ELSEVIER

Gene 295 (2002) 173–183

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

www.elsevier.com/locate/gene

Review

Heat shock protein genes and their functional significance in fish

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Received 30 July 2001; received in revised form 10 November 2001; accepted 7 May 2002

Abstract

Despite decades of intensive investigation, important questions remain regarding the functional, ecological, and evolutionary roles of heat shock proteins. In this paper, we discuss the utility of fish as a model system to address these questions, and review the relevant studies of heat shock protein genes and the regulation of their expression in fish. Although molecular studies of the heat shock proteins in fish are still in their early descriptive phase, data are rapidly being collected. More is known about the biotic and abiotic factors regulating heat shock proteins. We briefly review these studies and focus on the role of heat shock proteins in development, their regulation by the endocrine system, and their importance in fish in nature. Functional genomics approaches will provide the tools necessary to gain a comprehensive understanding of the significance of heat shock proteins in the cellular stress response, in the physiological processes at higher levels of organization, and in the whole animal in its natural environment.

Keywords: Stress proteins; hsp; Gene families; Functional significance; Fish

1. Introduction

Heat shock proteins are a family of highly conserved cellular proteins present in all organisms that have been examined (Morimoto et al., 1990; Welch, 1993; Feder and Hofmann, 1999), including fish (reviewed by Iwama et al., 1998). Extensive studies on model species have revealed three major families of heat shock proteins: Hsp90 (85–90 kDa), Hsp70 (68–73 kDa), and low molecular weight heat shock proteins (16–47 kDa). In the unstressed cell, these proteins have constitutive functions that are essential in various aspects of protein metabolism (reviewed by Morimoto et al., 1990; Hightower, 1991; Nover, 1991; Hendrick and Hartl, 1993; Welch, 1993; Fink and Goto, 1998). Hsp90 is active in supporting various components of the cytoskeleton and steroid hormone receptors (Csermely et al., 1998; Pearl and Prodromou, 2000; Young et al., 2001). Hsp70 is known to assist the folding of nascent polypeptide chains, act as a molecular chaperone, and mediate the repair and degradation of altered or denatured proteins (Kiang and Tsokos, 1998). The low molecular weight heat shock proteins have diverse functions that are species-specific. Unlike other heat shock proteins, these proteins have no known constitutive function and are only induced during stress (Ciocca et al., 1993).

Classical studies of stress in fish have focused on the organismal stress response. The characteristic feature of this organismal stress response is the rapid release of stress hormones, including cortisol and catecholamines, resulting in the mobilization of energy reserves in an attempt to re-establish homeostasis (reviewed by Wendelaar Bonga, 1997; Fabbri et al., 1998; Mommsen et al., 1999). In addition to this organismal stress response, a generalized stress response system exists at the cellular level, which includes the actions and functions of various heat shock proteins (Hightower, 1991; Iwama et al., 1999; Goligorsky, 2001). While the term 'heat shock protein' arose from early observations on *Drosophila* exposed to a severe heat stress, heat shock proteins can be up-regulated in cells that are exposed to a wide variety of stressors, particularly those that denature proteins (Welch, 1993; Freeman et al., 1999). In fish, the induction of heat shock protein families, a component of the cellular stress response, has been reported in cell lines, primary cultures of cells, as well as in various tissues from whole animals (Iwama et al., 1998). Most of these studies demonstrated a correlation between increased levels of heat shock proteins and exposure to stressors within an ecologi-

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0378-1119/02/$ - see front matter
PII: S0378-1119(02)00687-X

cally relevant range. These observations suggest that the cellular stress response is likely to be playing some role in enhancing the survival and health of the stressed fish.

The mechanisms underlying the sensing of a stressor and the induction of heat shock proteins are far from clear. Studies on Hsp70 are the most extensive and have demonstrated that the regulation of *hsp70* gene expression occurs mainly at the transcriptional level (Morimoto et al., 1990; Nover, 1991; Fink and Goto, 1998). Analysis of heat shock protein genes and a comparison of their promoter sequences from a variety of organisms led to the identification of a palindromic heat shock element (HSE), CNNGAANNTTCNNG (Bienz and Pelham, 1987). It has been demonstrated that heat shock protein induction results primarily from the binding of an activated heat shock transcription factor (HSF) to HSEs upstream of heat shock protein genes (Morimoto et al., 1992). Since most of the inducible heat shock protein genes do not contain introns, the mRNA is rapidly translated into nascent protein within minutes following exposure to a stressor.

While most of our knowledge regarding the biology of heat shock proteins has been derived from work on a limited number of model systems, fish represent an ideal organism in which to resolve the regulation and functional significance of heat shock proteins. In particular, fish offer an alternate and excellent model system in which to investigate the functional, ecological, and evolutionary genomics of heat shock proteins. Fish are ectothermic vertebrates that inhabit an aquatic environment with high temperature conductivity. As a result, temperature is an important factor influencing their biogeographic distribution over evolutionary time. In addition, daily and seasonal temperature fluctuations have an important impact during the lifetime of individual fish. Therefore, fish are a convenient model to study the effects of thermal stress in the intact organism on both short and long time scales. Fish have also emerged as an important developmental model (Kelly et al., 2000), since many species have external fertilization, and large manipulable eggs and embryos. Thus, heat shock protein expression and regulation can be studied at all life-history stages in fish. In this paper we review what is known regarding the sequence and genomic structure of the major heat shock protein gene families in fish. We then address the physiological roles of heat shock proteins in fish and their importance as part of the integrated response to environmental change. Studies into the functional genomics of heat shock proteins in fish will provide substantial insight into the physiological and ecological roles of these highly conserved proteins.

2. Heat shock protein genes in fish

In order for functional genomics to be successfully applied in fish, a substantial amount of basic molecular information must first be collected. Most studies of heat shock proteins in fish have been performed exclusively at the protein level, and thus relatively little is known about the sequence, genomic structure, or organization of the genes encoding heat shock proteins in fish. Indeed, heat shock protein genes have only been cloned from a modest number of different fish species. *hsp70* has been cloned from rainbow trout (*Oncorhynchus mykiss*; Kothary et al., 1984), medaka (*Oryzias latipes*; Arai et al., 1995), zebrafish (*Danio rerio*; Lele et al., 1997), tilapia (*Oreochromis mossambicus*; Molina et al., 2000), and pufferfish (*Fugu rubripes*; Lim and Brenner, 1999), and heat stress-related increases in mRNA levels have been documented for many of these genes. As is the case for *hsp70* genes in other organisms, the *hsp70* genes of fish are highly conserved at the amino acid level (see, for example, Molina et al., 2000). Zafarullah et al. (1992) isolated and characterized another member of the rainbow trout *hsp70* multigene family, the constitutively expressed heat-shock cognate, *hsc71*. Santacruz et al. (1997) cloned and characterized a zebrafish *hsc70*. Recently, a heat shock cognate (*hsc71m*) has also been isolated from the hermaphroditic teleost *Rivulus marmoratus* (Park et al., 2001). This gene is not induced by stress, but is enriched in *Rivulus* muscle, suggesting that there may be multiple isoforms of the heat-shock cognate, with differing tissue-specific distributions. A fragment of an *hsc70* gene has also been sequenced in carp (*Cyprinus carpio*; Yin et al., 1999).

Currie and Tufts (1997) detected a band corresponding to Hsp90 while profiling the Hsp70 response in rainbow trout red blood cells. Mammalian genomes encode two closely related *hsp90* genes (alpha and beta). Both have been sequenced in zebrafish and both have been shown to be differentially regulated in developing embryos (Krone and Sass, 1994). A complete sequence of an *hsp90α* has also been obtained from the chinook salmon (*Oncorhynchus tshawytscha*; Palmisano et al., 2000). The expression of this gene was studied in a chinook salmon embryonic cell line and it was shown to be heat inducible (Palmisano et al., 2000). A fragment of *hsp90α* has been cloned from the Japanese flounder (*Paralichtys olivaceus*; Nam, Hirono, and Aoki, unpublished data; accession number AU090921). An *hsp90* sequence from Atlantic salmon (*Salmo salar*) was characterized by Pan et al. (2000) that corresponded to the *hsp90β* of zebrafish with a 92% amino acid identity. Atlantic salmon *hsp90β* expression, in vitro and in vivo, was shown to be upregulated in gill and kidney tissues, but the magnitude of induction was not as great as for the inducible *hsp70* gene.

Several members of the low molecular weight heat shock protein family have been cloned in fish. An *hsp30* has been cloned from the chinook salmon (Hargis, Goff, Hickey and Weber, unpublished data; accession number U19370). Pearson et al. (1996) cloned and characterized an *hsp47* in zebrafish. Norris et al. (1997) cloned two low molecular weight heat shock proteins, *hsp27* and *hsp30*, in the desert pupfish, *Poeciliopsis lucida*. Sequence analysis indicated that these

genes are members of the α-crystallin/small heat shock protein superfamily. The *hsp30* genes appear to have diverged more rapidly than *hsp27* and were most similar to homologs in *Xenopus*, while *hsp27* was highly similar to mammalian and avian homologs (Norris et al., 1997).

The mechanisms regulating the expression of heat shock protein genes in fish have not yet been extensively studied, although some work has been initiated on *hsp70* genes. Currie and Tufts (1997) first suggested that Hsp70 in rainbow trout is regulated primarily at the level of transcription. Subsequently Airaksinen et al. (1998) reported that an *HSF1*-like factor was involved in the induction of *hsp70* mRNA in rainbow trout. Recently, this transcription factor has been cloned in zebrafish (Rabergh et al., 2000). These same authors also cloned a fragment of *HSF* from bluegill sunfish (*Lepomis macrochirus*). Interestingly, the *HSF* from bluegill sunfish was more similar to human, mouse and chicken *HSF* than it was to the zebrafish gene. This suggests that either the *HSF* genes of fishes are surprisingly divergent, or that fish genomes encode multiple genes for *HSF* (Rabergh et al., 2000). Two forms of *HSF1* transcript were detected using reverse transcription–polymerase chain reaction (RT–PCR) in zebrafish. Expression of both transcripts was confirmed by RT–PCR analysis of control and heat shocked hepatic, gonad, and gill tissue. There were differences in the amounts of these two transcripts among tissues, and in their responses to heat stress (Rabergh et al., 2000). The two transcripts were highly similar, except for a 78 bp insertion/deletion, and thus appear to be splice variants. However, there was a single nucleotide change that caused a substitution of lysine to asparagine adjacent to the alternative splice site, which opens the possibility that these two transcripts represent distinct isoforms of *HSF1* (Rabergh et al., 2000). However, the high degree of similarity between these two zebrafish *HSF* variants cannot account for the surprising degree of divergence between the zebrafish and bluegill sunfish genes.

3. Genomic structure of fish heat shock protein genes

At present remarkably little is known about the genomic organization of any of the genes encoding heat shock proteins in fish. For most species, there is little or no information regarding even the total number of heat shock family members encoded in the genome, or their linkage relationships. Lim and Brenner (1999) found five intronless *hsp70* genes in a region of approximately 42 kb in the pufferfish (*Fugu rubripes*). However, the linkage relationships of the *Fugu hsp70* genes bore no similarity to those in other organisms. Complete sequences were obtained only for *hsp70-2* and *hsp70-4*, while the partial sequences were obtained for *hsp70-1*, *hsp70-3*, and *hsp70-5*. The *Fugu hsp70-4* was most similar to the *hsp70-1* of mammals, the sequence linked to the major histocompatibility complex (MHC), but there was no evidence of this linkage relationship in *Fugu*.

The five *Fugu hsp70* genes were found to be arranged in a head to head, tail to tail, and head to tail orientation. The genes were fairly similar to each other (94% similarity at the amino acid level) and amino acid differences were distributed broadly across the molecules, with no obvious regions of lower similarity. The primary exception was the *hsp70-2* gene, which contained a substantial deletion at the 3′ end relative to the other copies. The significance of this deletion is not known. Putative HSEs were identified in the 5′ regions adjacent to each of the *Fugu hsp70* genes, suggesting the possibility that all of these genes are heat-inducible. There were multiple stretches of sequence similarity within the upstream regions of the genes, suggesting some additional similarity in their regulation. However, there were also substantial differences between genes in the promoter regions, suggesting the possibility of tissue or stressor-specific regulation. A functional genomics analysis has not yet been attempted for these genes, so the question of their relative roles and regulation remains to be addressed.

Little is known regarding the total number of *hsp70* genes in other fish species. Lele et al. (1997) used degenerate PCR from genomic DNA in an attempt to identify variants of *hsp70* in zebrafish (*Danio rerio*). Only two genes were identified: *hsp70-4* and *hsp70-15*. *Hsp70-4* was most similar to the *hsp70* of rainbow trout (*Oncorhynchus mykiss*; Kothary et al., 1984), and was strongly heat inducible during embryonic development, suggesting that this gene represents the inducible *hsp70* gene in zebrafish. *Hsp70-15* was similar to the heat shock cognate (*hsc71*) of rainbow trout (Zafarullah et al., 1992), but Lele et al. (1997) were not able to find any evidence that this gene was actually expressed in zebrafish, at least in the developing embryo. Santacruz et al. (1997) studied the expression of an *hsc70* gene in the developing zebrafish embryo, indicating that this gene was expressed. Although the evidence presented by Lele et al. (1997) is not conclusive, it does suggest that either there is only one copy of *hsp70* in the zebrafish genome, or that the sequences of these gene copies have been homogenized through gene conversion. This process has occurred in other species. For example, in the rat, the *hsp70-1* and *hsp70-2* gene products, although encoded by different genes, are identical at the amino acid level (Walter et al., 1994).

Genomic sequence information is available for a few other fish heat shock protein genes. The complete gene encoding an *hsp70* has been cloned in tilapia (*Oreochromis mossambicus*; Molina et al., 2000). Typical of the inducible *hsp70* genes in other species, this gene does not contain introns. Three copies of the HSE consensus sequence were present in the promoter of the gene, and transfection experiments were used to show that these elements could confer heat-induction on a reporter gene. The promoter of an *hsp70* gene from zebrafish has also been cloned (Halloran et al., 2000). These authors made stable transgenic lines of zebrafish expressing green fluorescent protein (GFP) under

the control of the zebrafish heat shock promoter. At normal temperatures GFP activity was not detectable in developing embryos (except in the eye lens), but was expressed in all tissues following heat shock.

Genomic sequences have been obtained for a heat shock cognate (*hsc71*) from *Rivulus marmoratus* (Park et al., 2001) and rainbow trout (Zafarullah et al., 1992). Like the homologous mammalian genes, the fish *hsc71* gene contained eight introns. Exon sizes were identical between these two fish species, although their intron sizes differed. The most striking difference was in the size of intron 4, which was 1580 bp in *Rivulus*, but only 225 bp in the rainbow trout. The first intron was larger in both the fish species than in the homologous genes in mammals (1.6–1.8 kb in the fish vs. 0.56–0.73 kb in mammals). The functional or evolutionary significance of these differences is not known.

Much remains to be studied regarding the functional genomics of heat shock proteins in fish, but sufficient sequence information is now available that these experiments can be productively attempted. Important questions to be addressed include: (a) what is the functional significance of the multiple somewhat divergent copies of heat shock protein family members in fish genomes; (b) does the regulation of, for example, multiple similar copies of *hsp70*, differ in response to heat stress or among tissues; and (c) what is the relationship between the thermal habitat of fish species and the structure, number, or regulation of heat shock protein genes?

Previous studies on the roles of heat shock proteins in fish provide important insights that can guide functional genomic investigation. In the remainder of this review we summarize this work on heat shock proteins in fish in the context of factors regulating heat shock proteins, the roles of heat shock proteins in development, the effects of hormones on heat shock proteins, and the importance of these proteins for environmental adaptation in fish. Taken together, these studies indicate that the regulation and roles of heat shock proteins in fish are complex. This complexity makes fish an ideal model for studying the significance of heat shock proteins in the cellular stress response using functional genomics.

4. Factors regulating heat shock proteins in fish

Understanding the factors that regulate heat shock proteins in fish is providing researchers with substantial insight into their functional significance and roles within the cellular and organismal stress responses. Heat shock proteins expression is influenced by a wide variety of abiotic and biotic factors, and in this section we discuss some of the factors that influence them.

4.1. Abiotic factors and their effects on heat shock proteins

The majority of studies on heat shock proteins in fish have been limited to in vitro examinations conducted in laboratory environments. Furthermore, most of these studies reported the induction of heat shock protein families following exposure to stress, without elucidating the functional significance underlying their observations (Iwama et al., 1998). Studies in fish have demonstrated that heat stress can induce various heat shock proteins in cell lines (Kothary and Candido, 1982; Kothary et al., 1984; Mosser and Bols, 1988), primary cell culture (Koban et al., 1987; Renfro et al., 1993; Currie et al., 1999; Sathiyaa et al., 2001), and in tissues from whole animals (Koban et al., 1991; Dietz and Somero, 1993; Dubeau et al., 1998; Ackerman et al., 2000). Osmotic stress has recently been demonstrated to induce *hsp90* mRNA in chinook salmon (*Oncorhynchus tshawytscha*; Palmisano et al., 2000) and Atlantic salmon (*Salmo salar*; Pan et al., 2000), and Hsp54 and Hsp70 in Atlantic salmon (Smith et al., 1999). Elevated levels of various heat shock proteins have been measured in tissues of fish exposed to environmental contaminants, such as heavy metals (Heikkila et al., 1982; Misra et al., 1989; Sanders et al., 1995; Williams et al., 1996; Duffy et al., 1999), industrial effluents (Janz et al., 1997; Vijayan et al., 1998), pesticides (Sanders, 1993; Hassanein et al., 1999), and polycyclic aromatic hydrocarbons (Vijayan et al., 1997; Vijayan et al., 1998). It is noteworthy that while many indicators of fish stress (e.g. plasma cortisol concentrations) are altered by handling and sampling procedures, Vijayan et al. (1997) demonstrated that handling stress does not alter levels of hepatic hsp70 in rainbow trout (*Oncorhynchus mykiss*). The effects of abiotic factors on heat shock protein expression in fish have been extensively reviewed (Sanders, 1993; Iwama et al., 1998).

4.2. Biotic factors and their effects on heat shock proteins

Less is known regarding the effects of biotic factors on heat shock proteins in fish. Kagawa et al. (1999) reported that levels of Hsp70 were significantly raised in the brains of goldfish (*Carassius auratus*) that were reared in the presence of a predator, the bluegill sunfish (*Lepomis macrochirus*). More is known about the effects of pathogenic exposure on heat shock proteins. Pathogens are common in natural environments and can have detrimental impacts on the health of fish populations. Heat shock proteins are known to be involved in the immune response following pathogenic exposures in mammals (Young, 1990). Cho et al. (1997) were the first to observe a cellular stress response (Hsp90) in fish cells, following exposure of cells to infectious haematopoietic necrosis virus (IHNV). Forsyth et al. (1997) observed increased Hsp70 in hepatic and head kidney tissues of coho salmon (*Oncorhynchus kisutch*) infected with *Renibacterium salmoninarum*, the causative agent of a slowly developing, chronic disease (bacterial kidney disease) of salmonids. Subsequent experiments demonstrated that juvenile rainbow trout (*Oncorhynchus mykiss*) infected with *Vibrio anguillarum*, the causative agent of the acute disease vibriosis, had elevated levels of

Hsp70 in hepatic and head kidney tissues prior to clinical signs of disease (Ackerman and Iwama, 2001). Collectively, these data provide early evidence that a relationship exists between heat shock proteins and disease in fish.

There are several plausible links between heat shock proteins and the immune system in organisms that are faced with bacterial challenges (Young, 1990), including fish. Perhaps the simplest connection is that virulent pathogens may damage components within a cell through the release of cytolytic substances, thus altering cellular homeostasis and inducing heat shock proteins. In fish, the inflammatory pathology caused by pathogenic exposure may alter physiological processes at the cellular level, such as ion regulation and acid-base balance. Host immunocytes (phagocytes and granulocytes) release extracellular substances such as reactive oxygen species, cationic peptides, lysozyme, and cytokines that are known inducers of various heat shock proteins (Jacquier-Sarlin et al., 1994). The exogenous administration of heat shock proteins can also upregulate two major macrophage/monocyte differentiation markers (Edgington, 1995), and studies have demonstrated that host cells can recognize small tumour-related peptides when complexed with Hsp70 (Blachere et al., 1993). Therefore, heat shock proteins may be an integral part of the MHC-class II peptide complex assembly (DeNagel and Pierce, 1993) lending support to the hypothesis that heat shock proteins are involved in antigen presentation (Bachelet et al., 1998). Heat shock proteins may also be important in providing maintenance (Polla et al., 1995) and protection to phagocytic cells (Maridonneau-Parini et al., 1993) by repairing damage or protecting against autolysis or apoptosis due to auto-oxidation brought about by the cells own internal defence systems. Further studies are necessary to elucidate the relationship between the immune system and heat shock proteins, and to resolve how their production assists fish in responding at the immunological level to an infectious challenge. Such studies can not only help us to understand disease states more clearly, but can assist in the formulation of strategies to protect against them.

5. Developmental regulation of heat shock proteins

Zebrafish (*Danio rerio*) are ideal candidates to explore the role of heat shock proteins in ontogeny because zebrafish development has been extensively studied and currently there is a substantial sequencing effort on this species (Kelly et al., 2000). Comparative studies on model organisms, such as *Xenopus* and *Drosophila*, suggest that heat shock proteins assume specific cellular functions during embryogenesis. Generally, those functions serve to sustain the development of the whole organism rather than solely maintaining cellular processes in a 'housekeeping' fashion (Morange, 1997). Based on the apparent ubiquity of heat shock protein function, observations in other organisms can serve as a good starting point to develop experimental hypotheses in fish. An understanding of the role particular heat shock proteins have during fish development may help researchers resolve the function of these heat shock proteins during later life history stages.

Krone and Sass (1994) observed low levels of constitutive *hsp90α* in developing zebrafish, but this gene was strongly induced following a heat stress in the gastrula and later stage embryos. On the other hand, constitutive levels of *hsp90β* were high, relative to *hsp90α*, but only weakly induced following a similar heat stress (Krone and Sass, 1994). Therefore, within a specific heat shock protein family (i.e. Hsp90), isoforms exist that are differentially regulated and as a consequence, may have different cellular functions during developmental and later life stages. Subsequent localization studies revealed that constitutive *hsp90α* was restricted primarily to regions of the developing zebrafish that are involved in myogenesis, including the pectoral fin buds and a subset of cells within the somites (Sass et al., 1996). Similar to *hsp90α*, *hsp47* was highly induced in response to stress (Pearson et al., 1996) and has been implicated in aiding the formation of fish embryonic tissues based on its association with procollagen (Krone et al., 1997; Lele et al., 1997). These data demonstrate that heat shock proteins may serve specific roles during development, such as myogenesis, in addition to their cellular 'housekeeping' functions.

Lele et al. (1997) demonstrated that basal levels of a stress-inducible *hsp70* were low during embryogenesis, but were elevated significantly in a tissue- and stress-dependent manner when zebrafish were exposed to various stressors. For example, embryos exposed to heat stress had elevated levels of *hsp70* throughout the embryo, but those exposed to ethanol had elevated levels of *hsp70* localized primarily in the head region. Contrary to the above findings, Santacruz et al. (1997) isolated a cognate member of the Hsp70 family, and demonstrated that this constitutive *hsp70* was: (a) expressed strongly during normal zebrafish development; (b) slightly increased following heat stress; and (c) significantly increased at the onset of somitogenesis and neurogenesis. Taken together, these two studies demonstrate that a constitutive and stress-induced form of *hsp70* exist in the developing zebrafish. Since these heat shock proteins are differentially expressed in a spatial, temporal, and stress-specific manner, they likely serve specific roles in embryonic development that need to be resolved. An interesting aspect of fish development was observed by Santacruz et al. (1997) who noticed that constitutive *hsp70* is maternally derived in zebrafish. This observation raises questions related to the physiological significance of maternal transfer, including: (a) does this transfer provides protection for the embryo; (b) are other families of heat shock proteins transferred; (c) how does the health status of the mother affects the amount of heat shock protein transferred; and (d) is this phenomenon observed in other species of fish?

6. Effects of hormones on heat shock proteins

Hormones affect a wide range of physiological systems, and recent data suggests that they may also regulate heat shock protein levels in fish (Deane et al., 1999; Iwama et al., 1999; Sathiyaa et al., 2001). A series of studies demonstrated that elevated levels of cortisol can suppress the heat stress-related induction of: (a) Hsp30 in gill tissues of cutthroat trout (*Oncorhynchus clarki clarki*; Ackerman et al., 2000); (b) Hsp70 in hepatic and gill tissues of rainbow trout (*Oncorhynchus mykiss*; Basu et al., 2001); (c) Hsp70 in gill tissues of Mossambique tilapia (*Oreochromis mossambicus*; Basu et al., 2001); and (d) *hsp90* mRNA in primary cultures of rainbow trout hepatocytes (Sathiyaa et al., 2001). Given that the glucocorticoid receptor mediates the physiological effects of cortisol (Wendelaar Bonga, 1997), and the function, assembly and transport of the glucocorticoid receptor depends on its association with Hsp56, Hsp70, and Hsp90 (Pratt, 1993; Pratt and Welsh, 1994), studies are required to understand the nature of the cortisol-glucocorticoid receptor-heat shock protein complex. It has recently been demonstrated that the glucocorticoid receptor heterocomplex contains Hsp70 in rainbow trout hepatic tissues (Basu, N., University of British Columbia, unpublished data). Elevated levels of cortisol in fish can down-regulate the number of cellular glucocorticoid receptor units (Pottinger, 1990; Maule and Schreck, 1991). After cortisol binds to the glucocorticoid receptor, Hsp70 is displaced from the mature receptor complex (Pratt, 1993) and this free Hsp70 may act through a negative feedback loop to inhibit heat shock transcription factor trimerization and subsequent heat shock protein induction (Morimoto et al., 1992). With increased amounts of circulating cortisol, perhaps due to physiological stress, the cellular levels of Hsp70 may be further depleted since levels of non-activated or unbound glucocorticoid receptor are reduced. However, these hypothetical scenarios of heat shock protein regulation require further experimental support and validation. Fish are ideal models to study the interaction between the organismal and cellular stress responses since: (a) they are a vertebrate species with complex physiological organ systems; (b) much information is already known regarding their stress physiology; and (c) they can easily be maintained in a laboratory setting.

In contrast to cortisol, adrenaline may augment levels of heat shock protein in fish. Rainbow trout hepatocytes exposed to adrenaline, with and without heat stress, had significantly elevated levels of Hsp70 protein. This response was abolished when cells were exposed to propranolol (a β-adrenoreceptor blocker; Ackerman et al., 2000). Furthermore, in vivo studies on the silver sea bream (*Sparus sarba*) demonstrated that administration of adrenaline increased *hsp70* expression, 1.8- and 3.2-fold in the branchial and hepatic tissues, respectively (Deane, E., Woo, N.Y.S., unpublished data presented at the 4th International Symposium on Fish Endocrinology). Considering the contrasting effects of cortisol and adrenaline on heat shock proteins in fish, further studies are required to understand why these endocrine stress responses mediate heat shock protein levels in apparently opposite manners.

Other hormones have recently been demonstrated to affect heat shock protein levels in fish. Deane et al. (1999) observed that exogenous administration of growth hormone and prolactin resulted in decreased levels of both hepatic *hsp70* mRNA (42 and 54%, respectively) and Hsp70 protein (76 and 64%, respectively) in the silver sea bream. Subsequent studies observed that sulpiride (a prolactin stimulant) suppressed *hsp70* expression, and bromocriptine (a prolactin suppressant) induced *hsp70* expression in the silver sea bream, suggesting levels of circulating prolactin mediate Hsp70 levels in this fish species (Deane et al., 2000). Collectively, these data provide an early indication that there are interactions between heat shock proteins and hormone-mediated, physiological processes such as the organismal stress response (cortisol and adrenaline), growth (growth hormone), and osmoregulation (prolactin and cortisol). A complete characterization and understanding of all the genes involved in these processes will greatly improve our understanding of the functional relationship between the endocrine system and heat shock proteins.

7. Heat shock proteins and environmental adaptation in fish

Although heat shock proteins have a relatively short half-life (6–9 h in *Drosophila*; Lindquist, 1986), their levels remain elevated in organisms long after the stressor is removed. Because of the persistence of heat shock proteins, it has been proposed that heat shock proteins play a role in the long-term adaptation of animals to their environment (Parsell and Lindquist, 1993; Morimoto and Santoro, 1998). It has been well documented that there is a correlation between the expression of heat shock proteins and thermotolerance (an increased resistance to heat; Li and Hahn, 1978; Hahn et al., 1985; Russotti et al., 1996). Studies in fish have shown that the appearance and decay of heat shock proteins share a close temporal relationship with the induction and disappearance of thermotolerance (Mosser et al., 1987; Mosser and Bols, 1988). The role of heat shock proteins in thermotolerance appears to be crucial, since the inhibition of heat shock protein synthesis prevents the development of thermotolerance in rainbow trout fibroblasts (*Oncorhynchus mykiss*; Mosser and Bols, 1988). Studies in transgenic *Drosophila* that over-express *hsp70* support the idea that heat shock proteins are responsible, at least in part, for induced thermotolerance (Feder and Krebs, 1998). Therefore, it is believed that heat shock proteins are the primary mediators of this thermotolerance, although the sensor for the stressor and the regulation of heat shock protein induction are not clearly understood.

Studies on fish in their natural environments also suggest a clear link between thermal stress and heat shock proteins. Hightower et al. (1999) mapped threshold temperatures for heat shock protein induction for two tropical fish species and six desert species onto a thermal preference profile. From that profile, they revealed that the threshold temperature (33 °C) for Hsp70 induction was closely linked to the temperature most preferred by these fish. On the other hand, they found that the threshold temperature (37 °C) for Hsp30 induction was closely linked to high temperatures that these fish rarely choose. Therefore, the correlation between the threshold for heat shock protein expression and the levels of stress that an animal naturally experiences is becoming well established (reviewed by Feder and Hofmann, 1999).

Some authors have reported that threshold temperatures for heat shock protein induction and thermal tolerance of fish may be strictly genetically controlled, while others have concluded that the threshold for heat shock protein induction can be modified by acclimation and acclimatization. Koban et al. (1987) demonstrated that different acclimation temperatures did not affect the threshold temperature for Hsp70 induction in catfish (*Ictalurus punctatus*) hepatocytes. In a series of studies on hemiclone hybrids of viviparous desert fish (genus *Poeciliopsis*), Dilorio et al. (1996) demonstrated that survival was greatest when *Poeciliopsis monacha* genomes were combined with a sympatric *Poeciliopsis lucida* genome. Quantification of constitutive and stress inducible forms of Hsp70 indicated that variation in survival among hybrid fish was best explained by the combined effects of these two proteins, suggesting that heat shock proteins contribute to thermal adaptation through genetic mechanisms. These reports support the idea that heat shock protein expression and the thermal tolerance of a species is genetically controlled. In contrast, it has been widely demonstrated that threshold temperatures for heat shock protein induction are primarily influenced by the recent thermal history of an animal. Dietz and Somero (1993) demonstrated that summer-acclimatized gobies (genus *Gillichthys*) had significantly higher levels of brain Hsp90 than winter-acclimatized fish, and that the threshold induction temperature for Hsp90 was 4 °C higher in the summer-acclimatized group. Fader et al. (1994) demonstrated that tissue levels of constitutive Hsp70 in four species of stream fish (*Pimephales promelas*, *Salmo trutta*, *Ictalurus natalis*, and *Ambloplites rupestris*) varied according to seasons, with the highest levels recorded in the spring, followed by the summer, fall, and winter. Furthermore, Norris et al. (1995) demonstrated that the threshold temperature for heat shock protein induction in many species of desert fish (genus *Poeciliopsis*) was closely linked to their thermal history. Collectively, these data suggest that heat shock protein expression is subject to acclimatization, and that heat shock proteins may fluctuate in response to normal variations in seasonal temperature in the aquatic environment.

There are clearly two different groups of studies, one of which suggests that the threshold induction temperatures for heat shock proteins is genetically controlled, while the other argues that this threshold is modified by the environment. Disagreement between these two groups of studies may have been caused by differences in species and in experimental design. In particular it is possible that differences in species' thermal history over recent or evolutionary time may have a profound influence on the relative importance of purely genetic or environmental regulation of heat shock protein induction.

Intertidal fish may be good models to study the effect of short-term changes in thermal history on the cellular stress response of fish, since they are exposed to daily and hourly fluctuations in water quality and temperature. We have recently observed that there is a disparity in the cellular stress response between laboratory- and field-acclimatized tidepool sculpins (*Oligocottus maculosus*; Nakano, 2000). Results showed that field-acclimatized sculpins had a higher survival rate than laboratory-acclimatized sculpins following a thermal challenge. Furthermore, basal levels of hepatic Hsp70 were the same between these two groups, but the field-acclimatized group had significantly higher inducible levels of hepatic Hsp70 than the laboratory-acclimatized group following a heat stress. These data suggested the possibility that recent short-term thermal history can influence the cellular stress response within a species, and that thermal tolerance of intertidal fish may not be genetically hard-wired, but is likely affected by environmental thermal conditions.

On the other hand, an extreme example of the effects of long-term stable thermal conditions has been demonstrated in an Antarctic fish, *Trematomus bernacchii*. Even though the cellular stress response is generally accepted as a common feature in a wide variety of organisms (Feder and Hofmann, 1999), some organisms under extreme conditions may have lost the ability to invoke a measurable cellular stress response. The levels of Hsp70 in the brain, white muscle, gill tissues (Carpenter and Hofmann, 2000) and hepatocytes (Hofmann et al., 2000) of *T. bernacchii* did not significantly increase when fish were subjected to a thermal challenge. Since the thermal environment of the Antarctic Ocean is extremely stable (Carratù et al., 1998) and very little chemical pollution exists in this region, Hofmann et al. (2000) concluded that there might not have been a strong selective pressure for these fish to retain a potentially energetically costly stress response. These data suggest that Antarctic fish may have lost the ability to respond to heat stress at a genetic level, and that changes in environmental temperature may not have a significant effect on the cellular stress response and thermal tolerance of *T. bernacchii*.

Most studies on heat shock proteins in an environmental context have focused on the effects of heat stress; however, natural environments are highly complex and fish are often exposed to multiple stressors. Cross-protection, also known

as cross-tolerance, is the ability of one stressor to transiently increase the resistance of an organism to a subsequent heterologous stressor. This cross-protection may be a critical feature of the cellular stress response in an environmental context.

The earliest studies of cross-protection in fish were performed in vitro in the winter flounder (*Pleuronectes americanus*). Exposure of the renal epithelium to a heat stress protected these cells against the deleterious effects of a subsequent extreme temperature or chemical challenge (Brown et al., 1992). These researchers showed that protection afforded by a mild heat shock coincided with increased levels of Hsp28, Hsp70, and Hsp90 in the cell. Subsequent work demonstrated that low levels of zinc had similar protective effects (Renfro et al., 1993). DuBeau et al. (1998) reported that heat stressed Atlantic salmon (*Salmo salar*) that had artificially elevated levels of branchial and hepatic Hsp70 were better able to tolerate an osmotic challenge, relative to control fish. This cross-protection was only observed during the two month period coinciding with parr-smolt transformation, suggesting a role for heat shock proteins in ionic and osmotic adaptation. This may provide evidence of a fish's ability to regulate their heat shock protein gene expression to enhance their tolerance to an upcoming environmental change. Whether fish can mediate changes in heat shock protein levels in anticipation of predictable changes in their environment is an interesting aspect of heat shock protein biology that needs further study. Taken together, these studies provide strong evidence of the ability of one stressor to condition a fish to better tolerate a subsequent, more severe stressor. Heat shock proteins appear to be integral in this process; however, the regulation of heat shock protein genes and their cellular functions that ultimately culminate in stress tolerance at the organismal level are unknown and warrant future investigation.

There is strong evidence suggesting that heat shock proteins have a critical role in helping fish cope with environmental change. Their involvement in inducible stress tolerance (thermotolerance and cross-protection) raises some fundamental questions regarding the regulation of this protection and whether fish in nature can be conditioned by one stressor to better tolerate a subsequent insult. Clearly the regulation of heat shock proteins in fish has both a genetic and an environmental component. By studying fish in their natural environments we can begin to tease out this complex and highly integrated relationship, and shed some light on the relative importance of recent or long-term environmental history in regulating the cellular stress response.

8. Conclusions

Heat shock proteins are known to play a pivotal role in protein homeostasis and the cellular stress response within the cell (Lindquist, 1986; Feder and Hofmann, 1999). However, despite decades of extensive investigations a number of outstanding questions remain. Feder and Hofmann (1999) suggested that future experiments are required to: (a) resolve how heat shock protein genes, their regulation, and function have co-evolved in response to environmental change, and (b) how the action of heat shock proteins at the molecular level leads to whole-organismal stress tolerance. We propose that one of the fundamental questions about the role of heat shock proteins is the functional relationship between the cellular stress response, the organismal stress response, and physiological processes at higher levels of biological organization. This linkage between genomics and physiology has seldom been addressed, but will be critical for understanding the responses of organisms to their environment.

Fish are ideal models for addressing this question as they are naturally exposed to thermal and other complex stressors in their natural environment, and offer an excellent vertebrate model to investigate the physiology, function, and regulation of heat shock proteins. DNA sequences are now becoming available for heat shock proteins in fish, providing the tools necessary to begin functional genomic research in fish. Even in a context where complete sequence information is not known, it is possible to carry out preliminary functional genomic experiments. Gracey et al. (2000) used DNA microarrays to study gene expression in *Gillichthys mirabilis*, even though little prior sequence information was available for this species. This indicates that functional genomics studies can be carried out in any species.

With the development of new tools, we will be able to revisit old questions regarding the roles and regulation of heat shock proteins and gain new insights into their functional importance in fish. Heat shock proteins are collectively only one of the molecular mechanisms that animals utilize to tolerate stress, and these proteins can have pleiotropic effects, interacting with multiple systems in diverse ways. Thus, the cellular stress response has impacts on, and is influenced by, processes at all levels of biological organization. Functional and evolutionary genomics approaches will be critical for understanding the complex and integrative regulatory mechanisms that animals invoke in order to cope with changes in their natural environment.

Acknowledgements

Operating grants from AquaNet (Networks of Centres of Excellence - Canada) and the Natural Sciences and Engineering Research Council (NSERC) of Canada to Drs George K. Iwama and Patricia M. Schulte supported the writing of this paper and unpublished data presented within. We would like to express a special thanks to Dr S. Samuels for critical comments on the manuscript.

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