....T..SN.D....M.STHGFSVDTTALLDL..P.MTVIDMN.PDL  cHSF1
345  SNTAPMDTTGAQAPAL..PS.PE.....V......N.......AM.SN.D....M.TSHGFSVDT.ALLD.  mHSF1
345  VTALTDARGHTDTEGRPP.P.P.S.PE.....V......-N.......AM.SN.D....M.SSHGFSVDT.ALLDL..P.VTVPDMS.PDL  hHSF1

414  -FSLDSIQDLLSSDPPKGAERSDNY--TAGKQLVQYTNQPMLLTDPLTNENGGADLPTLLE--GDFYFSQD--PEEEPTISLTS---DIA  rtHSF1a
420  -..F.C.......S.........C--.-......S......................LE..S..T..QD...D.....LTTDYHT.  rtHSF1b
408  -D..A.......P..V.FT.SGIDT--DS........S..IV.P...STDSSST...M...LQD.S...SE---.A.D...A.LNLQ---P  zHSF1
395  DS..A........QEQQKPSEA.AAAADT.....H..A..LF.V.SSAVDV.SG...IFF.LGEGS..TDGDEYN.D.....L.GT-EQP  cHSF1
413  -------.E...PQE.PRPIEAE.SNPDS.....H..A..LF.L..DAVDT.SSE..V.F.LGESS...EGDDYTDD.....LTGT-EPH  mHSF1
433  DS..A...E...PQE.PRPPEAE.SSPDS.....H..A..LF.L..GSVDT.SN...V.F.LGEGS...EGDGFA.D.....LTGS-EPP  hHSF1

494  APDKPKLS  rtHSF1a
506  .....E..  rtHSF1b
490  V.FD....  zHSF1
484  K.KD.IV.  cHSF1
496  KAKD.IV.  mHSF1
522  KAKD.IV.  hHSF1
```
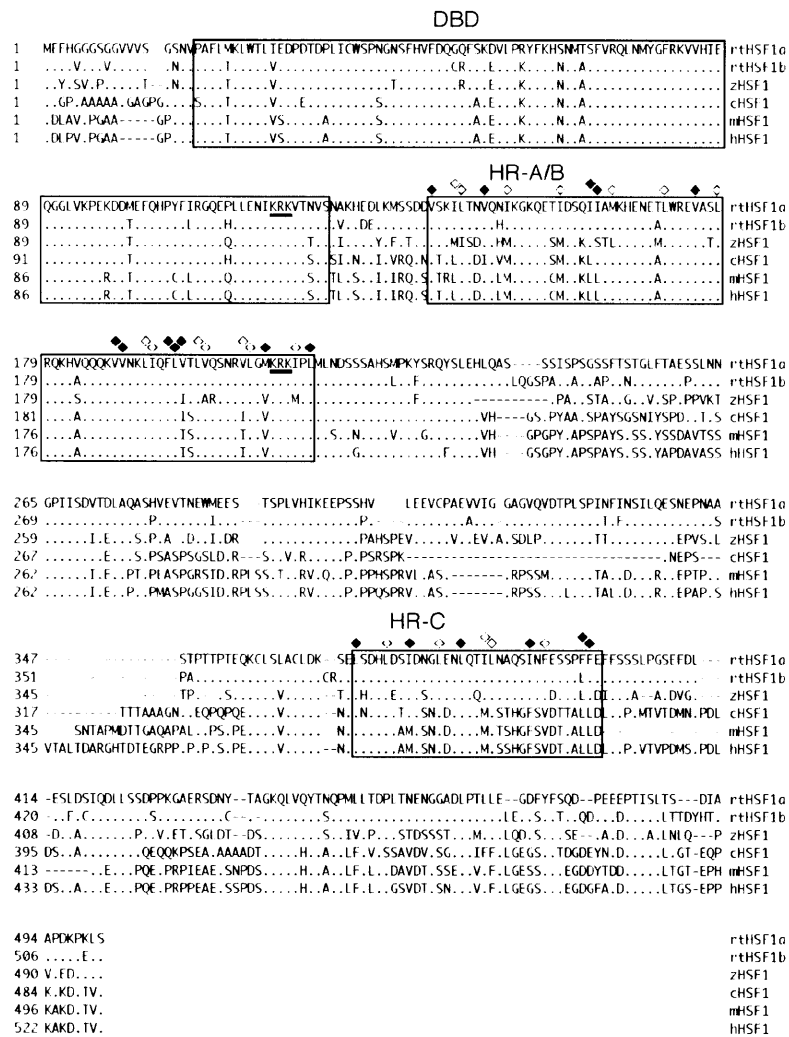
Fig. 3-1. Comparison of the predicted amino acid sequences of rainbow trout (rt) HSF1a and HSF1b with the sequences of zebrafish (z), chicken (c), mouse (m) and human (h) HSF1. The three domain structures, DBD, HR-A/B and HR-C, are boxed. Open and filled diamonds indicate the repeats of hydrophobic amino acids. The underlined KRK tripeptides are putative nuclear localization signals (NLSs). The numbers on the left indicate the amino acid positions of each protein.

The genomic organization of the rainbow trout *HSF1*s were examined by Southern blot analysis using the 3'-UTRs of *HSF1a* and *HSF1b* as probes. These two probes showed different hybridized patterns (Fig. 3-4), demonstrating that each HSF1 is encoded by a distinct gene in the genome.
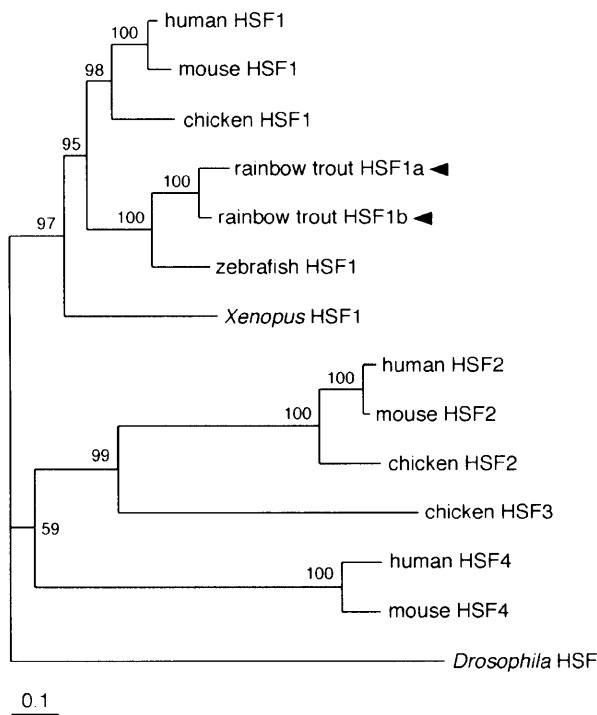

Fig. 3-2. Phylogenetic tree of the vertebrate HSF family based on the amino acid sequences. The tree was calculated by neighbor joining, with *Drosophila* HSF used as an outgroup. Arrowheads indicate the position of rainbow trout HSF1a and HSF1b. Numbers at the nodes indicate the percentage of bootstrap values (1000 replicates). The scale bar refers to a phylogenetic distance of 0.1 amino acid substitutions per site. GenBank accession numbers for the sequences are as follows: human HSF1 (M64673), HSF2 (M65217), HSF4 (D87673); mouse HSF1 (X61753), HSF2 (X61754), HSF4 (AB029350); chicken HSF1 (L06098), HSF2 (L06125), HSF3 (L06126); *Xenopus* HSF1 (L36924); zebrafish HSF1 (AB062117); rainbow trout HSF1a (AB062548), HSF1b (AB062549); *Drosophila* HSF (M60070).

Fig. 3-3. Schematic representation of HSF1 domain structures. The three regions of identity are denoted region I, corresponding to DBD; region II, corresponding to the amino-terminal hydrophobic heptad repeat (HR-A/B); and region IV, corresponding to the carboxyl-terminal hydrophobic heptad repeat (HR-C). Regions III and V roughly correspond to domains of mammalian HSF1, namely, the regulatory and transactivation domains, respectively.

Table 3-1. Comparison of rainbow trout HSF1s with other vertebrate HSF1

Species [a]	Percentage identity (%) [b]					
	ORF	I [c]	II [c]	III [c]	IV [c]	V [c]
rtHSF1a	100	100	100	100	100	100
rtHSF1b	86.4	89.8	96.0	83.9	97.0	78.8
zHSF1	68.4	89.8	73.3	61.6	78.8	50.5
cHSF1	55.7	88.0	78.7	40.3	39.4	40.0
hHSF1	55.3	85.2	78.7	41.8	42.4	39.1
rtHSF1b	100	100	100	100	100	100
zHSF1	69.5	95.4	74.7	59.9	81.8	52.1
cHSF1	55.9	92.6	81.3	38.0	42.4	40.2
hHSF1	56.4	90.7	81.3	40.0	48.5	41.7

[a] rt, rainbow trout; z, zebrafish; c, chicken; h, human.

[b] The percentage amino acid identity was calculated by the ALIGN program in LASERGENE software.

[c] The five regions, I–V, are indicated in Fig. 3-3.
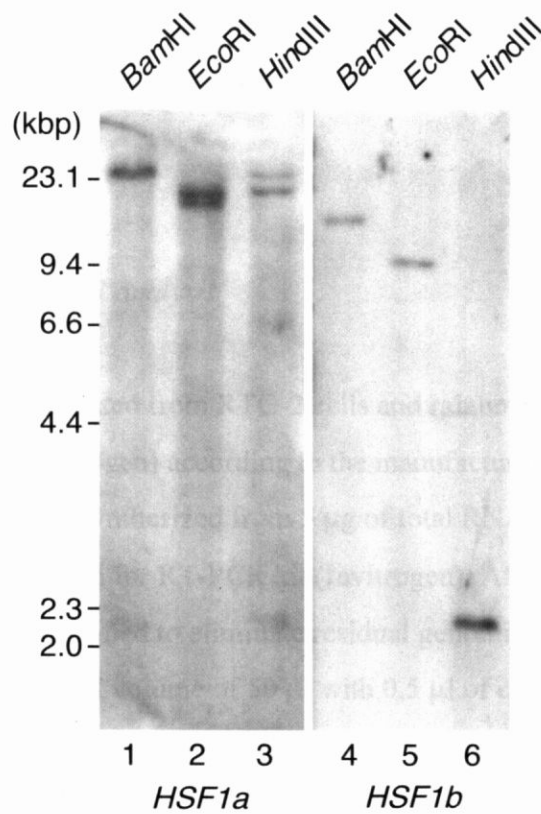
Fig. 3-4. Genomic Southern blot analysis of rainbow trout *HSF1*s. In the left panel, hybrization was carried out with a DIG-labeled *HSF1a* probe (a 377-base fragment of the 3'-UTR of *HSF1a* cDNA), whereas in the right panel, hybrization was carried out with a DIG-labeled *HSF1b* probe (a 362-base fragment of the 3'-UTR of *HSF1b* cDNA). λ DNAs digested with *Hin*dIII were used as molecular markers and are indicated on the left.

Section 2   mRNA expression pattern of HSF1 in various tissues

In the preceding section, it was shown that two distinct genes encoding HSF1 are present in rainbow trout cells. This raises the question that the two genes may have distinct expression patterns in various tissues. To answer this question, this section describes the expression of HSF1 mRNAs in various tissues of rainbow trout.

Materials and Methods

Isolation of RNA and RT-PCR analysis

Total RNA was isolated from RTG-2 cells and rainbow trout (6 months old) tissues with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 5 µg of total RNA by using a SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen). After reverse transcription, DNase I digestion was performed to eliminate residual genomic DNA from the RNA samples. PCR was carried out in a total volume of 50 µl with 0.5 µl of cDNA synthesis mixture containing HotStarTaq DNA Polymerase (Qiagen) in an automated thermal cycler (model 2400; PerkinElmer, Wellesley, MA, USA). PCR consisted of one initiation cycle of 15 min at 95 °C, amplification cycles of 0.5 min at 94 °C, 0.5 min at 50 °C and 0.5 min at 72 °C, and one termination cycle of 1 min at 72 °C, with 35 cycles in total for *HSF1a* and *HSF1b* and 30 for *Hsc70*. Hsc70 cDNA was amplified as a positive control, because Hsc70 mRNA is constitutively expressed in different rainbow trout tissues (Zafarullah *et al.*, 1992) and in RTG-2 cells as shown in the preceding chapters. The oligonucleotide primers were as follows: *HSF1a* forward, 5'-GAAGCAGCTTGTCCAGTACACCAA-3'; *HSF1a* reverse, 5'-TTCCAAGAGCTGAACAAACCATTG-3'; *HSF1b* forward, 5'-GAAGCAGCTGGTCCAGTACACCTC-3'; *HSF1b* reverse, 5'-GGCTGAATAAAC CATGCCAGTAGC-3'; *Hsc70* forward, 5'-ACATCAGCGACAACAAGAGG-3'; *Hsc70* reverse, 5'-AGCAGGTCCTGGACATTCTC-3'. The amplified products were visualized by

ethidium bromide staining.

Results

To determine whether the two *HSF1*s cloned from RTG-2 cells are actually transcribed in rainbow trout, RT-PCR was used to analyze total RNA isolated from unstressed RTG-2 cells and rainbow trout tissues. As a positive control, rainbow trout Hsc70 cDNA was analyzed. The PCR products were predicted to be 423 bp for *HSF1a*, 439 bp for *HSF1b* and 421 bp for *Hsc70*, and bands corresponding to these sizes were amplified (Fig. 3-5). *HSF1a* and *HSF1b* transcripts were both detected in all rainbow trout tissues examined, as well as in RTG-2 cells. These bands were not due to contamination by genomic DNA because no bands were amplified in the negative control reactions in which total RNA was used as the template without reverse transcription (data not shown). Taken together, these results demonstrate that the *HSF1a* and *HSF1b* mRNAs are coexpressed in unstressed rainbow trout cells without tissue specificity.
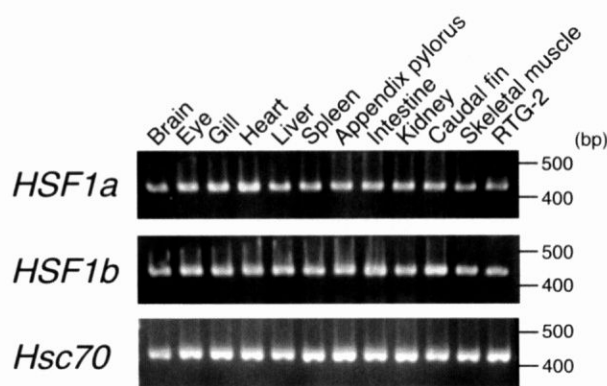
Fig. 3-5. RT-PCR analysis of the *HSF1a* and *HSF1b* genes in rainbow trout RTG-2 cells and tissues. Rainbow trout *Hsc70* was subjected to RT-PCR analysis as a positive control. Molecular size markers are indicated on the right (in base pairs).

Section 3   Construction of HSF1 expression vectors

In the preceding section, it was shown that two rainbow trout *HSF1*s are coexpressed in all tissues examined. To further characterize their function, the proteins themselves are required. To this end, this section describes the construction of plasmid vectors that express epitope-tagged HSF1s, and confirmation of the protein expression.

Materials and Methods

Plasmids

To distinguish between HSF1a and HSF1b in the following experiments, hemagglutinin (HA) tagged HSF1a (HSF1a–HA) and Protein C tagged HSF1b (HSF1b–Protein C) were constructed. The coding regions of both HSF1 cDNAs were amplified with the specific PCR primers possessing a *Hin*dIII or *Not*I restriction enzyme site. The primers were as follows: *HSF1a* forward, 5'-CCCAAGCTTGATATGGAGTTCCACGGTGG-3'; *HSF1a* reverse, 5'-TATGCGGCCGCGAGGATAATTTGGGCTTGTCTG G-3'; *HSF1b* forward, 5'-CCCAAGCTTGATAATGGAGTTTCACGTTGG-3'; *HSF1b* reverse, 5'-TATGCGGCCGCGGATAGTTCGGGCTTGTCTGG-3'. PCR was carried out in a total volume of 50 µl with KOD-Plus-DNA Polymerase (Toyobo) using 1 µl of the plasmid RTG-2 cDNA library described in Section 1 of Chapter II as the template. PCR consisted of one initiation cycle of 2 min at 94 °C, amplification cycles of 0.25 min at 94 °C, 0.5 min at 53.6 °C and 1.5 min at 68 °C, and one termination cycle of 1 min at 68 °C, with 37 cycles in total. The C terminus of HSF1a was fused to an HA epitope tag in plasmid pMH (Roche Diagnostics) at *Hin*dIII and *Not*I restriction enzyme sites. Likewise, the C terminus of HSF1b was fused to a Protein C epitope tag in plasmid pMX (Roche Diagnostics). In control experiments, pHMlacZ and pXMlacZ (Roche Diagnostics), which contain the β-galactosidase gene cloned in-frame with an N-terminal tag of either HA or Protein C, were used.

Coupled in vitro transcription and translation

Coupled *in vitro* transcription/translation was performed with a TNT Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's instructions. For the reaction, 1 μg each of the plasmids described above was used as a template in a 50-μl reaction mixture.

Western blot analysis

Ten microliters of *in vitro* translated products were separated by SDS–PAGE on 10% gels and transferred to poly(vinylidene difluoride) (PVDF) membranes (Hybond-P; Amersham Biosciences) by electrophoretic transfer. The membranes were blocked with Tris-buffered saline containing 5% skim milk for 1 h at room temperature. Antibodies against HA or Protein C (Roche Diagnostics) were used to detect epitope-tagged proteins at a working concentration of 1 μg/ml each. Incubation and washing procedures for these antibodies were performed according to the manufacturer's instructions. An ECL Western blotting analysis system (Amersham Biosciences) was used to detect the epitope-tagged proteins. Positive signals were detected by exposure on Hyperfilm-ECL (Amersham Biosciences).

Results

A coupled *in vitro* transcription/translation assay was performed for the two HSF1s using cDNAs encoding epitope-tagged HSF1 (HSF1a–HA and HSF1b–Protein C) to check for protein expression. As positive controls, cDNAs of epitope-tagged β-galactosidase (HA- and Protein C-βgal) were translated. The reaction mixtures were subjected to Western blotting,

and the translated products were detected by antibodies against the epitope tags. Specific translation products were detected in lanes containing those from the HSF1 expression vectors (Fig. 3-6, lanes 3 and 6). From their migration on the gel, the apparent molecular masses of HSF1a–HA and HSF1b–Protein C were estimated to be 70 kDa and 72 kDa, respectively (Fig. 3-6, lanes 3 and 6, respectively). These sizes were, however, larger than the expected molecular masses of 57 200 Da for HSF1a–HA and 58 590 Da for HSF1b–Protein C calculated from the predicted amino acid sequences.
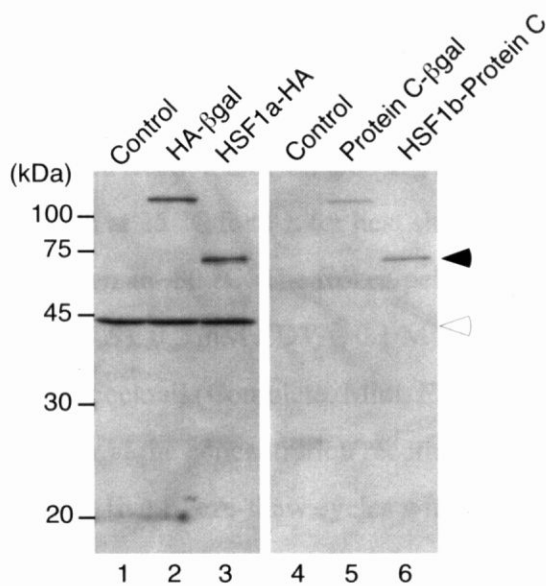
Fig. 3-6. Western blot analysis of *in vitro* translated expression vectors. Filled arrowhead indicates the position of the epitope-tagged rainbow trout HSF1a and HSF1b (lanes 3 and 6, respectively); open arrowhead indicates nonspecific bands (lanes 1–3). The TNT Quick Master Mix (Promega) used for *in vitro* translations was analyzed as a negative control (lanes 1 and 4), and vectors encoding epitope-tagged β-galactosidase (HA–βgal or Protein C–βgal) were translated *in vitro* as positive controls (lanes 2 and 5).

Section 4   DNA-binding ability of HSF1

In the preceding section, epitope-tagged HSF1s were successfully synthesized *in vitro*. It is known that specific binding to HSE in the promoter of *HSP*s is a common feature of mammalian and avian HSF1. Do HSF1s of rainbow trout have HSE-binding ability as in the case of other vertebrates? By using the *in vitro* translated HSF1s, this section describes the sequence-specific DNA binding ability of rainbow trout HSF1s.

Materials and Methods

Preparation of whole cell extracts

RTG-2 cells were cultured in a 100-mm dish (Asahi Techno Glass, Funabashi, Japan) at 15 °C as described in Section 2 of Chapter I. The dishes were sealed with Parafilm and immersed in a water bath at 25 °C for 1 h for heat shock. The cells were harvested, centrifuged, and rapidly frozen at −80 °C. The frozen pellets were suspended in extraction buffer [20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.1 M KCl, 1 mM dithiothreitol, 20% glycerol]. Protease inhibitor cocktail (Complete, Mini, EDTA-free; Roche Diagnostics) was added to the extraction buffer at the concentration recommended by the manufacturer. The pellets were homogenized by five freeze-thaw cycles with liquid nitrogen and pipetting. The homogenates were centrifuged at 10 000 *g* at 4 °C for 5 min. The supernatants were collected, and the protein concentrations were measured by a Protein Assay kit (Bio-Rad Laboratories).

Electrophoretic mobility shift assay (EMSA)

The DNA-binding ability of HSF1 was analyzed by EMSA as described previously (Mosser *et al.*, 1988) with the following modifications. The *in vitro* translated products and the whole-cell extracts from RTG-2 cells were used as the protein samples. Binding reaction

mixtures were incubated for 30 min on ice. Gels were run at 4 °C for 3 h at 150 V, dried, and

exposed on Hyperfilm-MP (Amersham Biosciences). A double-stranded synthetic HSE,

which contains four inverted nGAAn repeats (5'-tcgactaGAAgcTTCtaGAAgcTTCtag-3'),

was used as a probe and a competitor. The probe was end-labeled with [^{32}P]dCTP by the

Klenow fragment of DNA polymerase I. For competition experiments, a 50-fold molar excess

of unlabeled HSE oligonucleotides was added to the binding reaction mixtures.

Results

The DNA-binding ability of each HSF1 was examined by EMSA using the *in vitro*

translated proteins, and gel shift bands were observed in the lanes containing epitope-tagged

HSF1 (Fig. 3-7, lanes 5 and 8). The bands were detected at a position corresponding to the

gel-shift band of heat-shocked RTG-2 cell extract (Fig. 3-7, lane 2), and were abolished by

the addition of excess unlabeled HSE probe (Fig. 3-7, lanes 6 and 9). Moreover, these bands

were not detected in the lanes containing epitope-tagged β-galactosidase (Fig. 3-7, lanes 4 and

7). This means that the gel-shift bands were not due to a factor endogenous to the *in vitro*

translation mixture or to the epitope tags. Taken together, these results demonstrate that *in*

vitro translated HSF1a and HSF1b, as well as endogenous HSF1 in RTG-2 cells, bind

specifically to HSE consensus sequences.
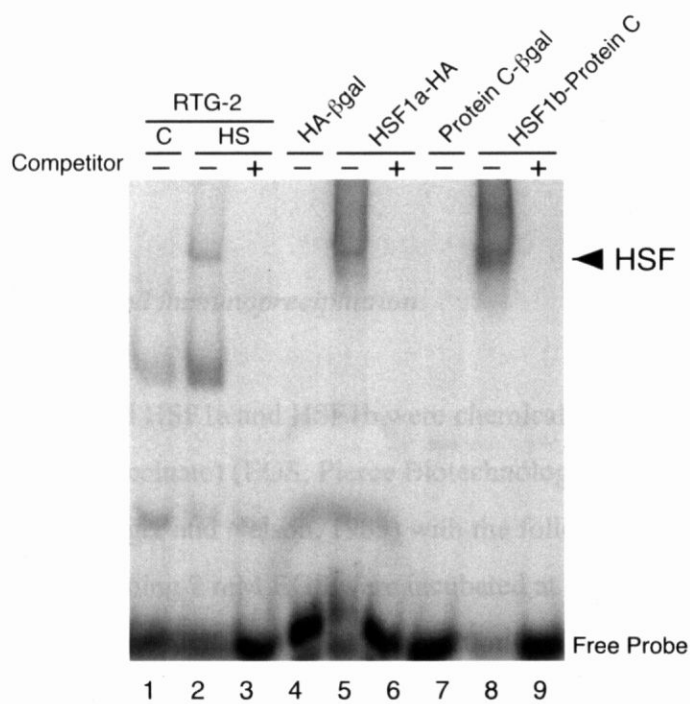
Fig. 3-7. EMSA of endogenous rainbow trout HSF1 and *in vitro* translated HSF1a and HSF1b. Unlabeled HSE oligonucleotides were used as a competitor and added to the binding reaction mixtures as indicated. RTG-2 cells were cultured at 15 °C (C) and heat shocked at 25 °C for 1 h (HS). *In vitro* translated HA–βgal and Protein C–βgal were used as negative controls.

Section 5   Oligomeric state of HSF1

In the preceding section, it was demonstrated that HSF1a and HSF1b both could bind to HSE consensus sequence. It is known that mammalian and avian HSF1 binds to HSE as a homotrimer; however, rainbow trout has two HSF1 isoforms. This raises the question whether the two HSF1s form a heterotrimer or not. Thus, this section describes the analysis of oligomeric state of rainbow trout HSF1 by using the differentially epitope-tagged proteins.

Materials and Methods

Chemical cross-linking and immunoprecipitation

In vitro translated HSF1a and HSF1b were chemically cross-linked using ethylene glycol bis(succinimidylsuccinate) (EGS; Pierce Biotechnology, Rockford, IL, USA) as described previously (Sorger and Nelson, 1989) with the following modifications. *In vitro* translated products containing 2 mM EGS were incubated at 22 °C for 30 min and then quenched by adding glycine to 50 mM at 22 °C for 20 min. The samples were immunoprecipitated with anti-HA or anti-Protein C Affinity Matrix (Roche Diagnostics) according to the manufacturer's instructions. The immunoprecipitates were separated by SDS–PAGE on 6% gels. The HSF1a–HA and the HSF1b–Protein C were detected by Western blot analysis using anti-HA and anti-Protein C Ig (Roche Diagnostics), respectively, as described in Section 3 of this chapter.

Results

To investigate whether rainbow trout HSF1s form oligomeric structures, chemical cross-linking with EGS, followed by immunoprecipitation with antibodies specific for the epitope tags, was performed. The immunoprecipitated proteins were analyzed by Western

blotting. In this experiment, three *in vitro* translated products were analyzed: HSF1a–HA, HSF1b–Protein C, and a mixture of both HSF1a–HA and HSF1b–Protein C.

Fig. 3-8A shows the immunoprecipitated proteins probed with anti-HA Ig. When the cross-linked proteins were immunoprecipitated with anti-HA Ig, two bands were detected in the lanes containing HSF1a–HA (Fig. 3-8A, lanes 1 and 3). The apparent molecular masses of the bands were ≈ 200 kDa and ≈ 70 kDa. These molecular masses correspond to the sizes of an HSF1 trimer and monomer, respectively. This result therefore suggests that the 200- and 70-kDa products are cross-linked trimers and monomers of HSF1a–HA, respectively. Moreover, when the same cross-linked proteins were immunoprecipitated with anti-Protein C Ig, two similar bands were detected in the lane containing both HSF1a–HA and HSF1b–Protein C (Fig. 3-8A, lane 6). This suggests that the ≈ 200-kDa product is a cross-linked HSF1 trimer containing both HA and Protein C epitope tags, i.e. an HSF1 heterotrimer. Because the ≈ 70-kDa product is an HSF1a–HA monomer that coimmunoprecipitated with HSF1b–Protein C, this provides evidence that the two isoforms interact with each other. By contrast, no bands were observed in the lanes containing only HSF1a–HA or HSF1b–Protein C (Fig. 3-8A, lanes 4 and 5), verifying that the epitope tags were not interacting with themselves. These results therefore indicate that HSF1a and HSF1b interact with each other and form heterotrimers.

To confirm further the above results, the same immunoprecipitated proteins were probed with anti-Protein C Ig by using a replica membrane from the Western blotting (Fig. 3-8B). This analysis indicated that HSF1b also formed homotrimers and heterotrimers with HSF1a. In addition, a band corresponding to an HSF1b dimer was detected (Fig. 3-8B, lanes 5 and 6). Taken together, these results demonstrate that rainbow trout HSF1s form homo- and heterotrimers *in vitro*.
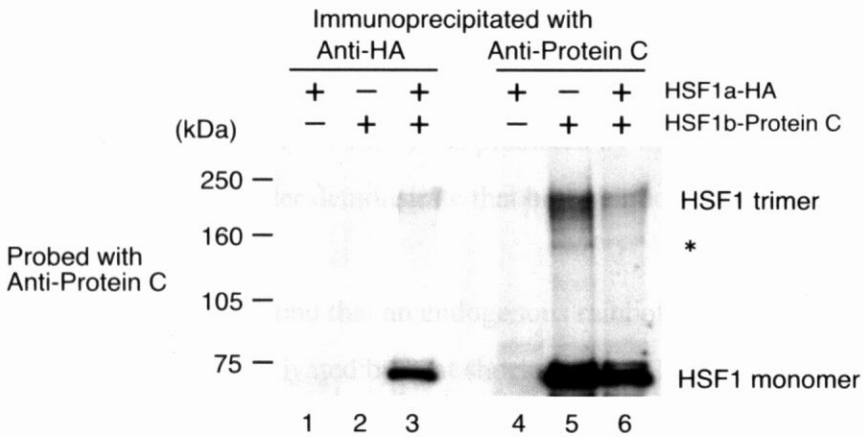
A
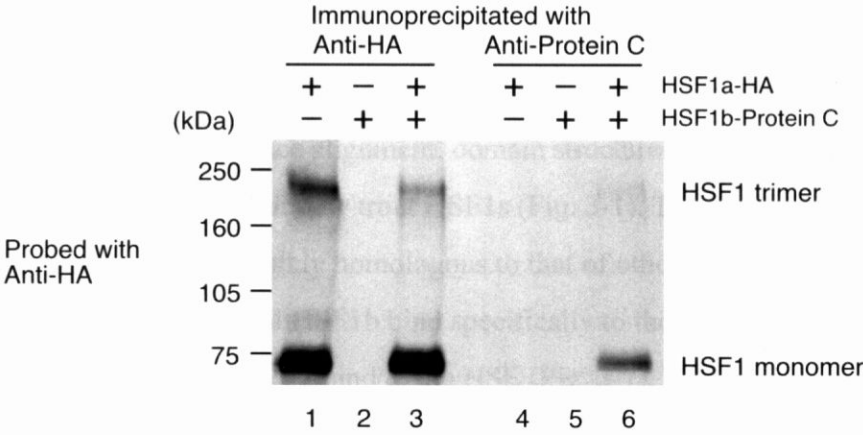
B

Fig. 3-8. Chemical cross-linking and immunoprecipitation. *In vitro* translated products containing either HSF1a or HSF1b, or both, were cross-linked using EGS and immunoprecipitated with anti-HA or anti-Protein C Ig. The immunoprecipitates were analyzed by Western blotting using antibodies against HA (A) or Protein C (B). Molecular mass markers are indicated on the left (in kDa). The asterisk indicates the band corresponding to a HSF1b dimer.

Section 6   Discussion

In this chapter, it was demonstrated that two distinct isoforms of HSF1 exist in rainbow trout cells. In vertebrates, *HSF1*s have been already isolated from human (Rabindran *et al.*, 1991), mouse (Sarge *et al.*, 1991), chicken (Nakai and Morimoto, 1993), *Xenopus* (Stump *et al.*, 1995), and zebrafish (Råbergh *et al.*, 2000); however, the present study is the first report of the cloning of a gene encoding HSF1 from cold-water fish species.

Using multiple sequence alignment, domain structures that are common to the HSF1 family were identified in the rainbow trout HSF1s (Fig. 3-1). The DNA-binding domain in both rainbow trout HSF1s is highly homologous to that of other vertebrate HSF1 (Table 3-1), suggesting that both HSF1a and HSF1b bind specifically to the HSE consensus sequence. As expected, both proteins did indeed bind to the HSE (Fig. 3-7). HSF1a and HSF1b also possess other domains conserved in the HSF1 family, i.e., HR-A/B and HR-C (Fig. 3-1). HR-A/B has been reported to be essential for forming HSF1 trimers through their α-helical coiled-coil structures (Clos *et al.*, 1990; Sorger and Nelson, 1989). The second hydrophobic repeat, HR-C, has been suggested to suppress trimer formation by interacting with HR-A/B under normal conditions (Rabindran *et al.*, 1993). As predicted by the presence of these domain structures, the data in this chapter demonstrate that both rainbow trout HSF1s form trimers (Fig. 3-8).

Furthermore, it was found that an endogenous rainbow trout HSF1 is suppressed under normal conditions but activated by heat shock in RTG-2 cells (Fig. 3-7, lanes 1 and 2). This stress-inducible activation of HSF1 has been observed in rainbow trout hepatocytes (Airaksinen *et al.*, 1998) and in the embryonic fibroblast cell line STE and male germ cells (Le Goff and Michel, 1999). Taken together, the results in this chapter suggest that rainbow trout HSF1s are activated to form DNA-binding trimers by heat shock in a manner similar to the activation of other vertebrate HSF1s. In addition to the conserved domain structures, both rainbow trout HSF1s contain two KRK tripeptides, which are also conserved among members of the HSF1 family (Fig. 3-1). The cluster of the basic residues preceding HR-A/B has been reported to be the major NLS of human HSF1 (Zuo *et al.*, 1995). Moreover, the basic peptide KRK has been reported to be a part of a bipartite type NLS in human HSF2 (Sheldon and

Kingston, 1993).

In contrast to the highly conserved domains discussed above, other regions of the rainbow trout HSF1s were poorly conserved in comparison with other vertebrate HSF1s. These poorly conserved regions are illustrated in Fig. 3-3 as regions III and V. Regions III and V roughly correspond to domains of mammalian HSF1 that have been described by several authors (Green et al., 1995; Shi et al., 1995; Zuo et al., 1995), namely, the regulatory and transactivation domains, respectively. Green et al. (1995) have shown that the central regulatory domain of human HSF1 regulates the function of the transactivation domain in a heat-shock inducible manner. Moreover, Newton et al. (1996) have suggested that the regulatory domain of human HSF1 alone is sufficient to sense heat stress. Thus, structural differences in regions III and V between rainbow trout and other vertebrate HSF1s may reflect differences in the activation temperature of HSF1. For example, human, mouse, and chicken HSF1 are activated at $\approx$ 42 °C (Morimoto, 1998), whereas rainbow trout HSF1 is activated at 25 °C in RTG-2 cells (Fig. 3-7). Notably, regions III and V of rainbow trout HSF1s even share low similarity with the corresponding regions of zebrafish HSF1. Again, this may be related to differences in the threshold temperature for HSP induction between cold- and warm-adapted fishes, as discussed at the beginning of this chapter. Moreover, because region V of rainbow trout HSF1a shows low similarity to that of HSF1b (Table 3-1), transactivation may differ between the two rainbow trout HSF1s.

It was demonstrated here that each rainbow trout HSF1 is encoded by a separate gene (Fig. 3-4). To date, two isoforms of HSF1 generated by alternative splicing have been reported for mouse (Fiorenza et al., 1995) and zebrafish (Råbergh et al., 2000); however, among vertebrates, rainbow trout HSF1 is the first case of having two genetically distinct isoforms. The HSF1a and HSF1b mRNAs are coexpressed in rainbow trout tissues (Fig. 3-5), which suggests that both are involved in the heat shock response of rainbow trout. However, since the existence of the proteins has not been checked in the same cell, the actual protein abundance remains to be elucidated.

To characterize rainbow trout HSF1 isoforms, in vitro translated HSF1s having distinct epitope tags were used in this chapter. Although migration of the products was retarded in SDS–PAGE, this phenomenon may result from the poor binding of SDS to the

proteins because of their acidic isoelectric point (HSF1a, 4.64; HSF1b, 4.63). As pointed out by Sarge *et al.* (1991), such retarded migration of HSF on SDS–PAGE seems to be characteristic of several HSFs that have been cloned to date. It was therefore concluded that the epitope-tagged HSF1s were successfully generated *in vitro*.

It was assumed that the *in vitro* translated HSF1s would be in the form of active trimers with DNA-binding ability because the *in vitro* translations were performed at 30 °C, at which rainbow trout endogenous HSF1 is already activated *in vivo* (Airaksinen *et al.*, 1998; Le Goff and Michel, 1999). As predicted, the *in vitro* translated HSF1s did indeed possess DNA-binding ability (Fig. 3-7, lanes 5 and 8).

Importantly, the chemical cross-linking and immunoprecipitation experiments in this chapter showed that the two HSF1 isoforms have the ability to form heterotrimers *in vitro* (Fig. 3-8A, lane 6 and Fig. 3-8B, lane 3). Given that the two HSF1 isoforms form both homo- and heterotrimers, there are four potential assemblies of HSF1 trimer, namely, two homotrimers (a_3 and b_3) and two heterotrimers (a_2b_1 and a_1b_2). The existence of the four types of trimer may be reflected in the broad band migrating at ≈ 200 kDa in Fig. 3-8. On the other hand, a band corresponding to an HSF1b dimer (denoted by the asterisk) was detected by Western blot analysis with anti-Protein C Ig (Fig. 3-8B, lanes 5 and 6). It remains unclear whether dimer formation is a feature only of HSF1b.

Although four HSFs, HSF1–HSF4, have been identified in vertebrates, it has been previously stated that HSF family proteins function as homotrimers. Sarge *et al.* (1991) pointed out, however, that mouse HSF1 and HSF2 are likely to co-oligomerize because they share highly homologous oligomerization domains. Likewise, Sistonen *et al.* (1994) raised the possibility that human HSF1 and HSF2 may associate to form heterotrimers for synergistic induction of the *HSP70*. The results of this chapter concerning rainbow trout HSF1 raise the same possibility of hetero-oligomerization. If hydrophobic interactions are the major stabilizing force of HSF trimerization, it is not surprising that HSF family proteins form heteromeric complexes because they possess similar heptad repeats of hydrophobic amino acids. However, since the *in vivo* state of rainbow trout HSF1 has not been examined, it remains to be elucidated whether the HSF1 isoforms form heterotrimers *in vivo*.

Why are there two isoforms of HSF1 in rainbow trout? Although the existence of the two genes may be explained simply by ancestral tetraploidization of salmonids, this does not rule out the possibility that the isoforms have acquired divergent functions during evolution. One possibility is that the distinct HSF1 isoforms contribute to the tissue specificity of the heat shock response. Airaksinen *et al.* (1998) reported that the induced expression of HSPs is both cell type- and tissue-specific in rainbow trout. Furthermore, it has been reported that rainbow trout HSF1, as well as mouse HSF1 (Goodson and Sarge, 1995), is activated at a lower temperature in male germ cells than in somatic cells (Le Goff and Michel, 1999). By contrast, the alternatively spliced isoforms of HSF1 are suggested to regulate the tissue-specific gene expression of HSPs in zebrafish (Råbergh *et al.*, 2000) and mouse (Sarge, 1995). In the present study, however, both HSF1a and HSF1b mRNAs were coexpressed in all rainbow trout tissues examined. Thus, the above-mentioned assemblies of HSF1 trimers, rather than transcriptional regulation of *HSF1*s themselves, may regulate the tissue specificity of the heat shock response in rainbow trout. Another possibility is that the two homotrimers and/or two heterotrimers play the role of other HSF family members, i.e., HSF2, HSF3, and HSF4. For instance, the relationship between rainbow trout HSF1a and HSF1b may be similar to that between chicken HSF1 and HSF3. Tanabe *et al.* (1998) reported that HSF3 has a dominant role in regulating the heat shock response and directly influences HSF1 activity in chicken cells. Unfortunately, in the present study, a cDNA encoding HSF members other than HSF1 was not found. However, a cDNA encoding rainbow trout HSF2 has been reported (Le Goff *et al.*, 2004), then the relationship between HSF1 and HSF2 in this species is needed to be elucidated in future studies.

SUMMARY AND GENERAL DISCUSSION

In Chapter I, full-length cDNA clones encoding Hsp70 were isolated from rainbow trout and investigated for their mRNA expression profiles during heat stress. Consequently, two *Hsp70*s, *Hsp70a* and *Hsp70b*, were identified and found to have 98.1% identity in their deduced amino acid sequences. Southern blot analysis indicated that the two Hsp70s are encoded by distinct genes in the genome. Northern blot analysis showed that each of *Hsp70a* and *Hsp70b* expressed two mRNA species having different sizes by heat stress in rainbow trout RTG-2 cells. The induction levels of total Hsp70b mRNAs were consistently higher than Hsp70a counterparts during heat stress, although the expression profiles of the two genes were similar to each other in temperature shift and time course experiments. Interestingly, an mRNA species with a larger molecular size was expressed only under severe heat stress not less than 28 °C irrespective of *Hsp70a* and *Hsp70b*.

In Chapter II, multiple *HSP*s were isolated from RTG-2 cells and quantitatively compared for their mRNA levels between unstressed and heat-shocked cells using real-time RT-PCR analysis. Consequently, nine cDNAs encoding HSPs were isolated from heat-shocked RTG-2 cells, namely, Hsp90βa, Hsp90βb, Grp78, Hsp70a, Hsc70a, Hsc70b, Cct8, Hsp47, and DnaJ homolog. Quantitative RT-PCR analyses, in which *Hsp70b* isolated previously was included, showed that the mRNA accumulation levels of *Hsp70a*, *Hsp70b*, *Hsc70a*, *Hsc70b*, and *Hsp47* were significantly increased after heat shock, and the increased levels of two *Hsp70*s, *Hsp70a* and *Hsp70b*, were most conspicuous. In the case of *Hsc70*s, the increased level of *Hsc70b* was more remarkable than that of *Hsc70a*.

In Chapter III, genes encoding HSF1 were cloned from RTG-2 cells. Consequently, two distinct *HSF1*s, named *HSF1a* and *HSF1b*, were identified. The predicted amino acid sequence of *HSF1a* showed 86.4% identity to that of *HSF1b*. The two proteins contained the general structural motifs of HSF1, i.e. a DNA-binding domain, hydrophobic heptad repeats and nuclear localization signals. Southern blot analysis showed that each HSF1 is encoded by a distinct gene. The two HSF1 mRNAs were coexpressed in unstressed RTG-2 cells and in various tissues. In EMSA, each *in vitro* translated HSF1 bound to the HSE. Chemical cross-linking and immunoprecipitation analysis showed that HSF1a and HSF1b form

heterotrimers as well as homotrimers. Taken together, these results demonstrate that in rainbow trout cells there are two distinct HSF1 isoforms that can form heterotrimers, suggesting that a unique molecular mechanism underlies the stress response in tetraploid and/or cold-water fish species.

In rainbow trout, the present study demonstrated that at least some *HSP* and *HSF* have duplicate genes attributed to ancestral tetraploidization. The genes found to be duplicated in the present study are summarized as follows: *Hsp90β*, *Hsp70*, *Hsc70* and *HSF1*. Since the other *HSP* may have possible paralogs as discussed in Section 3 of Chapter II, it is apparent that the comprehensive identification of duplicate genes is prerequisite to accurately examining gene expression profiles of rainbow trout cells. Meanwhile, HSPs are induced in fish cells by a variety of stresses, including industrial effluents, polycyclic aromatic hydrocarbons, several metals such as copper, zinc and mercury, pesticides, and arsenite as well as heat (Iwama *et al.*, 2004). Thus, although the present study focused on heat stress, it is likely that the mRNA expression profiles of HSPs differ among stressors. It is interesting to compare the differences of the profiles for utilizing *HSP*s as a biomarker of various environmental stresses.

Further, the present study demonstrated that two distinct isoforms of HSF1 can form heterotrimers as well as homotrimers *in vitro*. This finding suggests that a unique molecular mechanism, which functions through two distinct HSF1 isoforms, underlies the stress response in rainbow trout. However, the *in vivo* functional difference between HSF1a and HSF1b remains to be elucidated. Since the lower activation temperature of rainbow trout HSF1 is a unique feature among vertebrate counterparts, a detailed comparison of rainbow trout and other vertebrate HSF1s will lead to further insight into the activation mechanism of the transcription factor.

ACKNOWLEDGMENTS

REFERENCES

AIRAKSINEN, S., RÅBERGH, C. M. I., SISTONEN, L. & NIKINMAA, M. (1998) Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) cells. *J. Exp. Biol.*, **201**, 2543–2551.

BASU, N., TODGHAM, A. E., ACKERMAN, P. A., BIBEAU, M. R., NAKANO, K., SCHULTE, P. M. & IWAMA, G. K. (2002) Heat shock protein genes and their functional significance in fish. *Gene*, **295**, 173–183.

CLOS, J., WESTWOOD, J. T., BECKER, P. B., WILSON, S., LAMBERT, K. & WU, C. (1990) Molecular cloning and expression of a hexameric *Drosophila* heat shock factor subject to negative regulation. *Cell*, **63**, 1085–1097.

CURRIE, S., TUFTS, B. L. & MOYES, C. D. (1999) Influence of bioenergetic stress on heat shock protein gene expression in nucleated red blood cells of fish. *Am. J. Physiol.*, **276**, R990–R996.

DELLAVALLE, R. P., PETERSEN, R. & LINDQUIST, S. (1994) Preferential deadenylation of Hsp70 mRNA plays a key role in regulating Hsp70 expression in *Drosophila melanogaster*. *Mol. Cell. Biol.*, **14**, 3646–3659.

FIORENZA, M. T., FARKAS, T., DISSING, M., KOLDING, D. & ZIMARINO, V. (1995) Complex expression of murine heat shock transcription factors. *Nucleic Acids Res.*, **23**, 467–474.

GETHING, M.-J. (Ed.) (1997a) *Guidebook to Molecular Chaperones and Protein-Folding Catalysts*, Oxford, Oxford University Press.

GETHING, M.-J. (1997b) Mammalian BiP. IN GETHING, M.-J. (Ed.) *Guidebook to Molecular Chaperones and Protein-Folding Catalysts*. Oxford, Oxford University Press, pp. 59–64.

GOODSON, M. L. & SARGE, K. D. (1995) Regulated expression of heat shock factor 1 isoforms with distinct leucine zipper arrays via tissue-dependent alternative splicing. *Biochem. Biophys. Res. Commun.*, **211**, 943–949.

GREEN, M., SCHUETZ, T. J., SULLIVAN, E. K. & KINGSTON, R. E. (1995) A heat shock-responsive domain of human HSF1 that regulates transcription activation

domain function. *Mol. Cell. Biol.*, **15**, 3354–3362.

HAWKINS, A. J. S. (1996) Temperature adaptation and genetic polymorphism in aquatic animals. IN JOHNSTON, I. A. & BENNETT, A. F. (Eds.) *Animals and Temperature: Phenotypic and Evolutionary Adaptation.* Cambridge, Cambridge University Press, pp. 103–125.

HIGHTOWER, L. E. & LEUNG, S.-M. (1997) Mammalian Hsc70 and Hsp70 proteins. IN GETHING, M.-J. (Ed.) *Guidebook to Molecular Chaperones and Protein-Folding Catalysts.* Oxford, Oxford University Press, pp. 53–58.

HIGHTOWER, L. E. & RENFRO, J. L. (1988) Recent applications of fish cell culture to biomedical research. *J. Exp. Zool.*, **248**, 290–302.

HIRAYOSHI, K., KUDO, H., TAKECHI, H., NAKAI, A., IWAMATSU, A., YAMADA, K. M. & NAGATA, K. (1991) HSP47: a tissue-specific, transformation-sensitive, collagen-binding heat shock protein of chicken embryo fibroblasts. *Mol. Cell. Biol.*, **11**, 4036–4044.

IWAMA, G. K., AFONSO, L. O. B., TODGHAM, A., ACKERMAN, P. & NAKANO, K. (2004) Are hsps suitable for indicating stressed states in fish? *J. Exp. Biol.*, **207**, 15–19.

KAYA, C. M. (1978) Thermal resistance of rainbow trout from a permanently heated stream and two hatchery strains. *Prog. Fish-Cult.*, **40**, 138–142.

KOTHARY, R. K. & CANDIDO, E. P. M. (1982) Induction of a novel set of polypeptides by heat shock or sodium arsenite in cultured cells of rainbow trout, *Salmo gairdnerii. Can. J. Biochem.*, **60**, 347–355.

KOTHARY, R. K., BURGESS, E. A. & CANDIDO, E. P. M. (1984a) The heat-shock phenomenon in cultured cells of rainbow trout hsp70 mRNA synthesis and turnover. *Biochim. Biophys. Acta*, **783**, 137–143.

KOTHARY, R. K., JONES, D. & CANDIDO, E. P. M. (1984b) 70-Kilodalton heat shock polypeptides from rainbow trout: characterization of cDNA sequences. *Mol. Cell. Biol.*, **4**, 1785–1791.

KUBOTA, H., MATSUMOTO, S., YOKOTA, S., YANAGI, H. & YURA, T. (1999) Transcriptional activation of mouse cytosolic chaperonin CCT subunit genes by heat

shock factors HSF1 and HSF2. *FEBS Lett.*, **461**, 125–129.

KUBOTA, H. & WILLISON, K. (1997) CCTθ. IN GETHING, M.-J. (Ed.) *Guidebook to Molecular Chaperones and Protein-Folding Catalysts.* Oxford, Oxford University Press, pp. 225–226.

LE GOFF, P., LE DREAN, Y., LE PERON, C., LE JOSSIC-CORCOS, C., AINOUCHE, A. & MICHEL, D. (2004) Intracellular trafficking of heat shock factor 2. *Exp. Cell Res.*, **294**, 480–493.

LE GOFF, P. & MICHEL, D. (1999) HSF1 activation occurs at different temperatures in somatic and male germ cells in the poikilotherm rainbow trout. *Biochem. Biophys. Res. Commun.*, **259**, 15–20.

LINDQUIST, S. & CRAIG, E. A. (1988) The heat-shock proteins. *Annu. Rev. Genet.*, **22**, 631–677.

LUND, S. G., PHILLIPS, M. C., MOYES, C. D. & TUFTS, B. L. (2000) The effects of cell ageing on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) red blood cells. *J. Exp. Biol.*, **203**, 2219–2228.

MIAO, B., DAVIS, J. & CRAIG, E. A. (1997) The Hsp70 family–an overview. IN GETHING, M.-J. (Ed.) *Guidebook to Molecular Chaperones and Protein-Folding Catalysts.* Oxford, Oxford University Press, pp. 3–13.

MORIMOTO, R. I. (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.*, **12**, 3788–3796.

MOSSER, D. D., HEIKKILA, J. J. & BOLS, N. C. (1986) Temperature ranges over which rainbow trout fibroblasts survive and synthesize heat-shock proteins. *J. Cell. Physiol.*, **128**, 432–440.

MOSSER, D. D., THEODORAKIS, N. G. & MORIMOTO, R. I. (1988) Coordinate changes in heat shock element-binding activity and HSP70 gene transcription rates in human cells. *Mol. Cell. Biol.*, **8**, 4736–4744.

MOSSER, D. D., VAN OOSTROM, J. & BOLS, N. C. (1987) Induction and decay of thermotolerance in rainbow trout fibroblasts. *J. Cell. Physiol.*, **132**, 155–160.

MUNRO, S. & PELHAM, H. R. B. (1987) A C-terminal signal prevents secretion of luminal

ER proteins. *Cell*, **48**, 899–907.

NAGATA, K. (1997) Vertebrate Hsp47. IN GETHING, M.-J. (Ed.) *Guidebook to Molecular Chaperones and Protein-Folding Catalysts*. Oxford, Oxford University Press, pp. 465–467.

NAKAI, A. & MORIMOTO, R. I. (1993) Characterization of a novel chicken heat shock transcription factor, heat shock factor 3, suggests a new regulatory pathway. *Mol. Cell. Biol.*, **13**, 1983–1997.

NEWTON, E. M., KNAUF, U., GREEN, M. & KINGSTON, R. E. (1996) The regulatory domain of human heat shock factor 1 is sufficient to sense heat stress. *Mol. Cell. Biol.*, **16**, 839–846.

OHNO, S., WOLF, U. & ATKIN, N. B. (1968) Evolution from fish to mammals by gene duplication. *Hereditas*, **59**, 169–187.

PAGE, R. D. (1996) TreeView: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.*, **12**, 357–358.

PALMISANO, A. N., WINTON, J. R. & DICKHOFF, W. W. (1999) Sequence features and phylogenetic analysis of the stress protein hsp90α in chinook salmon (*Oncorhynchus tshawytscha*), a poikilothermic vertebrate. *Biochem. Biophys. Res. Commun.*, **258**, 784–791.

PALTI, Y., GAHR, S. A., HANSEN, J. D. & REXROAD, C. E., III (2004) Characterization of a new BAC library for rainbow trout: evidence for multi-locus duplication. *Anim. Genet.*, **35**, 130–133.

PEARSON, D. S., KULYK, W. M., KELLY, G. M. & KRONE, P. H. (1996) Cloning and characterization of a cDNA encoding the collagen-binding stress protein hsp47 in zebrafish. *DNA Cell Biol.*, **15**, 263–272.

PIRKKALA, L., NYKÄNEN, P. & SISTONEN, L. (2001) Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB J.*, **15**, 1118–1131.

RABINDRAN, S. K., GIORGI, G., CLOS, J. & WU, C. (1991) Molecular cloning and expression of a human heat shock factor, HSF1. *Proc. Natl. Acad. Sci. USA*, **88**, 6906–6910.

RABINDRAN, S. K., HAROUN, R. I., CLOS, J., WISNIEWSKI, J. & WU, C. (1993) Regulation of heat shock factor trimer formation: role of a conserved leucine zipper. *Science*, **259**, 230–234.

RISE, M. L., VON SCHALBURG, K. R., BROWN, G. D., MAWER, M. A., DEVLIN, R. H., KUIPERS, N., BUSBY, M., BEETZ-SARGENT, M., ALBERTO, R., GIBBS, A. R., HUNT, P., SHUKIN, R., ZEZNIK, J. A., NELSON, C., JONES, S. R. M., SMAILUS, D. E., JONES, S. J. M., SCHEIN, J. E., MARRA, M. A., BUTTERFIELD, Y. S. N., STOTT, J. M., NG, S. H. S., DAVIDSON, W. S. & KOOP, B. F. (2004) Development and application of a salmonid EST database and cDNA microarray: data mining and interspecific hybridization characteristics. *Genome Res.*, **14**, 478–490.

RYAN, J. A. & HIGHTOWER, L. E. (1996) Stress proteins as molecular biomarkers for environmental toxicology. *EXS*, **77**, 411–424.

RÅBERGH, C. M. I., AIRAKSINEN, S., SOITAMO, A., BJÖRKLUND, H. V., JOHANSSON, T., NIKINMAA, M. & SISTONEN, L. (2000) Tissue-specific expression of zebrafish (*Danio rerio*) heat shock factor 1 mRNAs in response to heat stress. *J. Exp. Biol.*, **203**, 1817–1824.

SAITOU, N. & NEI, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406–425.

SAMPLES, B. L., POOL, G. L. & LUMB, R. H. (1999) Polyunsaturated fatty acids enhance the heat induced stress response in rainbow trout (*Oncorhynchus mykiss*) leukocytes. *Comp. Biochem. Physiol.*, **123B**, 389–397.

SARGE, K. D. (1995) Male germ cell-specific alteration in temperature set point of the cellular stress response. *J. Biol. Chem.*, **270**, 18745–18748.

SARGE, K. D., ZIMARINO, V., HOLM, K., WU, C. & MORIMOTO, R. I. (1991) Cloning and characterization of two mouse heat shock factors with distinct inducible and constitutive DNA-binding ability. *Genes Dev.*, **5**, 1902–1911.

SATHIYAA, R., CAMPBELL, T. & VIJAYAN, M. M. (2001) Cortisol modulates HSP90 mRNA expression in primary cultures of trout hepatocytes. *Comp. Biochem. Physiol.*, **129B**, 679–685.

SATOH, M., HIRAYOSHI, K., YOKOTA, S., HOSOKAWA, N. & NAGATA, K. (1996)

Intracellular interaction of collagen-specific stress protein HSP47 with newly synthesized procollagen. *J. Cell Biol.*, **133**, 469–483.

SCHEIBEL, T. & BUCHNER, J. (1997) The HSP90 family–an overview. IN GETHING, M.-J. (Ed.) *Guidebook to Molecular Chaperones and Protein-Folding Catalysts.* Oxford, Oxford University Press, pp. 147–151.

SCHEUFLER, C., BRINKER, A., BOURENKOV, G., PEGORARO, S., MORODER, L., BARTUNIK, H., HARTL, F. U. & MOAREFI, I. (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell*, **101**, 199–210.

SHELDON, L. A. & KINGSTON, R. E. (1993) Hydrophobic coiled-coil domains regulate the subcellular localization of human heat shock factor 2. *Genes Dev.*, **7**, 1549–1558.

SHI, Y., KROEGER, P. E. & MORIMOTO, R. I. (1995) The carboxyl-terminal transactivation domain of heat shock factor 1 is negatively regulated and stress responsive. *Mol. Cell. Biol.*, **15**, 4309–4318.

SISTONEN, L., SARGE, K. D. & MORIMOTO, R. I. (1994) Human heat shock factors 1 and 2 are differentially activated and can synergistically induce hsp70 gene transcription. *Mol. Cell. Biol.*, **14**, 2087–2099.

SOMORJAI, I. M. L., DANZMANN, R. G. & FERGUSON, M. M. (2003) Distribution of temperature tolerance quantitative trait loci in Arctic charr (*Salvelinus alpinus*) and inferred homologies in rainbow trout (*Oncorhynchus mykiss*). *Genetics*, **165**, 1443–1456.

SONNA, L. A., FUJITA, J., GAFFIN, S. L. & LILLY, C. M. (2002) Effects of heat and cold stress on mammalian gene expression. *J. Appl. Physiol.*, **92**, 1725–1742.

SORGER, P. K. & NELSON, H. C. (1989) Trimerization of a yeast transcriptional activator via a coiled-coil motif. *Cell*, **59**, 807–813.

SREEDHAR, A. S., KALMÁR, É., CSERMELY, P. & SHEN, Y.-F. (2004) Hsp90 isoforms: functions, expression and clinical importance. *FEBS Lett.*, **562**, 11–15.

STUMP, D. G., LANDSBERGER, N. & WOLFFE, A. P. (1995) The cDNA encoding *Xenopus laevis* heat-shock factor 1 (XHSF1): nucleotide and deduced amino-acid sequences, and properties of the encoded protein. *Gene*, **160**, 207–211.

TAKECHI, H., HOSOKAWA, N., HIRAYOSHI, K. & NAGATA, K. (1994) Alternative 5' splice site selection induced by heat shock. *Mol. Cell. Biol.*, **14**, 567–575.

TANABE, M., KAWAZOE, Y., TAKEDA, S., MORIMOTO, R. I., NAGATA, K. & NAKAI, A. (1998) Disruption of the HSF3 gene results in the severe reduction of heat shock gene expression and loss of thermotolerance. *EMBO J.*, **17**, 1750–1758.

THOMPSON, J. D., HIGGINS, D. G. & GIBSON, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**, 4673–4680.

THORGAARD, G. H., BAILEY, G. S., WILLIAMS, D., BUHLER, D. R., KAATTARI, S. L., RISTOW, S. S., HANSEN, J. D., WINTON, J. R., BARTHOLOMEW, J. L., NAGLER, J. J., WALSH, P. J., VIJAYAN, M. M., DEVLIN, R. H., HARDY, R. W., OVERTURF, K. E., YOUNG, W. P., ROBISON, B. D., REXROAD, C. & PALTI, Y. (2002) Status and opportunities for genomics research with rainbow trout. *Comp. Biochem. Physiol.*, **133B**, 609–646.

WOLF, K. & QUIMBY, M. C. (1962) Established eurythermic line of fish cells *in vitro*. *Science*, **135**, 1065–1066.

WU, C. (1995) Heat shock transcription factors: structure and regulation. *Annu. Rev. Cell Dev. Biol.*, **11**, 441–469.

YAMASHITA, M., HIRAYOSHI, K. & NAGATA, K. (2004) Characterization of multiple members of the HSP70 family in platyfish culture cells: molecular evolution of stress protein HSP70 in vertebrates. *Gene*, **336**, 207–218.

YOKOYAMA, Y., HASHIMOTO, H., KUBOTA, S., KINOSHITA, M., TOYOHARA, H., SAKAGUCHI, M., TANAKA, M., SEIKAI, T. & KANAMORI, M. (1998) cDNA cloning of heat-inducible HSP70, a 70.6 kDa heat shock protein, in Japanese flounder *Paralichtys olivaceus*. *Fish. Sci.*, **64**, 964–968.

ZAFARULLAH, M., WISNIEWSKI, J., SHWORAK, N. W., SCHIEMAN, S., MISRA, S. & GEDAMU, L. (1992) Molecular cloning and characterization of a constitutively expressed heat-shock-cognate hsc71 gene from rainbow trout. *Eur. J. Biochem.*, **204**, 893–900.

ZUO, J., RUNGGER, D. & VOELLMY, R. (1995) Multiple layers of regulation of human heat shock transcription factor 1. *Mol. Cell. Biol.*, **15**, 4319–4330.