

Heat shock protein expression in fish

GEORGE K. IWAMA^{1,*}, PHILIP T. THOMAS[‡], ROBERT B. FORSYTH¹
and MATHILAKATH M. VIJAYAN^{1,§}

¹*Department of Animal Science, and the Canadian Bacterial Diseases Network, University of British Columbia, Vancouver, BC, Canada V6T 1Z4*

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Abstract

Heat shock proteins (HSP) are a family of proteins expressed in response to a wide range of biotic and abiotic stressors. They are thus also referred to as stress proteins. Their extraordinarily high degree of identity at the amino acid sequence level and the fact that this cellular stress response has been described in nearly all organisms studied, make this group of proteins unique. We provide a brief historical overview of HSP research, as a background to summarizing what is known about HSP expression in fish.

The expression of HSPs in fish has been described in cell lines, primary cultures of various cells, and in the tissues of whole organisms. Collectively, the data show that the expression of HSPs are affected in a wide variety of fish cells and tissues, in response both to biological stressors such as infectious pathogens, as well as to abiotic stressors such as heat and cold shock, and environmental contaminants. HSP research in fish is in its early stages and many studies are describing the expression of proteins in response to various stressors. Several studies have contributed to our understanding of the molecular nature and the molecular biology of HSPs in fish. Recent studies have shown a relationship between HSP expression and the generalized stress response in

* Author to whom correspondence should be addressed. E-mail: giwama@unixg.ubc.ca

‡ Present address: Union Christian College, Dept. of Zoology, Alwaye-683 102, Kerala, India.

§ Present address: Bio-Stress Research, Vancouver, BC, Canada.

fish, but further research is needed to clarify the complex relationships between stress hormones and the cellular HSP response. In general, the HSP response seems to be related to the sensing of the stressor and the subsequent cellular effects which may adapt the cells to cope with the stressors. Consequently, such data may be of central importance in understanding the significance of HSP expression to the whole organism. We conclude with sections on laboratory methods used in HSP research and on potential applications of this knowledge in biomonitoring.

Keywords: heat shock proteins, HSP, stress proteins, stress, generalized stress response, biomonitoring

General background

Heat shock proteins (HSPs) are a group of intracellular proteins that have an unusually high degree of identity at the amino acid level, among diverse organisms. As this family of proteins is induced by stressors other than heat, they are also commonly referred to as 'stress proteins' in the literature. The term stress proteins also may refer to several other groups of proteins that respond to stressors. For example, metallothioneins, which are expressed in response to heavy metal exposure, or cytochrome P450 enzymes, or HSPs, all may be considered as stress proteins. In contrast to the general nature of the term stress proteins, the HSP nomenclature is more commonly used in naming, and in reference to, specific HSPs. This review is concerned only with stress proteins that, to our knowledge, are HSPs. Thus, the HSP nomenclature is used. In the last three decades, there has been an exponential increase in the interest and research activity concerning the description, classification and functional significance of these proteins. Heat shock proteins are constitutively expressed in cells to maintain a number of critical cellular processes relating to protein folding, fidelity and translocation. These proteins also are induced in cells in response to a variety of stressors and enhance survival by protecting vital cellular functions.

The discovery of HSPs is often attributed to the reporting of chromosomal puffs in the salivary gland cells of the fruit fly, *Drosophila busckii*, soon after a heat shock (Ritossa, 1962). As Mitchell and Peterson (1982) state in their review, stress effects on specific gene expression leading to certain phenotypes have been reported since the mid-1930s. The special nature of the HSPs came to light in the decade after Ritossa's observations, as various investigators described special characteristics of this group of proteins. Investigators such as Berendes *et al.* (1965), Ashburner (1970), and Mitchell *et al.* (1974) showed that the heat-induced puffs in the *Drosophila* salivary gland chromosomes occurred within minutes of the heat shock, and that the novel group of proteins were associated with those puffs. Mitchell *et al.* (1974) also reported that a number of constitutive proteins disappeared as the novel HSPs were being produced. Tissieres *et al.* (1974) showed that these proteins did not occur exclusively in the salivary glands. They reported HSP expression also in brain, Malpighian tubules, and wing marginal discs of the fruit fly. Definitive evidence that specific HSPs are expressed from genes in the chromosome puffs came in the late 1970s when it was shown that purified mRNA which hybridized to the heat shock puffs were translated to specific HSPs when added to *in vivo* systems (Lindquist-McKenzie *et al.*, 1975; Spradling *et al.*, 1977; Mirault *et al.*, 1978). A similar heat shock response has been found in cells and tissues of many other organisms such as chick embryo fibroblasts (Kelly and Schlesinger, 1978), *E. coli* (Lemeux *et al.*, 1978; Yamamori *et al.*, 1978),

yeast (McAlister and Finkelstein, 1980), and plants (Barnett *et al.*, 1980). By using such purified heat shock mRNA fractions, HSP genes were among the first to be cloned in eukaryotes. Subsequently, nucleotide sequences became available and the unusually high degree of similarity in certain HSP groups, among diverse organisms, became known. For example, HSP70 is one of the most highly conserved of HSP groups. It has been found in all studied cells, except for one species of hydra (*Hydra oligactis*; Bosch *et al.*, 1988), and the amino acid sequence identity values range from 42% to more than 57% between organisms as diverse as *E. coli* and humans (Gupta and Golding, 1993). Given that equivalent comparisons for other highly conserved proteins such as ATPase, glutamine synthetase and RNA polymerase are in the range of 13–58%, 7–41% and 22–30%, respectively (Gupta and Golding, 1993), the high degree of amino acid sequence identity among the HSP70 in such divergent organisms is remarkable. The DNA sequences for HSP70 are more than 50% similar among bacteria, yeast and *Drosophila* (Craig *et al.*, 1979).

The naming of HSPs are generally based on their molecular mass (kilodaltons, kDa) as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Heat shock proteins are also grouped according to function (e.g. chaperonin), DNA sequence, and antibody cross-reactivity. The commonly used categories are: 100 kDa; 90 kDa; 70 kDa; 60 kDa; and the 16–30 kDa group, and are usually referred to as HSP100, HSP90, HSP70, HSP60, and the low molecular weight (LMW) class of proteins, respectively (Morimoto *et al.*, 1994).

The HSPs are known to play vital cellular roles including protein assembly, correct folding, and translocation, as well as regulating interactions between hormones and their receptors (Welch, 1993). Research in this area continues to grow at an ever-increasing rate, and applications to problems and opportunities in human health and environmental monitoring are developing rapidly.

Heat shock protein studies in fish are still in the early stages compared with those in bacteria, yeast and mammals. Studies in fish are still in the descriptive stages of documenting novel proteins that are produced in various tissues in response to a variety of biological and abiotic stressors. The primary objective of this review is to summarize what is currently known about HSP expression in fish cell lines, in fish cells in primary culture, and in whole animals. The current models of the molecular processes that are involved in HSP expression as well as the molecular characteristics of fish HSPs are described. There is a discussion of what is known about the relationship between the generalized stress response and HSP expression in fish and other animals. A section on the commonly used methodology in HSP research is included to benefit those new to this field. The review concludes with speculations as to the feasibility of using the expression of HSPs in biomonitoring of the aquatic environment, through the detection of stressed states in fish.

Molecular processes related to HSP expression

We are far from an unequivocal understanding of the molecular processes that underlie the sensing of a stressor and the regulation of HSP production. It is clear, however, that HSP induction results mainly from the binding of activated heat shock transcription factor (HSF) to key promoter regions, called the heat shock elements (HSE), in front of the heat shock genes. The major heat shock genes do not contain introns and so the messenger RNA can be immediately translated into new proteins within minutes of

exposure to the stressor. Although yeasts and fruit flies have only one HSF, two or more HSFs have been reported in the tomato, humans, mice and chicken. Only one of these HSFs, HSF1, is apparently able to regulate the heat shock genes (Baler *et al.*, 1993; Sarge *et al.*, 1993; Voellmy, 1996). Heat shock factors from the various plants and animals studied show a high degree of structural similarity. There is a DNA-binding domain of about 100 amino acids at the amino terminus (Fig. 1). Adjacent to the DNA-binding domain is a region containing three leucine zipper repeats that are key to the trimerization of HSF1 (see below). Both the DNA-binding and leucine zipper 1–3 (oligomerization domain) regions are highly conserved across diverse organisms. There is 85–95% amino acid sequence identity between human, mouse and chicken HSF1 (Morimoto *et al.*, 1994). In the fruit fly and higher eukaryotes, there is an additional leucine zipper 4 at the carboxy-terminus of HSF1. The vertebrate HSF has a short element known as the carboxy-terminal repeat (CTR) (Rabindran *et al.*, 1993). Stress does not change the amount of HSF1 (Rabindran *et al.*, 1991), but changes the inactive form of HSF1 to a transcriptionally active state that results in the expression of HSP70. What is yet unknown is the cause and mechanism of HSF1 activation and the nature of the sensor of the stressor in the cell.

A model for the regulation of HSP expression based on proteotoxicity has been supported by experimental evidence (Morimoto *et al.*, 1996; Voellmy, 1996). The proteotoxicity model is based on the observations that denatured or foreign proteins are potent inducers of HSPs. The model is described briefly here and represented in Fig. 2. As mentioned above, a wide range of stressors cause the expression of HSPs in the cell (Welch, 1993). According to this model, all such stressors would act by damaging native proteins inside the cell, and involve HSP70 as the key sensor and mediator of the essential events leading to further HSP70 production. HSF1 is found as a monomer (Westwood *et al.*, 1991; Baler *et al.*, 1993; Sarge *et al.*, 1993) or as a heterodimer complexed with HSP70 (Baler *et al.*, 1992, 1995; Rabindran *et al.*, 1994) in the cytosol of unstressed cells. As the result of a stressor, the misfolded or damaged proteins sequester HSP70 and other chaperone proteins to aid their repair or destruction.

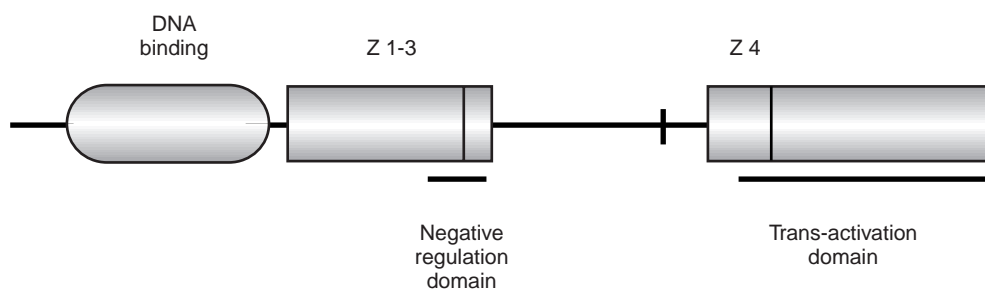


Fig. 1. Diagram of heat shock factor (HSF1) structure showing DNA binding (carboxyl-terminus), leucine zippers (Z) 1–3, and leucine zipper 4 structures. Thick bars indicate areas of constitutive phosphorylation. Evidence supports a model whereby interactions between Z1–3 and Z4 result in an inert monomeric form of HSF1 under control conditions (see Fig. 2). Heat shock and other stressors lead to unfolding of this inert form and new interactions between Z1–3 regions of separate monomeric HSF1 units, leading to trimerization and other events depicted in Fig. 2. Drawing was based on a diagram in Morimoto *et al.* (1997). See text and Morimoto *et al.* (1996) for details.

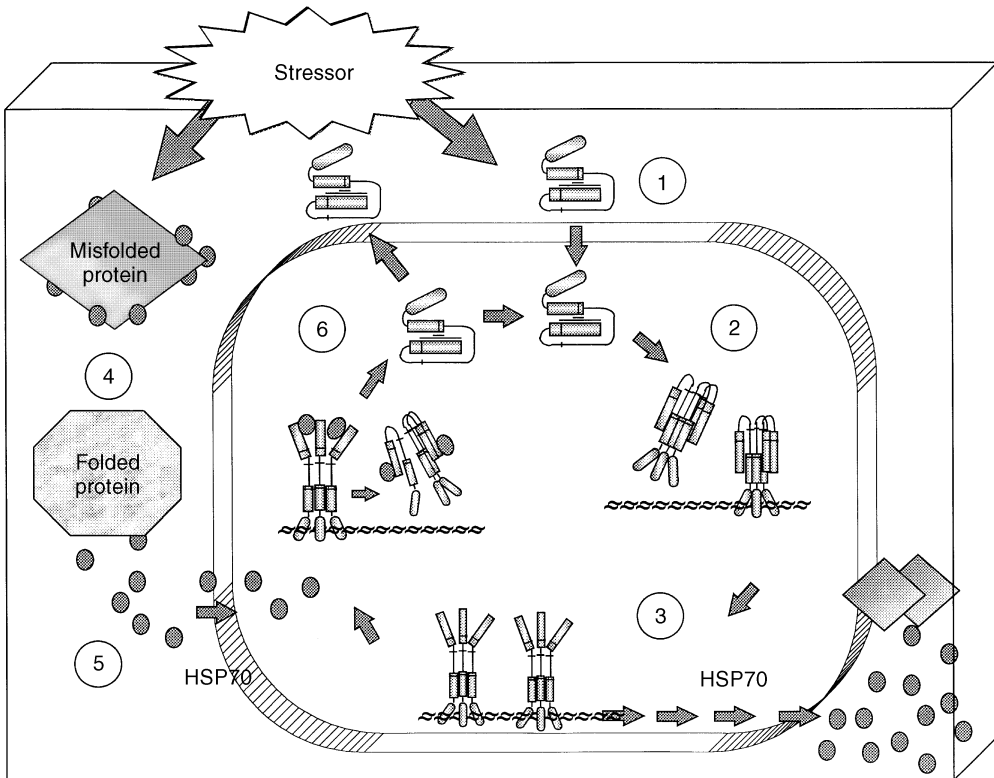


Fig. 2. Model of the role of heat shock factor (HSF1) in the regulation of heat shock protein expression. Drawing was made after diagrams in Morimoto *et al.* (1996). (1) Latent monomeric form of HSF1 in cytoplasm or nucleus. (2) Activation by stressor leads to trimerization of HSF1, which facilitates binding to heat shock element of the HSP70 gene promoter. (3) Phosphorylation and activation of HSF1 transcription results in HSP70 expression. (4) Increase in cytosolic HSP70 repairs misfolded proteins, along with other HSPs. (5) Subsequent repair of damaged proteins results in higher levels of free HSP70 in cytosol. (6) Higher concentration of HSP70 results in binding to HSF1, facilitating release from DNA, and dissociation of HSF1 back to the monomeric form.

According to this proteotoxicity model, HSP70 that is normally bound to the monomeric form of cytosolic HSF1, possibly as part of larger multiprotein aggregates, is dissociated away from such protein complexes (Morimoto *et al.*, 1996). This results in the translocation of HSF1 into the nucleus, and a change from the latent state of the monomeric HSF1 to a trimeric state, owing to interactions among the oligomerization domains of HSF1. The trimeric form is able to bind to the promoter regions of the heat shock genes, but remains transcriptionally inactive (Jurvich *et al.*, 1992; Bruce *et al.*, 1993) until stress-induced phosphorylation of the HSF1 takes place (Cotto *et al.*, 1996; Morimoto *et al.*, 1996). Upon phosphorylation of HSF1, HSP70 is expressed through the normal transcription and translation processes. There is evidence to suggest that the binding of free HSP70 to HSF1 is needed for the release of the HSF1 bound to HSE of the DNA, and for subsequent dissociation of the trimeric form back to the inactive

monomers that move back into the cytoplasm. During recovery, such free HSP70 would come both from the newly formed protein as well as from those that were bound by the damaged proteins that may have released them as a result of repair or destruction. The release of HSF1 from the DNA occurs at a much faster rate (approximately 10×) *in vivo* than *in vitro*, suggesting some active releasing mechanism in the intact organism (Abravaya *et al.*, 1991).

There are several other possible signal transduction mechanisms for sensors of the stressors that initiate and/or regulate HSP production. For example, redox changes in the cell might serve as a regulator of HSP production (Voellmy, 1996). Huang *et al.* (1994) have shown that reducing agents prevent the induction of HSF oligomerization and DNA-binding activity that would otherwise take place in HeLa cells exposed to stressors such as mild heat shock, hypoxia, hydrogen peroxide or dinitrophenol. Another possibility is a role that mitogen-activated protein (MAP) kinase cascades in the cell play in the heat shock response (Bensaude *et al.*, 1996). Various stressors that cause a cellular stress response also cause an increase in intracellular calcium concentration and the stimulation of enzymes such as phospholipases C and A2, both of which are important second messengers in the heat shock response (Calderwood *et al.*, 1993; Kiang and McClain, 1993), and thus in triggering intracellular signal transduction cascades. These processes may then affect the phosphorylation of the HSF1, described above, which is key to the regulation of HSP production. Furthermore, heat sensors that trigger MAP kinase cascades seem to be located in the plasma membranes, thereby suggesting that physical changes to the membrane (e.g. stretch, fluidity) may also be involved in the heat shock response (Bensaude *et al.*, 1996).

Heat shock protein studies in fish

Studies of HSPs in aquatic organisms other than fish have recently been reviewed by Sanders (1993). That review summarized studies in algae, Protozoa, Cnidaria, Rotifera, Nematoda, Mollusca, Arthropoda and Echinodermata, and discussed the possible physiological roles of HSPs in aquatic organisms in relation to environmental perturbations. Studies of HSPs in fish may be divided into three categories: those that involve work with fish cell lines; experiments that involve primary cell culture preparations; and those that involve whole fish exposed to stressors. The vast majority of studies are focused on the effects of heat shock: cataloguing the various HSPs induced, determining the onset and duration of HSP expression, and studying the role of HSP expression in thermotolerance. However, the emerging data are giving some insights into the significance of HSP expression in fish at the organismal level.

CELL LINE STUDIES

A number of fish cell lines have been used in HSP studies and the HSP responses of these cell lines to different stressors are listed in Table 1. Although the induction of identical HSPs within a particular cell line for a particular treatment, such as heat shock, has not been found by different investigators, there are some noteworthy trends. In all cases where it was measured, the induction of HSPs is a reversible process. In a comprehensive study of the HSP response of the CHSE cell line to heat shock and heavy metal exposure, Heikkila *et al.* (1982) found that the new proteins of 68, 70 and 84 kDa increased as a general response to all stressors, while new proteins of 65 and 41 kDa, and

Table 1. Heat shock proteins expressed in fish cell lines exposed to various stressors

Treatment	Cell lines ¹	HSP -kDa	Reference
Heat shock	RTG-2	19, 27, 30, 39, 60, 68, 70, 87, 100, 200	Kothary and Candido (1982), Kothary <i>et al.</i> (1984a), Mosser <i>et al.</i> (1986), Mosser and Bols (1988)
Heat shock	RTH-149	28, 70, 74, 84, 95	Misra <i>et al.</i> (1989)
Heat shock	CHSE-214	41, 65, 68, 70, 80; *12 proteins from 21 to 95; †90	Heikkila <i>et al.</i> (1982), *Gedamu <i>et al.</i> (1983), Misra <i>et al.</i> (1989), †Cho <i>et al.</i> (1997)
Heat shock	RBCF-1	30, 42, 70, 90	Mitani <i>et al.</i> (1989)
Heat shock	FHM	22, 36, 39, 47, 57, 60, 70	Merz and Laudien (1987)
Heat shock	TO-2	27, 28, 70, 87, 100	Chen <i>et al.</i> (1988), Wang <i>et al.</i> (1989)
Cold shock	RTG-2	70	Yamashita <i>et al.</i> (1996)
Infectious haematopoietic necrosis virus	CHSE-214	90	Cho <i>et al.</i> (1997)
Copper sulphate	CHSE-214	90	Cho <i>et al.</i> (1997)
2-Mercaptoethanol	CHSE-214	90	Cho <i>et al.</i> (1997)
Zinc chloride, cadmium chloride; ‡cadmium sulphate	RTH-149 and CHSE-214; ‡CHSE-214	14, 28, 46, 50, 51, 65, 67, 68, 70, 84, 95; ‡90	Heikkila <i>et al.</i> (1982), Misra <i>et al.</i> (1989), ‡Cho <i>et al.</i> (1997)
Sodium arsenite	RTG-2	70	Kothary and Candido (1982), Burgess (1984), Kothary <i>et al.</i> (1984a)

¹RTG-2, rainbow trout (*Oncorhynchus mykiss*) gonadal cells; RTH-149, rainbow trout hepatoma cells; CHSE-214, chinook salmon (*O. tshawytscha*) embryonic cells; RBCF-1, goldfish (*Carassius auratus*) caudal fin cells; FHM, fathead minnow (*Pimephales promelas*) epithelial cells; and TO-2, tilapia (hybrid of *Tilapia mossambica* and *T. nilotica*) ovary cells.

others of 28, 46 and 51 kDa, increased specifically in response to heat shock and metal exposure, respectively. Cho *et al.* (1997), also working with a CHSE cell line (CHSE-214), showed the expression of a novel 90 kDa HSP in response to infection by a rhabdovirus, heat shock, as well as exposure to 2-mercaptoethanol, copper sulphate, and cadmium sulphate. Most investigators report that the increase in HSP70 was the most prominent response in most heat shock experiments. Mosser and Bols (1988), for example, found that 63% of the new proteins induced by a +6 °C heat shock to RTG-2 cells was in the 70 kDa class. In subjecting RTH and CHSE cell lines to both heat stress and heavy metal exposure, Misra *et al.* (1989) found the increase in HSP70 as a general response in both cell lines to all the stressors applied, but found the metallothionein protein to increase only in response to metal exposure, and only in the CHSE cell line. There is only one study showing the expression of HSPs to cold shock in fish cells. Yamashita *et al.* (1996) have shown the expression of a 70 kDa in the RTG-2 cell line, that is different from the heat shock HSP70 protein, but identical in some residues to the mammalian valosin-containing protein (VCP) and the yeast CDC48p, in response to acclimation at 4 °C. Because VCP and CDC48p are proteins associated with cell division, these investigators suggested that this response was a metabolic compensatory response to the reduction in cell division owing to the cold. The observed differences in the molecular masses of individual HSPs may well be attributable to the slight differences in techniques, such as in the experimental set-up, isolation of tissues and the molecular weight markers used in each study. Such factors may also be particularly important for the various reported time courses (e.g. time to: significant increase; maximum increase in concentration; return to control levels) for HSP induction. Our opinion is that, focusing on classes of HSPs, rather than on minor differences between individual HSPs, is more useful in gaining a reasonable understanding of which HSPs are expressed in a particular cell line for a given stressor. Several studies with transcription blockers such as actinomycin D have provided data which suggest that the transcription step of protein synthesis plays a major role in regulating the cellular HSP response to stressors (Heikkila *et al.*, 1982; Currie and Tufts, 1997). There is an apparent pattern for HSPs of high molecular masses (e.g. 60, 70, 90 kD) to be more highly conserved between different taxa of organisms, compared with those with low molecular masses (e.g. 16–30 kDa). The latter may be more species-specific, and as such may offer reasonable potential for diagnostic purposes among individual species, whereas the former may be used as indicators of non-specific stressors in a wide range of organisms.

The use of cell lines to study HSP expression has several advantages, including simplicity of maintenance, uniformity, and abundance of cells, and thus they are excellent experimental systems for examining different stressors. These characteristic benefits of working with cell lines have enabled detailed work to be conducted, such as: the separation of various isoforms within families or groups of induced proteins; the description of the time course of novel protein induction; and the study of possible functions of HSPs in the cell. The use of cell lines in this research has been vital to the knowledge of the roles HSPs play in normal homeostatic processes of the cell, such as ensuring proper protein folding, facilitating protein translocation between intracellular compartments, and the vital role they play in steroid–receptor interaction and function (Welch, 1993). Such research has also shown the importance of HSPs in inducing thermotolerance against severe heat shock in fish cell lines as in many other animal cells (Subjeck *et al.*, 1982; Mosser *et al.*, 1987; Mosser and Bols, 1988). However, one

must exercise caution in extrapolating the results from cell line experiments to whole fish because these cell lines are un-differentiated and cancerous, and thus may not truly represent the metabolic state of the cells *in vivo*. Also, stress is a phenomenon that is perceived and responded to by behaviour as well as all levels of physiological organization in the whole animal, and therefore, information from cell lines alone may not give a true picture of what occurs in the whole animal. Nevertheless, cell line work is useful in obtaining a general perspective of the range of HSPs that may be induced in response to a particular stressor. Cell lines can also be used for large-scale induction and subsequent isolation of relevant HSPs, which can be purified for the production of primary antibodies.

PRIMARY CELL CULTURE

Several studies have described the expression of HSPs in primary cultures of fish cells. The major advantage of primary culture over cell lines lies in the fact that the cells in primary culture still maintain their differentiated characteristics. Thus, they are being used as model systems for basic studies on the nature of the cellular stress response, as well as for metabolic and toxicological studies. Using primary cultures of hepatocytes from different species of the desert topminnows (*Poeciliopsis*, Poeciliidae), White *et al.* (1994) have shown that heat shock induces HSP30, HSP60, HSP70 and HSP100. Each of the six species studied showed 5–7 isoforms of the inducible HSP70, whereas the constitutive form of this protein was identical among the species. They found that the induced HSP60 and HSP100 was identical in all but one species, and that the form of the HSP30 was species-specific. In an examination of the variability of the HSP70 response, Norris *et al.* (1995) also found the constitutive HSC70 to be identical among individuals of the species *Poeciliopsis gracilis*; there was significant polymorphism in the heat-inducible HSP70.

Brown *et al.* (1992) used a primary culture of renal proximal tubule cells of the winter flounder (*Pleuronectes americanus*, Pleuronectidae) to study the protective effects of mild heat shock against the possible damaging effects of extreme temperature on transepithelial transport in this tissue. The glucose and sulphate fluxes were significantly reduced by a severe heat shock (+10 °C for 1.5 h), but those fluxes were completely protected by pre-treating the cells with a mild heat stress (+5 °C for 6 h). There was an increase in the three groups of HSPs, 28, 68–70, and 90 kDa, above constitutive levels in response to heat shock. Inhibition of protein synthesis by cyclohexamide abolished this protective effect, suggesting that these HSPs play an important physiological role in maintaining vital transepithelial transport properties of this tissue during stressful episodes. Subsequent work with fish renal tissue preparations has shown that HSPs may play an important role in the transport of sulphate as well as cytotoxic chemicals across the renal epithelium of fish. Renfro *et al.* (1993) showed that low levels (100 µM) of zinc can have similar effects as the above mild heat shock in protecting sulphate transport against the negative effects of severe heat shock (+10 °C), or of 0.5 mM 2,4-dichlorophenoxyacetic acid, on this epithelium. They also report the induction of HSP70 in that tissue with both zinc addition and mild heat shock treatments. Sussman-Turner and Renfro (1995) have shown that such a mild heat shock can even enhance the transport of the cytotoxic organic cation daunomycin across primary monolayer culture of this flounder proximal tubule tissue.

Koban *et al.* (1987) determined that acclimation temperatures (7, 15 and 25 °C) of a

primary culture of channel catfish (*Ictalurus punctatus*, Ictaluridae) hepatocytes had no effect on the threshold temperature for the induction of HSPs. New proteins of 65, 74, 76, 91 and 94 kDa mass were induced when the increase in temperature reached the threshold of 32.5 °C. Environmental contaminants also elicit HSP expression in primary cultures. Using primary cultures of hepatocytes from rainbow trout (*Oncorhynchus mykiss*, Salmonidae), Vijayan *et al.* (in preparation) have shown that HSP70 increases in response to several contaminants, including β -naphthoflavone (BNF), bleached kraft pulp mill effluent (BKME) and cadmium chloride exposure. The HSP response was more characteristic of a threshold response rather than a graded dose-related response. This is similar to the threshold type of response observed in other tissues stressed with a range of heat shock increments (e.g. erythrocytes of rainbow trout; Currie and Tufts, 1997). It is noteworthy that the increase in HSP70 in response to SDS and BKME exposure *in vivo* occurred at concentrations that were several-fold lower than the LC_{50} values (lethal concentration to 50% of the population in 96 h LC_{50} bioassay with rainbow trout), showing that the HSP response can be a sensitive indicator of sublethal effects of contaminants (Vijayan *et al.*, 1997a).

WHOLE ANIMAL STUDIES

Heat shock protein studies involving the whole fish are few, and the majority of these have investigated the effects of heat shock. While most of these studies have subjected the organism to various treatments, some have experimentally treated tissues such as blood cells or fin cells extracted from the fish. Koban *et al.* (1991) described the induction of HSPs in the gill, liver, heart, erythrocytes, skeletal muscle and brain of mummichog (*Fundulus heteroclitus*, Cyprinodontidae) that were exposed to elevated temperatures. Dyer *et al.* (1991) studied HSP induction in the gill, striated muscle and brain of the fathead minnow subjected to heat shock. Dietz and Somero (1992) observed enhanced synthesis of HSP90 in the brain of two species of gobiid fishes (*Gillichthys mirabilis* and *G. seta*) held at elevated temperatures. Dietz and Somero (1993) also examined HSP90 synthesis patterns in response to heat shock of four marine species: buffalo sculpin (*Enophrys bison*, Cottidae); staghorn sculpin (*Leptocottus armatus*, Cottidae); speckled sanddab (*Citharichthys stigmaeus*, Bothidae); and English sole (*Parophrys vetulus*, Pleuronectidae). Recently, Mazur (1996) showed increased levels of HSP70 in gill, liver, anterior kidney, and muscle tissues of cutthroat trout (*Oncorhynchus clarki*, Salmonidae) exposed to a 2 h heat shock of 10 °C (12.4–22.4 °C). HSP70 increased in all tissues after recovery for 1 h (except the muscle), and 48 h after being returned to the control temperature. In that study, a significant increase in HSP30 also was observed in gill tissue after recovery for 1 h. In a separate experiment, HSP70 in the red blood cells from cutthroat trout exposed to a 2 h heat shock of 16.2 °C (6.2–22.4 °C) were unchanged after recovery for 1 h, but were significantly elevated at 5 days after being returned to the control temperature (Mazur, 1996). This rather prolonged response also was seen in gill tissue of the same species exposed to a 2 h, 15 °C heat shock (7.4–22.4 °C), where increased HSP70 levels were observed up to 3 weeks after being returned to control temperatures (Mazur, 1996). In contrast to acute studies of step-changes in temperature, goldfish (*Carassius auratus*, Cyprinidae) that were acclimated for at least 5 weeks to 10 °C and 30 °C, showed higher concentrations of a new 65 kDa protein in the brain, liver and muscle tissues only in the warmer 30 °C water (Kikuchi *et al.*, 1993). That novel 65 kDa protein was distinct in characteristics from the HSP70 family of

proteins, in that it had no affinity to ATP and did not react to antibodies raised against this novel protein.

In vitro work with rainbow trout erythrocytes, from fish acclimated to 10 °C, showed a significant increase in HSP70 at 25 °C, but not at 15 °C nor at 20 °C (Currie and Tufts, 1997). This heat shock response was blocked by actinomycin D, suggesting that the regulation of HSP70 production was at the transcriptional level. Anoxia did not induce HSP production in the trout erythrocytes. As in other studies that have shown high levels of HSPs under control conditions (Misra *et al.*, 1989; Koban *et al.*, 1991; Yu *et al.*, 1994), their study showed a high level of apparent constitutive HSP70 content in the red cells (Currie and Tufts, 1997). The peripheral leukocytes of the channel catfish also have been shown to markedly express HSP70 when heat shocked from the control temperature of 23 °C to 37 °C, 2–3 h after treatment (Luft *et al.*, 1996). Oda *et al.* (1991) described the synthesis of three groups of new proteins of 28, 66 and 84 kDa, using autoradiography, in cells from the isolated tail fin of medaka (*Oryzias latipes*, Adrianichthyidae), in response to increases in ambient temperature from a range of temperatures (4–36 °C) to which the fish were acclimated.

Cauley and Sherman (1986) reported a 30 kDa protein obtained by translating the mRNA induced in goldfish as a result of physical ‘crush’ of the optic nerve. In response to a bacterial challenge of *Renibacterium salmoninarum*, Forsyth *et al.* (1997) have shown an induction of HSP70 in liver and head kidney tissues of coho salmon. It was clear in that study that the HSP70 originated in the host cells and not from the pathogen. These data corroborate the *in vitro* CHSE cell line work of Cho *et al.* (1997) showing the expression of a novel 90 kDa HSP in response to infection by the infectious haematopoietic necrosis virus of fish (see above).

Environmental contaminants such as heavy metals, BKME, SDS and BNF all have been shown to induce HSP70 in fish tissues. Juvenile rainbow trout exposed to metals in the water or feed showed significantly higher HSP70 levels in the gill tissue (Williams *et al.*, 1996). Liver HSP70 concentrations were higher in rainbow trout exposed to BNF (Vijayan *et al.*, 1997a). Exposure of rainbow trout to sublethal concentrations of BKME or SDS, for 96 h, caused significant increases in hepatic total HSP70 concentration (Vijayan *et al.*, 1997c). Furthermore, Vijayan *et al.* (1997c) showed that swimming juvenile chinook salmon (*Oncorhynchus tshawytscha*, Salmonidae) exposed to sublethal concentrations of BKME had higher levels of hepatic HSP70 at all concentrations of the contaminant. Janz *et al.* (1997) have shown that exposure of the white sucker (*Catostomus commersoni*, Catostomidae) to BKME results in increased HSP70 levels in the ovarian follicles. That response was accompanied by elevated ovarian cell apoptosis, reduced ovary size, as well as decreased plasma testosterone and increased plasma 17 β -oestradiol concentrations. The study of Vijayan *et al.* (1997a) showed that physical handling did not induce HSP70 expression nor modify the BNF-induced expression of HSP70 in the liver of rainbow trout. Also, the expression of HSP70 in the liver of rainbow trout with BNF correlated with decreased metabolic capacity, suggesting that higher HSP70 expression in tissues may be at the expense of other biochemical pathways (Vijayan *et al.*, 1997a). These recent data support the potential for using HSP70 as a biomarker of cellular stress, and consequently, the increase in tissue concentrations of these proteins may be indicative of physiological stress. However, the relationship between the cellular HSP response and the physiological stress response appears to be a complex one that requires further

investigation (see section below on the generalized stress response and the heat shock protein response).

MOLECULAR CHARACTERISTICS OF FISH HEAT SHOCK PROTEINS

The molecular biology of HSP expression in fish is another area that is lacking in basic knowledge. Kothary *et al.* (1984b) isolated and characterized two cDNA sequences encoding partial information for two distinct species of HSP70 from RTG-2. Zafarullah *et al.* (1992) described, for the first time, the complete structure and regulation of a constitutively expressed HSP70 gene from rainbow trout testes. Arai *et al.* (1995) isolated cDNA corresponding to two HSP70-related genes from two cell lines, derived from two species of medaka (OLHSC70 and CEHSC70 from *Oryzias latipes* and *O. celebensis*, respectively). The OLHSC70 cDNA was 2262 bp (base pairs) in length and encoded a 76 120 Da protein of 686 amino acids. The CEHSC70 cDNA was 2114 bp in length and lacked the 5' region found in the OLHSC70 cDNA. The *O. latipes* cell line expressed three HSP70 proteins. One of the HSP70 proteins was barely expressed constitutively, but highly inducible with heat, whereas the other two HSP70 proteins were expressed constitutively and showed only slight induction with heat. Luft *et al.* (1996) identified and characterized an HSP70 member in the channel catfish. De Jong *et al.* (1988) found homologies of the dogfish (*Squalus acanthias*, Squalidae) crystallin sequences with human, bovine, *Xenopus* and *Drosophila* low molecular weight HSPs, and with blood fluke (*Schistosoma* sp.) egg antigen. Such studies not only provide detailed structure of the HSPs, which may be useful in making synthetic peptides, but also reveal homologies between HSPs from different organisms. This may be profitably used in the development of antibodies either to homologous domains (providing cross-reactivity of the antibody against a variety of organisms) or to non-homologous domains (useful in species-specific diagnostics). These studies also are valuable in assessing the phylogenetic relationships between different groups of organisms.

THE GENERALIZED STRESS RESPONSE AND HSP EXPRESSION

Few studies have examined the question of how cellular HSP expression is related to the generalized stress response in animals. The generalized stress response in fish has been broadly categorized into the primary, secondary and tertiary responses (Mazeaud *et al.*, 1977; Wedemeyer *et al.*, 1990). The 'primary response' is the initial response; this represents the perception of an altered state and initiates a neuroendocrine/endocrine response that forms part of the generalized stress response in fish (Gamperl *et al.*, 1994). This response includes the rapid release of stress hormones, catecholamines and cortisol, into the circulation. Catecholamines are released from the chromaffin tissue situated in the head (anterior) kidney of teleosts, and from the endings of adrenergic nerves (Randall and Perry, 1992). Cortisol is released from the interrenal tissue, located in the head kidney, in response to several pituitary hormones, but most potently to adrenocorticotrophic hormone (ACTH) (Wendelaar Bonga, 1997). A recent study showed that ACTH may also stimulate adrenaline release, and that chronic cortisol treatment may affect catecholamine storage and release in trout (Reid *et al.*, 1996). As both the chromaffin tissue and the interrenal tissue lie in close proximity in fish, these results point to the possibility for a paracrine control of stress hormone regulation in fish (Reid *et al.*, 1996). The resting and stressed levels of adrenaline and cortisol concentrations in the plasma of salmonids are: adrenaline, <3 and 20–70 (nmole l⁻¹) and cortisol, <10 and 40–200

(ng ml⁻¹), respectively. These values should serve only as general guidelines because individual conditions, including genetic characteristics, prior rearing history and local environment can affect the true plasma values for control and stressed states.

The 'secondary response' comprises the various biochemical and physiological effects associated with stress, and mediated to a large extent by the above stress hormones. The stress hormones activate a number of metabolic pathways that result in alterations in blood chemistry including plasma glucose concentration and haematology (Maule *et al.*, 1989; Barton and Iwama, 1991; Randall and Perry, 1992; Vijayan *et al.*, 1994, 1996, 1997b). In addition to the measurement of these stress hormones, the measurement of plasma glucose concentration has been used as an indicator of stressed states in fish. It is probably the most commonly measured secondary change that occurs during the stress response in fish (Wedemeyer *et al.*, 1990). The 'tertiary response' represents whole-animal and population-level changes associated with stress. If the fish is unable to acclimate or adapt to the stressor, whole-animal changes may occur as a result of the repartitioning of energy by diverting energy substrates to cope with the enhanced energy demand associated with stress and away from vital life processes such as reproduction and anabolic processes such as growth. Decreased recruitment and productivity may alter community species abundance and diversity.

Some work on cell lines as well as whole animals provide some insights into how the generalized stress response, as described above, and HSP expression may be related. The interaction of HSP70 and HSP90 with the steroid receptor has been characterized using cell line models (Bohen and Yamamoto, 1994). A number of studies on heat-stressed and restraint-stressed rats have shown that physiological stress can and does elicit HSPs. Blake *et al.* (1990) demonstrated HSP gene expression in rats exposed to heat shock. Blake *et al.* (1991) and Udelsman *et al.* (1993) have shown the expression of HSP70 after 3–6 h of restraint stress in adrenal cortical tissue and thoracic aorta tissue of rats. The activation of the same heat shock transcription factor, HSF1, in response to both heat shock and restraint stress suggests that both responses utilize the same signal pathway for expression (Blake *et al.*, 1991). Furthermore, this suggests that the stressor may not be acting directly on the cells to induce HSP expression, but that it may be acting through a neuroendocrine pathway in the whole animal. The observation that hypophysectomized rats did not show the HSP-gene expression in response to restraint stress, and that addition of ACTH to those rats induced HSP70 expression in the adrenals (Blake *et al.*, 1991), indeed supports the possibility that a functional relationship between HSP expression and the hypothalamus–pituitary–adrenal axis exists. Udelsman *et al.* (1994a) have shown an α_1 -mediated adrenaline effect on HSP70 expression in aortic tissue of rats. Until recently, there has been no work relating physiological stress and the cellular HSP response in fish.

Some data on the relationship between the stress hormones adrenaline and cortisol are just emerging. Udelsman *et al.* (1994b) showed that the glucocorticoid dexamethasone attenuated the induction of HSP70 mRNA expression in the adrenal, but not in the aortic tissue of rats undergoing restraint stress. Mazur (1996) showed that adrenaline at physiological (10^{-7} M) and pharmacological (10^{-5} M) levels caused an increase in HSP70 concentration in primary cultures of rainbow trout hepatocytes. The absence of this response in the presence of propranolol suggests a possible role for a β -adrenoreceptor in the HSP response. The relationship between stress hormones and HSP70 induction may not be of a simple cause–effect nature, but may be modulated by

several factors *in vivo*. For instance, Mazur (1996) has shown that prior handling of rainbow trout (45 s dip-net stress which caused an increase in plasma cortisol concentration) significantly reduced the heat (+10 °C) shock-related increase of both HSP30 and HSP70 in the gill tissue. Furthermore, when exogenous cortisol was introduced via oil implants in the peritoneal cavity, the increase in gill tissue HSP70 levels in response to the heat shock was not affected, but the increase in HSP30 was significantly attenuated (Mazur, 1996). Some of these data contrast with the results of Vijayan *et al.* (1997a), which show that physical handling – which caused an increase in circulating cortisol levels – did not cause an increase in liver HSP70 levels or affect the BNF-induced HSP70 increase. Clearly, further studies are needed to understand the complex interaction between hormones and other environmental stressors on HSP expression in fish. It would be most interesting to identify the various signal pathways that regulate HSP production.

Methods of analysis

There have been numerous, and increasing, inquiries from fish physiologists and biologists for information about measuring HSP expression in fish. Requests for antibodies and information are often, and naturally, accompanied by questions about how the assays are conducted, and how samples should be taken, prepared and stored for analyses. Because most of the methods used for HSP analyses are generic to work of this nature, the detailed analytical methods can be found in many excellent methodology books on this subject.

The various HSPs are located in the cytoplasm, endoplasmic reticulum, Golgi complex, mitochondria, or within the nucleus, and there is increased trafficking between the various compartments during a stress event (Welch *et al.*, 1991). It is important to isolate cells or tissues for HSP analysis with as little protein degradation as possible. Tissue samples which are to be analysed for HSP content should be frozen immediately. When the tissues are to be subjected to stressors *in vitro* or used for metabolic labelling, aseptic techniques should be followed throughout all procedures. The samples should be quickly placed in tissue culture media and maintained at appropriate temperatures (e.g. ambient temperature of the fish).

One-dimensional SDS–PAGE is one of the most common techniques for protein analysis. The proteins from the sample, solubilized in and complexed with SDS, are applied in wells on top of the stacking gel, followed by the application of an electric field. The proteins, with a net negative charge, form a moving zone compressed between a trailing high-voltage glycine zone and a leading chloride ion zone. The mobility of the proteins is restricted by the sieving action of the small pores in a resolving gel, with large proteins remaining close to the top and small ones migrating to the bottom. Gels which contain no radiolabelled samples are usually stained using Coomassie brilliant blue or Silver stain to visualize the separated proteins as dark bands on a clear background. The amount of protein in the various bands can be quantified by scanning the gel in a densitometer. **Two-dimensional SDS–PAGE** is more powerful than one-dimensional SDS–PAGE in the further resolution of proteins in the samples, owing to a combination of isoelectric focusing and SDS–PAGE. In the first dimension, the proteins are separated to their isoelectric points within a polyacrylamide gel cast within a narrow tube. In the second dimension, the proteins are separated according to

molecular mass on SDS–PAGE. The separated proteins in the tube gel, arranged across the top of the SDS–PAGE gel according to their respective isoelectric points, are then separated in the resolving gel according to their molecular masses, in a direction perpendicular to the pH gradient established in the tube gel.

Metabolic labelling is a technique that is extremely useful in studying protein synthesis patterns in cells in response to any stimuli. The labelling is usually done by either: (1) steady-state labelling, in which cells are incubated in the presence of the radiolabel for relatively long periods of time (≥ 2 h); or (2) pulse-chase labelling, in which cells are incubated with the radiolabel (pulse) for shorter periods of time (e.g. 15 min), the radiolabel removed, and the cells either immediately harvested, or incubated in fresh medium with excess unlabelled amino acids (chase) for longer periods before harvest. Steady-state labelling is useful in obtaining a general picture of overall protein synthesis in cells, whereas pulse-chase labelling enables one to determine changes in the protein during the course of its maturation, such as post-translational modification or translocation into various cell compartments.

Gels containing radioactive proteins are subjected to **autoradiography** or **fluorography** to detect newly synthesized proteins. After SDS–PAGE, gels that contain radiolabelled proteins can be dried immediately, or washed and dried using a gel dryer. The dried gel is then exposed to X-ray film kept in an autoradiographic cassette. After exposure of the film for 1–2 weeks at -80°C , the developed film shows proteins synthesized during the experimental treatment as dark bands. In our lab we routinely use AGFA CURIX X-ray film which we pre-flash. In this case, good images can be obtained in about 4–6 days of exposure at -80°C (Fig. 3(a)). Pre-flashing the film reduces the required exposure time and improves the sensitivity of the film to low radiation levels (Laskey and Mills, 1975). Fluorography is a faster method than autoradiography, requiring only 24 h to obtain an image of the gel. The gels containing radiolabel are incubated in dimethyl sulphoxide (DMSO) and diphenyl oxazole (PPO) solutions, and then added to the gels and incubated for 1 h. The PPO in the gel is then precipitated, and the gels are then dried, applied to film and stored at -80°C . The film is processed after 24 h. Both DMSO and PPO are toxic substances, and caution (extreme caution in the case of PPO) must be exercised when handling these chemicals. Chamberlain (1979) has described an alternative method using the water-soluble flour, sodium salicylate, which is much less toxic than DMSO and PPO. This latter technique has the added advantage of being faster and less expensive than the former method.

Western blot enables the detection and quantification of HSPs in cells and tissues, using specific antibodies (polyclonal or monoclonal) to the respective HSPs (Fig. 3(b)). The detailed procedure for this very common analytical method is well described in any laboratory book on molecular biology techniques.

Applications

The potential application of HSPs as biomarkers continues to be an important subject of research in environmental physiology. Their universality, extraordinary conservation in structure, and the consistency with which they are induced by a broad spectrum of stressors have made HSPs good candidates for biomonitoring of the environment. The use of antibodies produced against conserved domains of inducible members of HSPs appears promising in evaluating the HSP levels in a variety of organisms. The stress

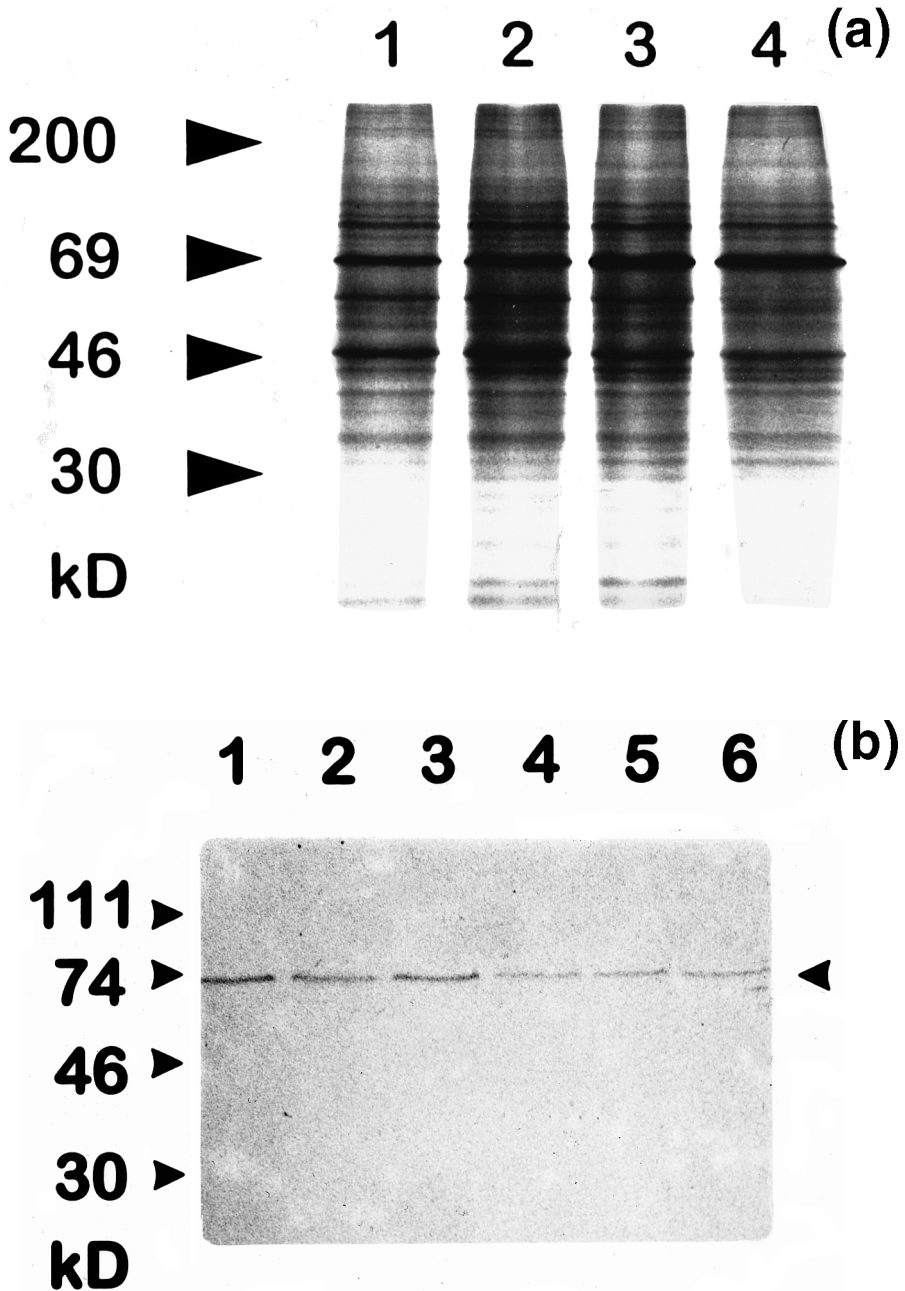


Fig. 3. (a) Autoradiograph of CHSE cell line exposed to hydrogen peroxide for 30 min, and labelled for 2 h with ^{35}S methionine. Lanes: 1, control; 2 = $10\ \mu\text{M}$; 3 = $100\ \mu\text{M}$; 4 = $1000\ \mu\text{M}$. Apparent molecular weights in kDa on the left. (b) Western blot liver tissue from coho salmon, *Oncorhynchus kisutch*, infected with *Renibacterium salmoninarum*, the causative agent for bacterial kidney disease (BKD). Lanes 1–3, liver tissues from infected fish with clinical signs of BKD; 4–6, liver tissues from controls. Apparent molecular weights in kDa on the left. From Forsyth *et al.* (1997).

profile of the biotic components (e.g. plants, pelagic animals, benthic animals) of a given ecosystem thus obtained may, then, be integrated with data on the quality of the abiotic components (e.g. water quality, oxygen content). The suitability of HSPs as biomarkers is extensively discussed in Bradley (1990), Sanders (1990) and Sanders and Martin (1993). However, more information is needed about the functional relationship between HSP expression and the physiological stress response, particularly the generalized stress response in fish (see text above, and Barton and Iwama, 1991). Information about the dose–response relationships, time course of the response, species specificity, and the effects of developmental stage are some examples of the knowledge required to assess, and possibly implement, the use of HSPs as biomarkers of environmental quality, through an assessment of the stressed states of the organisms that live in that environment. It would be very important to eventually describe the relationship between HSP expression and population-level changes. Descriptive studies such as those above help to identify tissues that show consistent patterns of HSP expression in response to a variety of stressors, and that are also amenable to easy isolation and processing. Externally exposed tissues such as gills, adipose fins, or better, mucus may be potential candidates for such sampling. A further advantage with those tissues is that the same fish may be monitored repeatedly, and with minimally invasive techniques. If physiological stress and HSP expression are closely related, then the potential exists for using specific antibody probes as indicators of stressed states in fish. Antibody-based ELISA or dot-blot kits could be developed as alternatives to the more complex and expensive methods now used, such as determining the concentrations of cortisol or other metabolites using radioimmunoassays which require sophisticated procedures and specialized instruments. This is an area which, in our opinion, requires more attention from researchers.

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