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Quantitative mRNA expression profiling of heat-shock protein families in rainbow trout cells

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Abstract

We isolated multiple *HSP*s from rainbow trout *Oncorhynchus mykiss* RTG-2 cells and quantitatively compared their mRNA levels between unstressed and heat-shocked cells using real-time RT-PCR analysis. Consequently, we isolated nine cDNAs encoding HSPs 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 *Hsp70s*, *Hsp70a*, and *Hsp70b*, were most conspicuous. In the case of *Hsc70*s, the increased level of *Hsc70b* was more remarkable than that of *Hsc70a*. These results demonstrate the importance of a comprehensive expression analysis of *HSP*s for better understanding of the cellular stress response in fish, especially in tetraploid species such as rainbow trout.

Keywords: Gene expression; Gene family; Heat-shock proteins; Heat stress; mRNA; Northern blotting; Quantitative RT-PCR; Rainbow trout; RTG-2 cell

Heat-shock proteins (HSPs) are present in all organisms studied to date and their synthesis is induced in cells by heat and a variety of stresses [1]. HSPs are traditionally classified by molecular weights into several families [2]. For example, a 70-kDa heat-shock protein belongs to the HSP70 family. HSP family members possess three principal biochemical functions, namely, molecular chaperone activity, regulation of cellular redox state, and regulation of protein turnover [2]. The inducible HSP expression is regulated by the heat shock transcription factors (HSFs) [3]. 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 [3].

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 [4]. 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 [4]. 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 Tier 1 biomarkers to perceive sublethal cellular damages as the result of an environmental stress [5].

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However, the use of HSPs as indicators for stress states of fish in general is premature [6], in part because little information is available about a comprehensive stress response profile of HSP genes in fish cells.

Among fish species, cold-water fish such as rainbow trout, which receive heat stress at moderately low temperatures, are thought to be particularly suitable for investigating the cellular heat-shock response. Moreover, rainbow trout have evolved by tetraploidization from a diploid ancestor [7], raising the possibility that duplicate HSP genes are expressed in cells and their induction levels differ from each other. Indeed, in a previous study, we identified two paralogous genes encoding Hsp70 in rainbow trout [8]. Hence, it is interesting to interpret the possible difference in the induction levels of the duplicate genes in terms of molecular evolution.

Thus, the purpose of the present study was to isolate multiple HSP genes from rainbow trout and to analyze their expression profiles at the mRNA levels. In this regard, we quantitatively compared the mRNA levels between unstressed and heat-shocked cells using real-time RT-PCR analysis. According to the nomenclature used in the previous publication [9], we used fully capitalized names to denote the protein family (e.g., HSP90, HSP70, HSP60, etc.) and initial capital letter for specific family members (e.g., Hsp90, Hsc70, Cct8, etc.) in the present paper.

Materials and methods

Cell culture. RTG-2 cells, which are immortalized rainbow trout gonadal fibroblasts [10], were cultured at 15 °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).

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 manufacturer's instructions. Poly(A) RNA was isolated from the total RNA using an Oligotex-MAG mRNA Purification kit (Takara). 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 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). 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). 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/).

RNA preparation. Control RTG-2 cells were cultured at 20 °C. 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 our previous study [8]. 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) in a 50-μl reaction volume containing 20 U RNase inhibitor (Toyobo). RNAs were extracted with phenol:chloroform:isoamyl alcohol (25:24:1 mixed, pH 5.2, Nakarai) followed by ethanol precipitation.

Northern blot analysis. Five micrograms of total RNA was 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 digoxigenin (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 1). 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).

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). 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 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) 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

Cloning of HSP genes expressed in heat-shocked RTG-2 cells

To clone multiple HSP genes at the same time, we arbitrarily isolated 200 cDNAs from heat-shocked RTG-2 cells and sequenced their 5′ ends. The readable sequence length was approximately 340 bp. The data

Table 1
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.

Table 2
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 1.
[b] F, forward primer; R, reverse primer.

are summarized in Table 3, where we defined the BLAST hits with an E value $\geqslant 10^{-5}$ 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%)

Table 3
List of cDNAs isolated from heat-shocked RTG-2 cells

Putative genes	No. of clones	% of total clones
HSP family members		
Heat shock cognate protein 70	3	
Heat shock protein 90 β	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

were classified as "chaperone-related proteins," which we designated, including proteins with chaperone or catalytic activity for protein folding.

Next, we completely sequenced the cDNA clones encoding HSP family members, resulting in identification of nine genes (Table 4). We designated these clones as in Table 4, 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*. We designated them as *Hsp90βa* and *Hsp90βb*, and *Hsc70a* and *Hsc70b*, respectively. Both Hsp90βa and Hsp90βb cDNAs contained complete 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 has an important role in cofactor binding mediated by a tetratricopeptide repeat (TPR) domain [11]. A BLAST search indicated that both proteins had the highest amino acid identity with Atlantic salmon *Salmo salar* Hsp90β (Table 4). Meanwhile, Hsc70b cDNA contained 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* [12]. For *Hsp70*, one clone was isolated and identified as *Hsp70a* that we previously cloned from a juvenile rainbow trout [8]. The two Hsp70a proteins 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

Table 4
Summary of cloned cDNAs encoding HSP family members

HSP family	Rainbow trout RTG-2 cDNA			The best matching sequence using 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 our previous study [8].
[d] Genomic clone.
[e] cDNA clone containing putative intron(s).

reticulum (ER) [13]. Other than 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 [14].

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

To investigate whether the cloned HSP genes are actually transcribed in rainbow trout cells, we performed Northern blot analysis using DNA probes specific to 3′-UTR of respective genes. In this regard, we excluded the DnaJ homolog gene from this analysis because its cDNA was incomplete where 3′-UTR was uncertain. Meanwhile, we included *Hsp70b* isolated in our previous study [8]. 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 in our previous study [8] (Fig. 1). Therefore, we concluded that our 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. 1). Therefore, to accurately determine the changes, we next performed quantitative RT-PCR analysis. In this regard, we used the β-actin gene 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 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 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 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 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 5).

Table 5
mRNA accumulation levels of HSP genes 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] Student's unpaired t test was used for group comparisons.
[c] Two-sided test.
[d] One-sided test.

Discussion

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

In preliminary experiments, we 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, we considered that the ratios of mRNAs of the HSP genes to those of the housekeeping gene would be relatively high in the heat-shocked cells, and attempted to use the cells as a source of a cDNA library to isolate as many HSP genes as

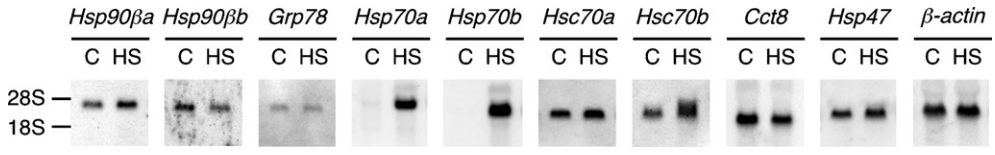

Fig. 1. mRNA accumulation levels of the HSP genes in control (C) and heat-shocked (HS) RTG-2 cells as revealed by Northern blot analyses. The probes are complementary to sequences in 3′-UTR of respective genes. The positions of 28S and 18S rRNAs are indicated on the left as size markers. The blots are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

possible. As expected, we identified multiple HSP genes by arbitrarily isolating cDNAs from the RTG-2 cells (Table 3). 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 [8] and heat shock transcription factor 1 (HSF1) genes [15]. Although we could identify only one clone for the other HSP family genes in the present study, these may have possible paralogs because it has been inferred that most of the rainbow trout genes are duplicated [7,16].

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: Hsp90α and Hsp90β [17]. 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 [18]. Our two Hsp90 genes, designated *Hsp90βa* and *Hsp90βb*, were constitutively expressed in RTG-2 cells irrespective of heat shock treatment (Fig. 1 and Table 5), suggesting that both genes actually encode a β isoform of Hsp90. On the other hand, Sathiyaa et al. [19] 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 our Hsp90s, raising the possibility that there exists at least one α isoform of Hsp90 besides our 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* [20].

In contrast to other HSP family members examined, the accumulation levels of Hsp70 mRNAs were dramatically increased after heat shock (Fig. 1 and Table 5). In Northern blot analysis (Fig. 1), two mRNA species having different sizes were detected in heat-shocked cells, irrespective of *Hsp70a* and *Hsp70b* as in our previous study [8]. 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 *Hsp70s* are the most useful biomarkers of heat stress among HSP family members in fish, at least in rainbow trout.

The present study demonstrated that there exist two *Hsc70*s in rainbow trout cells like *Hsp70*s in our previous study [8]. Zafarullah et al. [12] isolated a genomic clone encoding Hsc70 and designated it as *Hsc71*, which corresponds to our *Hsc70a* (Table 4). Hsc70 is a member of the Hsp70 family and exhibits constitutive expression [21]. It has been reported that Hsc71 mRNA levels did not significantly increase after heat shock in RTG-2 cells [12], and similar results were obtained by Northern blot analysis in our previous study [8]. 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 5). Considering that several putative heat-shock elements (HSEs) have been found in the promoter region of *Hsc71* [12], it is likely that there exist functional HSEs in 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 [22]. 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 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 [23]. However, as discussed by Kubota et al. [23], 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 5). It is known that *Hsp47* expression is induced by heat shock at the transcriptional level in zebrafish embryos [24] and chicken embryonic fibroblasts [25]. In vertebrate cells, Hsp47 is located in the lumen of ER [26], as is BiP/Grp78 [27]. 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 in mammalian cells [27], our results indicate that rainbow trout Grp78 mRNA was constitutively expressed in unstressed cells (Fig. 1 and Table 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 [28]. Under the same heat-shock conditions, the enhanced syntheses of a similar set of HSPs have also been found by the other research group [29], 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 the HSP100, HSP90, HSP70, HSP60, HSP40, and HSP27 families, respectively. Among these families, we cloned the genes encoding

the HSP90, HSP70, HSP60, and HSP40 families in the present study. Unfortunately, however, we could not find cDNAs encoding HSP100 and HSP27 families in our 200 cDNAs arbitrarily isolated.

In conclusion, the accumulation levels of Hsp70a and Hsp70b mRNAs were the most remarkably increased after heat shock in rainbow trout RTG-2 cells among HSP family members identified. Furthermore, Hsc70a, Hsc70b, and Hsp47 mRNA levels were significantly elevated after heat shock, though much weaker than those of *Hsp70*s. These results were demonstrated by the accurate quantitation of mRNA accumulation levels using real-time RT-PCR.

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