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Regulation of heat shock genes in isolated hepatocytes from an Antarctic fish, *Trematomus bernacchii*

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Summary

The Antarctic fishes, isolated over evolutionary history in the sub-zero waters of the Southern Ocean, are an ideal group for studying the processes of cold adaptation. One species of Antarctic notothenioid fish, *Trematomus bernacchii*, has lost the ability to induce heat shock proteins (Hsps) in response to exposure to acute thermal stress or to the toxic heavy metal cadmium, an important part of the cellular defense response to such stressors. To elucidate the mechanism responsible for the lack of Hsp induction, we examined several stages of the *hsp* gene expression pathway, including transcription factor activity, Hsp70 mRNA production and protein synthesis patterns, in hepatocytes from *T. bernacchii*. Hsp70 mRNA was detected, as was heat shock factor 1 (HSF1) with DNA-binding activity. However, exposure to elevated temperature and to chemical inducers of the heat shock response failed to increase Hsp70 mRNA levels, HSF1 activity or the concentration of any size class of Hsps. These results suggest that Hsps, inducible in nearly every other species, are expressed constitutively in the cold-adapted *T. bernacchii*.

Key words: heat shock protein, Hsp70, hepatocyte, constitutive expression, *Trematomus bernacchii*, Notothenioid, Antarctica.

Introduction

The endemic Antarctic fishes, confined by the establishment of the Antarctic Circumpolar Current, have evolved under near-freezing water temperatures for between 14 and 25 million years (Eastman, 1993). During this period, these fishes have acquired a truly cold-adapted physiology, marked by pronounced stenothermality. Typical upper lethal temperatures for Antarctic fish species are 8–12°C (Somero and DeVries, 1967). Therefore, these fishes provide an ideal system in which to study the biochemical and molecular mechanisms of adaptation to low temperature, including the regulation of thermally sensitive genes. Our research focuses on a well-characterized example of environmentally controlled gene expression, the heat shock response (HSR), in one common notothenioid species, *Trematomus bernacchii*.

This species may represent a rare exception to the taxonomic conservation of the HSR. This response is an important part of the cellular defense against proteo-toxic stress and is defined by the coordinated upregulation of several, functionally related genes following exposure of the protein pool to denaturing stressors such as elevated temperature or heavy metals (Lindquist, 1986; Parsell and Lindquist, 1993). The upregulated genes encode heat shock proteins (Hsps), which act as molecular chaperones, stabilizing heat-denatured polypeptides and disassociating unfolded proteins that have begun to interact with one another in potentially cytotoxic aggregations (Fink, 1999). Those Hsps induced by acute stress are related to isoforms of Hsps that are constitutively expressed and perform essential chaperoning functions in the routine synthesis of new polypeptides and the transport of proteins across membranes (Hartl, 1996; Hartl and Hayer-Hartl; 2002). Owing to the species-independent effects of elevated temperatures on protein structure, these genes have been maintained, at high levels of sequence conservation, in the genomes of every eukaryotic organism examined to date (Feder and Hofmann, 1999). As testament to the ecological importance of the HSR, the rapid induction of Hsps at temperatures between 5 and 10°C above a given organism's body temperature is nearly ubiquitous among all taxa (reviewed in Feder and Hofmann, 1999).

Given a habitat temperature that is constantly subzero (estimated at –1.86°C; except see Hunt et al., 2003), an induction of Hsps in the tissues of *T. bernacchii* would be expected in response to exposure to 3–8°C. However, in a previous study (Hofmann et al., 2000), we observed no induction of Hsps in any of five tissues that were exposed to up to 12°C, a lethal temperature for this species (Somero and DeVries, 1967). The constitutive isoforms of Hsps (such as Hsc71) are present in *T. bernacchii* (Carpenter and Hofmann, 2000; Hofmann et al., 2000), consistent with the important role that constitutive Hsps play in normal cellular protein

chaperoning. Interestingly, the exposure of isolated hepatocytes to cadmium chloride, a chemical inducer of the HSR (Lai et al., 1993; Ovelgonne et al., 1995; Liao and Freedman, 1998), also failed to stimulate the production of Hsps (Hofmann et al., 2000). This result suggests that an anomaly exists in the expression of *hsp* genes in this species, rather than insensitivity to temperature *per se*.

Such an anomaly could be due to numerous mechanisms, including irregularities in the transcriptional machinery controlling Hsp expression. All inducible size classes of Hsps are under the transcriptional control of heat shock factor 1 (HSF1), a latent cytoplasmic transcription factor that becomes activated through trimerization in response to stress (Wu, 1995; Morimoto, 1998; Pirkkala et al., 2001). HSF1 binds specifically to the heat shock element (HSE), an inverted heptad repeat (5′-nGAAn-3′) in the promoters of inducible *hsp* genes (Pelham, 1982; Xiao and Lis, 1988). The temperature and kinetics of HSF1 activation in poikilotherms can be dependent upon recent thermal history (Buckley and Hofmann, 2002, in press). Likewise, the Hsp induction temperature is not fixed for a given species but varies with acclimation or acclimatization (e.g. Buckley et al., 2001; Tomanek and Somero, 2002; Buckley and Hofmann, 2002).

Here, we test the following hypotheses concerning the underlying mechanism responsible for the absence of Hsp induction in *T. bernacchii*. First, the lack of protein synthesis may be due to an inability to produce stable mRNA from *hsp* genes. Second, a functional HSF1 with HSE-binding competency may be absent. Third, *hsp* genes may be expressed at a constant level and are non-responsive to either heat or chemical stressors. To test these hypotheses, we chose to study isolated hepatocytes, as this approach allowed us to examine multiple stages of *hsp* gene expression in cells from the same individual, exposed independently to a range of heat and chemical stresses. We investigated protein synthesis patterns, Hsp70 mRNA production and the presence, specificity and DNA-binding activity of HSF1. Consistent with the findings of a related study from our laboratory (Place et al., 2004), the results support the constitutive expression of *hsp* genes in this cold-adapted species.

Fig. 1. McMurdo Sound, Antarctica. McMurdo Station (United States Antarctic Program) and Cape Evans are marked with red dots; the collection site for *Trematomus bernacchii* is marked with a green square. Inset, Antarctica. The white box represents the area depicted on the larger map.

Materials and methods

Animal collection

Specimens of *Trematomus bernacchii* (Boulenger) were caught by hook and line in November 2002 in the nearshore water of Cape Evans in McMurdo Sound, Antarctica (77°53′ S, 166°40′ W; Fig. 1). Fish were collected from a depth of approximately 10 m. *T. bernacchii* is one of the most common species in the region (Gon and Heemstra, 1990), where the water temperature is consistently near –1.86°C with rare fluctuations to near 0°C (Hunt et al., 2003). Fish were transported to McMurdo Station in coolers and maintained in running seawater aquaria. Survivorship during transport was 100%. Fish were allowed to adjust to the tank conditions for 48 h before use in the following experiments.

Hepatocyte preparation

The preparation of hepatocytes was conducted at –1.8°C in a temperature-controlled room (protocol after Hofmann et al., 2000). Four individuals were anesthetized *via* immersion in MS-222 (at a concentration of 1 g l^{-1} in seawater). The hepatic portal vein was cannulated and the livers perfused with a buffer containing 290 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 10 mmol l^{-1} Hepes, 0.5 mmol l^{-1} EGTA and 25 mmol l^{-1} Tricine (pH 7.8), to remove red blood cells. Livers were then removed and incubated in a cell suspension buffer (SB) (292.5 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 2.5 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} $NaHCO_3$, 2 mmol l^{-1} NaH_2PO_4, 5 mmol l^{-1} glucose and 50 mmol l^{-1} Hepes, pH 7.8) that contained 5 units ml^{-1} collagenase (Sigma, St Louis, MO, USA) for 1 h, to separate cells from one another. This concentration of collagenase was determined empirically, to dissociate cells while avoiding cytolysis. Cells were sieved through 60 and 200 μm mesh screens and were then pelleted *via* centrifugation at 100 ***g*** for 10 min. Cells were counted on a hemocytometer and resuspended in SB at a concentration of 3×10^6 cells ml^{-1}. Hepatocyte viability was determined *via* bromophenol exclusion and was always >95%.

Three 1 ml aliquots of hepatocytes from each individual were exposed for 1 h to 0, 2, 4, 8 or 12°C or to 100 μmol l^{-1} $CdCl_2$ (Sigma) or 100 μmol l^{-1} MG132 (Sigma). The $CdCl_2$ and MG132 solutions were prepared in dimethylsulfoxide (DMSO). Three 1 ml aliquots were also exposed to neat DMSO as a control. Following stress exposure, for each treatment, one aliquot from each individual was assayed for (1) *de novo* synthesis of Hsps, (2) the presence of Hsp70 mRNA or (3) the activity of HSF1, as described below.

In vivo *metabolic labeling*

The *de novo* synthesis of protein was detected by metabolic labeling with radiolabeled amino acids (after Hofmann et al., 2000). Hepatocytes were incubated with 3.7×10^6 Bq of ^{35}S-labeled cysteine/methionine (specific activity 3.7×10^{11} mBq ml^{-1}; NEN, Torrance, CA, USA) for 2 h at –1.8°C. Homogenates were heated at 100°C for 5 min and centrifuged at 12 000 ***g*** for 10 min. Total counts of incorporated radiolabel in each extract were determined on a scintillation counter. Proteins were loaded onto 10% acrylamide gels and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), with an equal amount of radioactivity (75 000 c.p.m.) loaded in each lane. Dried gels were exposed overnight to X-ray film (Kodak).

Northern blotting

Northern blots were used to assay for the presence of mRNA from both the inducible Hsp70 and its constitutive cognate Hsc71. The probe for Hsc71 was generated from *T. bernacchii* tissues using primers from trout Hsc71 (for complete description of construction of all probes, see Place et al., 2004). Probes for Hsp70 were constructed from heat-shocked liver tissue from two species of notothenioid fishes, *T. bernacchii* and a species endemic to New Zealand, *Bovichtus variegatus*. Primers were consensus sequences from multiple alignment of several fish Hsp70 sequences. Probes were tested for cross-reactivity with heat shock and chemically stressed hepatocytes from *T. bernacchii* as well as with heat-shocked liver tissue from other species of teleost fishes known to display typical HSRs: *B. variegatus*, the New Zealand black cod (*Notothenia angustata*) and *Gillichthys mirabilis*, a temperate, eurythermal goby. The goal was to determine the specificity of the probes and their ability to detect the inducible expression of Hsp70 mRNA in species possessing such a response, and thus provide a context against which to contrast any lack of inducibility observed in *T. bernacchii*. In all cases, probes detected single bands on northern blots, with the Hsc71 band being clearly distinct from the Hsp70 band.

Following exposure to heat or chemical stress, hepatocytes were pelleted at 100 ***g*** and the supernatant discarded. Pellets were flash frozen in liquid nitrogen and stored at –80°C. Pellets were thawed in 1 ml of Trizol® reagent (Invitrogen, Carlsbad, CA, USA) and RNA extracted according to the manufacturer's protocol. For northern blotting, 10 μg total RNA was denatured with glyoxal/DMSO and blotted onto Zeta Probe nitrocellulose membrane (BioRad, Hercules, CA, USA) using a BioDot slot blotter (BioRad) under gentle vacuum. The wells were washed twice with 300 μl of 10 mmol l^{-1} $NaPO_4$ buffer (pH 7.0). The membranes were UV cross-linked once at 120 000 μJ cm^{-2} using a CL-1000 UV cross-linker (UVP, Upland, CA, USA) prior to hybridization with probes.

The probes were labeled with [α-^{32}P]-dCTP (1×10^{-6} Ci ng^{-1} DNA; specific activity 3000 Ci $mmol^{-1}$) using the Ready-to-Go labeling system (Amersham Pharmacia BioTech, Piscataway, NJ, USA). Glyoxal adducts were removed from the membranes by incubation in 20 mmol l^{-1} Tris-HCl (pH 8.0) at 65°C for 5 min immediately prior to prehybridization. Membranes were prehybridized in 20 ml Church's buffer (0.5 mol l^{-1} $NaPO_4$, 10 mmol l^{-1} EDTA, 7% SDS) at 60°C for 3 h, followed by hybridization with labeled probe at 60°C for 18 h. Following hybridization, membranes were washed twice at room temperature for 15 min with a low-stringency wash buffer (1×SSC/0.1% SDS) and once at 60°C for 20 min with a high-stringency wash buffer (0.25×SSC/0.1% SDS). Following washing, membranes were wrapped in plastic wrap and exposed to a phosphor storage screen (Molecular Dynamics, Piscataway, NJ, USA) for 12–18 h. Phosphor storage screens were scanned using the BioRad Personal FX imager, and densitometry was performed with Quantity One software (BioRad). Relative levels of mRNA were background corrected and standardized across successive northern blots.

Electrophoretic mobility shift assays (EMSAs)

After the 1 h exposure, hepatocytes were pelleted by centrifugation at 100 ***g*** at –1.8°C. The supernatant was discarded and the pellet was frozen in liquid nitrogen and stored at –80°C. Frozen pellets were thawed in 200 μl of extract buffer containing 25% (v/v) glycerol, 20 mmol l^{-1} Hepes (pH 7.9), 420 mmol l^{-1} NaCl, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} EDTA, 0.5 mmol l^{-1} PMSF and 0.5 mmol l^{-1} dithiothreitol. Homogenized extracts were centrifuged at 22 000 ***g*** at 4°C for 10 min. Pellets were discarded and the supernatants frozen at –80°C, after an aliquot was taken for total protein content determination by Bradford assay (Pierce, Rockford, IL, USA). EMSAs were conducted according to Buckley and Hofmann (2002). Briefly, 25 μg of total protein from each sample was incubated, on ice, with 15 pmol of ^{32}P-labeled oligonucleotide, containing the heat shock element (HSE) – an inverted heptad repeat sequence (5′-GCCTCGAATGTTCGCGAAGTTT-3′; Airaksinen et al., 1998). After a 20 min incubation, HSF1–HSE complexes were resolved on 5% acrylamide non-denaturing gels by electrophoresis for 2 h at 250 V. Gels were dried and exposed to a phosphoscreen. Densitometry was conducted on a scanning phosphoimager (BioRad). All densitometry values were normalized to that of a control run on all blots. Competitor/non-competitor assays were run to establish the specificity of the HSE probe used in the EMSAs. In these assays, a 200 mol l^{-1} excess of either unlabeled HSE (competitor) or radiolabeled AP2 oligo (non-competitor) was added to reactions containing extract and radiolabeled HSE probe.

Statistics

The effect of heat or chemical treatment on the concentrations of Hsp70 and Hsc71 mRNA, or on the activity of HSF1, was determined *via* one-way analysis of variance (ANOVA) at a confidence level of $P<0.05$ (SysStat software, Point Richmond, CA, USA).

Results

Protein synthesis patterns

To characterize the effects of exposure to elevated temperature or to chemical inducers of the HSR in hepatocytes from *Trematomus bernacchii*, *in vivo* metabolic labeling was conducted on cells that had been exposed for 1 h to 0, 2, 4, 8 or 12°C or to $CdCl_2$ or MG132 (Fig. 2). Although protein synthesis was robust under all treatments, in none of the four individuals examined did heat or chemical stress result in the detectable induction of any molecular mass size class of Hsps.

Hsp70 and Hsc71 mRNA analysis

Northern blotting was used to detect mRNA from Hsp70 and Hsc70 in treated hepatocytes. Both Hsp70 and Hsc71 mRNA was detected in all individuals (Fig. 3A). However, levels of neither Hsp70 nor Hsc71 mRNA varied significantly among treatments (P=0.076 and P=0.575, respectively; ANOVA; Fig. 3B). This same Hsp70 probe successfully detected the induction of Hsp70 in the tissues of the three other species on which it was tested (Table 1). The same result was obtained using the second Hsp70 probe, generated from *B. variegatus* (Table 1).

HSE-binding specificity

The HSE-binding activity of HSF1 was examined in *T. bernacchii*, a species for which the specificity of the HSE probe used in EMSAs had not been previously determined. Competitor/non-competitor assays were run to determine probe specificity, and a single HSE-specific band was observed (Fig. 4). This band was visible in the presence of non-competitor DNA and was absent in the presence of a $200\,mol\,l^{-1}$ excess of unlabeled competitor HSE oligonucleotide probe.

HSF1 activity

EMSAs were used to determine whether the HSE-binding activity of HSF1 in these species was responsive to temperature or chemical stressors (Fig. 5A). While there was HSF1 present with DNA-binding activity in all samples, there was no significant effect of treatment on HSF1 activity (Fig. 5B; P=0.23; ANOVA).

Discussion

The purpose of this study was to extend our investigation into the regulation of the HSR in the Antarctic notothenioid fish *Trematomus bernacchii*, a species that appears to lack heat-inducible Hsp expression (Hofmann et al., 2000). The fishes endemic to the Southern Ocean display a range of biochemical and molecular processes that allow them to tolerate their extreme environment and, in some cases, have lost attributes common to other fish (Somero et al., 1998). These changes include the production of anti-freeze proteins (DeVries, 1988), the loss of hemoglobin and/or myoglobin in some species (Cocca et al., 1997; Sidell et al., 1997; Somero et al., 1998) and the storage of extra lipid reserves for energy (Sidell et al., 1995), buoyancy (Eastman, 1988) and for aiding in oxygen delivery to tissues (Londraville and Sidell, 1990). To address the possibility that such a cold-adapted species could lose an otherwise highly conserved cellular response to heat stress, we examined the expression of *hsp* genes in *T. bernacchii* at multiple levels, including transcription factor activity, mRNA production and the synthesis of protein products. Our results suggest that part of the unique physiology that characterizes this species is the constitutive production of Hsps at a constant level and a lack of inducibility in these genes.

In almost all cells, an exposure to

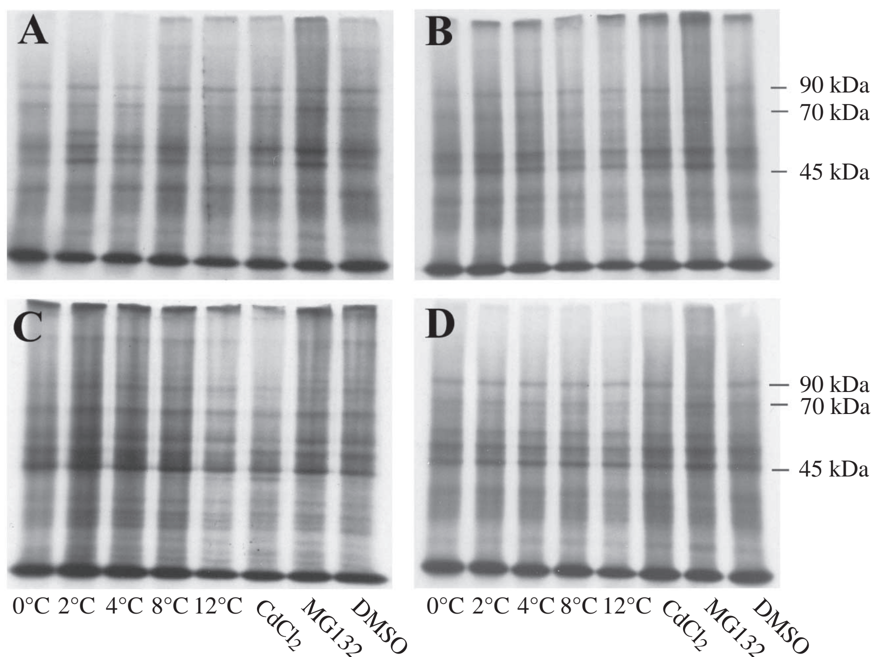

Fig. 2. Protein synthesis patterns in isolated hepatocytes from *Trematomus bernacchii* exposed to heat and chemical stress. (A–D) Patterns from four individuals exposed for 1 h to 0–12°C or $100\,\mu mol\,l^{-1}$ $CdCl_2$ or $100\,\mu mol\,l^{-1}$ MG132 are depicted. Following exposure, newly synthesized proteins were labeled with ^{35}S-radiolabeled cysteine/methionine for 2 h. Proteins were resolved on 10% polyacrylamide gels. Dried gels were then exposed to X-ray film. No visible induction of any size class of protein was observed.

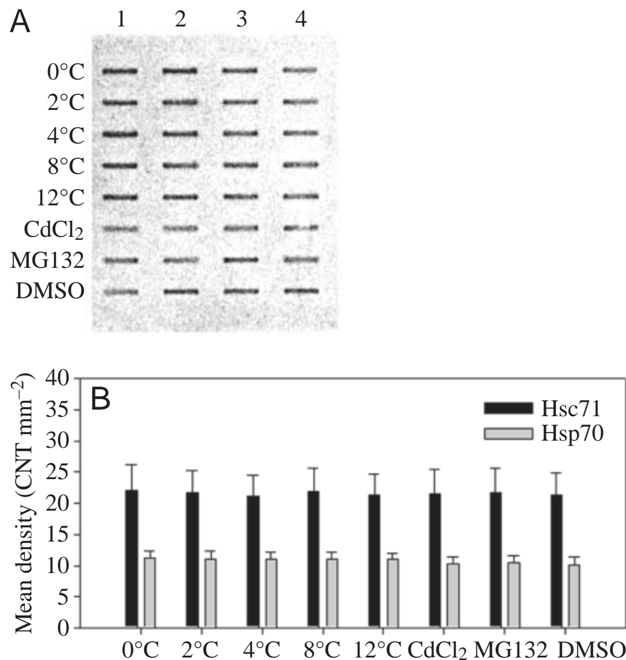

Fig. 3. Hsp70 and Hsc71 mRNA expression in hepatocytes of *Trematomus bernacchii*, exposed to heat and chemical stress. (A) Northern slot blots of Hsp70 mRNA in hepatocytes from four individuals (lanes 1–4) exposed for 1 h to 0–12°C or to 100 μmol l^{-1} $CdCl_2$ or 100 μmol l^{-1} MG132. Also depicted are samples of hepatocytes exposed to DMSO controls ($CdCl_2$ and MG132 were delivered in DMSO solutions; see Materials and methods). Samples were probed with Hsp70 mRNA probes generated in *T. bernacchii*. (B) Levels of Hsp70 and Hsc71 mRNA in the hepatocytes from four individuals. No significant effect of treatment on the concentration of either message was observed. Values are means ± S.E.M.

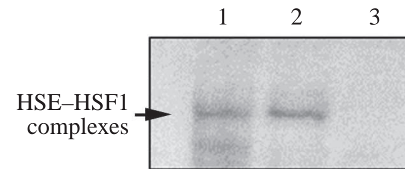

Fig. 4. HSF1–HSE complexes in the hepatocytes of *Trematomus bernacchii,* visualized *via* electrophoretic mobility shift assay (EMSA). A competition assay was run to determine HSE probe specificity. 25 μg of homogenized hepatocyte preparation was incubated with 15 pmol of ^{32}P-labeled HSE probe for 20 min at 0°C and then separated on a 5% non-denaturing polyacrylamide gel. Gels were dried and exposed to a phosphoscreen. HSF1–HSE complexes were visualized on a phosphoimager. Lane 1: hepatocyte sample and radiolabeled HSE probe only. Lane 2: identical to lane 1 except for the addition of a 200 mol l^{-1} excess of an unlabeled non-competitor DNA probe (AP2 from Promega). Lane 3: identical to lane 1 except for addition of a 200 mol l^{-1} excess of unlabeled HSE probe.

temperatures 5–10°C above body temperature results in the preferential expression of multiple classes of Hsps (Feder and Hofmann, 1999). In hepatocytes from *T. bernacchii*, we observed no preferential production of any polypeptide in response to heat (Fig. 2), although overall protein synthesis was observed even at temperatures (8–12°C) that would have been lethal to the intact organism. These results from isolated liver cells are consistent with similar *in vivo* experiments on sections of metabolically active brain, spleen, liver, heart and gill tissue (Hofmann et al., 2000). Furthermore, in time-course experiments, no induction of Hsps was observed in response to heat treatments of up to 12 h (S.P.P. and G.E.H., unpublished data); therefore, the lack of induction here is not simply a factor of the duration of exposure to elevated temperature. It should be noted that two-dimensional gel electrophoresis might reveal the expression of novel isoforms of Hsps in response to heat; however, if that is the case, the induction of such isoforms would be minimal, as the data presented here in the one-dimensional gels demonstrate that no significant increase in any size class of protein was detectable.

Two chemical inducers of the HSR ($CdCl_2$ and MG132) also failed to increase production of Hsps (Fig. 2). Cadmium causes protein denaturation (Abe et al., 1994) and the creation of reactive oxygen species (Manca et al., 1991), while MG132 inhibits proteasome activity, resulting in the accretion of misfolded or damaged proteins that would normally be degraded (Bush et al., 1997; Lee and Goldberg, 1998; Kim et al., 1999). As the presence of abnormally unfolded proteins is a trigger of the HSR (Hightower, 1980; Ananthan et al., 1986), MG132 can be a potent chemical Hsp inducer. The inability of these chemical insults to initiate Hsp induction supports the

Table 1. *Target species in which Hsp70 mRNA was detected with probes generated in either* Bovichtus variegatus *or* Trematomus bernacchii[a]

Probe	Target species	Hybridization[b]	Upregulation[c]
B. variegatus Hsp70	*G. mirabilis*	+	+
	B. variegatus	+	+
	N. angustata	+	+
	T. bernacchii	+	–
T. bernacchii Hsp70	*G. mirabilis*	+	+
	T. bernacchii	+	–

[a]Probes used in this study were developed in a related study (Place et al., 2004).
[b]+ indicates that probe detected a single band on northern blots when hybridized against listed species.
[c]Presence (+) or absence (–) of an induction in Hsp70 mRNA in response to heat shock.

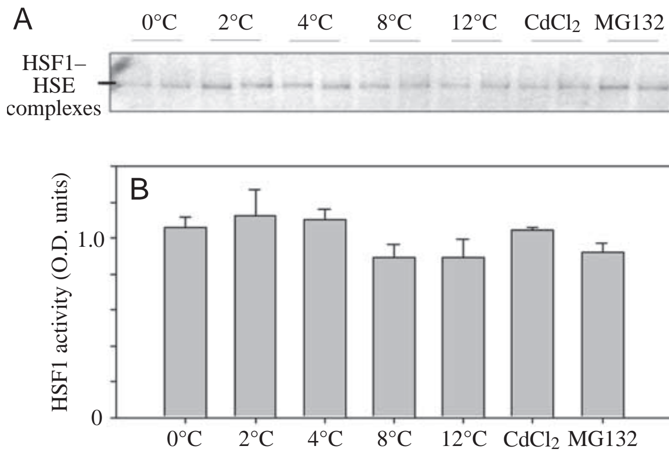

Fig. 5. HSF1 activity in *Trematomus bernacchii* hepatocytes. (A) Representative electrophoretic mobility shift assay (EMSA) image of HSF1–HSE complexes in hepatocytes from one individual, exposed for 1 h to 0–12°C or to 100 μmol l^{-1} $CdCl_2$ or 100 μmol l^{-1} MG132. Each sample was run in duplicate in two lanes. (B) Average (mean ± S.E.M.) HSF1 activity for four individuals exposed to heat and chemical stressors outlined above. While HSF1 activity was always detected in these cells, no significant effect of treatment was observed.

existence of an irregularity in the *hsp* gene expression pathway rather than the loss of temperature sensitivity in this cellular defense mechanism.

Preliminary sequencing of regions of the *hsp70* gene in this species demonstrates that this gene is present in its genome (Maresca et al., 1988; S. Lund and A. Whitmer, personal communication), and, in the current study, Hsp70 mRNA was detected in isolated hepatocytes (Fig. 3A). However, we observed no treatment-related increase in the concentration of Hsp70 mRNA (Fig. 3B), consistent with the lack of induced protein synthesis in the *in vivo* metabolic labeling experiments (Fig. 2). It should be noted that levels of Hsp70 mRNA in treated hepatocytes were equivalent to levels in field-caught specimens sacrificed immediately upon capture, precluding handling stress as the cause of the observed Hsp70 mRNA expression (Place et al., 2004). Furthermore, it has been demonstrated that handling stress does not affect hepatic Hsp70 expression in other teleost species (Vijayan et al., 1997). The results from the hepatocyte preparations were obtained with two Hsp70 mRNA probes, one generated from *T. bernacchii* tissue and one from the related New Zealand notothenioid *B. variegatus*. While both of these probes cross-reacted with Hsp70 mRNA from *T. bernacchii*, in neither case did they detect inducible expression of this transcript, although these same probes successfully detected typical heat induction of Hsp70 mRNA in other species of fishes (Table 1). This suggests that, despite sequence similarity in the coding regions of this gene, transcriptional regulation of *hsp70* in *T. bernacchii* differs from that of these non-polar species.

To characterize the regulation of *hsp* genes in *T. bernacchii*, we examined the DNA-binding activity of HSF1. All inducible *hsp* genes are regulated by HSF1, a cytoplasmic transcription factor that becomes activated in response to heat stress (Wu, 1995). Inactive monomeric HSF1 acquires the ability to bind to the HSE in *hsp* promoters through trimerization and phosphorylation, a requisite step in the transactivation of *hsp* genes (Morimoto, 1998). It has been shown that cadmium stress displays a similar mechanism of action to thermal perturbation, inducing Hsp expression through the activation of latent monomeric HSF1 (Hung et al., 1998; Gordon et al., 1997). Here, we detected an HSE-specific HSF1 (Fig. 4). Therefore, the absence of an induction of Hsps in this species is not due to the lack of an HSF1 with HSE-binding competency. However, the HSE-binding activity of this molecule did not change in response to heat or chemical treatment (Fig. 5).

The absence of inducible *hsp* gene expression is rare but not unprecedented. For instance, another Antarctic organism, the ciliate *Euplotes focardii*, also lacks the ability to appreciably increase the expression of *hsp70* in response to thermal stress, despite the fact that the gene is intact in this species (LaTerza et al., 2004). As in *T. bernacchii*, *hsp70* is constitutively expressed in *E. focardii* (LaTerza et al., 2001). Taken together, these findings suggest that adaptation to stable low temperatures may at times involve the production of molecular chaperones at a constant level, coupled with the loss of thermal sensitivity in the genes that encode them.

An additional species lacking a typical HSR is the freshwater hydra *Hydra oligactis*, which does not induce any size class of Hsp in response to elevated temperature and is deficient in acquired stress tolerance (Bosch et al., 1988; Gellner et al., 1992). The more thermotolerant *Hydra attenuata* and *Hydra magnipapilata* exhibit robust stress responses and persist during periods of warming sufficient to eliminate *H. oligactis* from certain environments. The lack of an inducible HSR in *H. oligactis* was determined to be due not to an inactive HSF1 or other upstream transcriptional dysfunction but rather has been linked to the instability of Hsp mRNA in this species (Brennecke et al., 1998). We cannot rule out that a similar mechanism is working synergistically with the constitutive expression of Hsp70 mRNA in *T. bernacchii*, as we do not know to what extent the message we detect is translated.

It is unknown whether other Antarctic notothenioids also lack inducible Hsp expression. In a study using differential display, Hsp70 mRNA was induced in the livers of another member of the suborder, the icefish *Chionodraco hamatus*, in response to whole-animal injections of cadmium (Carginale et al., 2002). The source of the disparity between the effect of cadmium on Hsp70 induction in *T. bernacchii* and that in *C. hamatus* is unclear but may be due to the different methods of cadmium exposure employed. In the *C. hamatus* study, cadmium was injected intramuscularly every other day for 7 days. It is possible that this longer-term exposure to the toxic heavy metal may have a different effect than the direct exposure of liver cells conducted here in *T. bernacchii*. However, it has been shown that exposure to concentrations of cadmium as low as 5 μmol l^{-1} are sufficient to induce Hsp70 expression in fish cells (Heikkila et al., 1982). The

concentrations used here were an order of magnitude greater, yet failed to cause Hsp induction. Another technical difference between the two studies lies in the preparation of liver tissue. We first perfused the liver to remove red blood cells, which are nucleated in fishes, to obtain unadulterated hepatocyte suspensions. The *C. hamatus* study examined gene expression in livers that had not been perfused, possibly resulting in the inclusion of erythrocytes.

Our findings suggest that the *hsp* genes in *T. bernacchii* are constitutively expressed and that their transcription is regulated by an HSF1 that is active even at nearly ambient environmental temperatures. The constant expression in *T. bernacchii* of what are stress-inducible genes in other species may reflect an elevated need for protein chaperoning in this cold-adapted fish. Alternatively, an alteration may have occurred in the transcriptional apparatus controlling *hsp* genes (including but not necessarily limited to the DNA-binding activity of HSF1) that resulted in the constitutive expression of Hsps. If this is the case, the constant production of Hsps must be relatively non-deleterious. Certainly, the cold and generally uncontaminated waters of the nearshore Antarctic ecosystem may have rendered the inability to induce Hsps above a constant level selectively neutral over evolutionary time. Studies are ongoing to characterize the HSR in other species of Antarctic notothenioids, including congeners of *T. bernacchii*, in order to establish the extent to which the pattern of Hsp expression observed in this species is shared with other members of the suborder.

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References

Abe, T., Konishi, T., Katoh, T., Hirano, H., Matsukuma, K., Kishimura, M. and Higashi, K. (1994). Induction of heat shock 70 mRNA by cadmium is mediated by glutathione suppressive and non-suppressive triggers. *Biochim. Biophys. Acta* **1201**, 29-36.

Airaksinen, S., Rabergh, C. M. I., Sistonen, L. and Nikinmaa, M. (1998). Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhyncus mykiss*). *J. Exp. Biol.* **201**, 2543-2551.

Ananthan, J., Goldberg, A. L. and Voellmy, R. (1986). Abnormal proteins serve as eucaryotic stress signals and trigger the activation of heat shock genes. *Science* **232**, 522-524.

Bosch, T. C. G., Krylow, S. M., Bode, H. R. and Steele, R. E. (1988). Thermotolerance and synthesis of heat shock proteins: These responses are present in *Hydra attenuata* but absent in *Hydra oligactis*. *Proc. Natl. Acad. Sci. USA* **85**, 7927-7931.

Brennecke, T., Gellner, K. and Bosch, T. C. G. (1998). The lack of a stress response in *Hydra oligactis* is due to reduced hsp70 mRNA stability. *Eur. J. Biochem.* **255**, 703-709.

Buckley, B. A., Owen, M.-E. and Hofmann, G. E. (2001). Adjusting the thermostat: the threshold induction temperature for the heat-shock response in intertidal mussels (genus *Mytilus*) changes as a function of thermal history. *J. Exp. Biol*. **204**, 3571-3579.

Buckley, B. A. and Hofmann, G. E. (2002). Thermal acclimation changes DNA-binding activity of heat shock factor 1 (HSF1) in the goby *Gillichthys mirabilis*: implications for plasticity in the heat-shock response in natural populations. *J. Exp. Biol.* **205**, 3231-3240.

Buckley, B. A. and Hofmann, G. E. (in press). Magnitude and duration of thermal stress determine kinetics of Hsp gene regulation in the goby, *Gillichthys mirabilis*. *Physiol. Biochem. Zool*. **77**.

Bush, K. T., Goldberg, A. L. and Nigam, S. K. (1997). Proteasomic inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. *J. Biol. Chem*. **272**, 9086-9092.

Carginale, V., Capasso, C., Scudiero, R. and Parisi, E. (2002). Identification of cadmium-sensitive genes in the Antarctic fish *Chionodraco hamatus* by messenger RNA differential display. *Gene* **299**, 117-124.

Carpenter, C. M. and Hofmann, G. E. (2000). Expression of 70 kDa heat shock proteins in Antarctic and New Zealand notothenioid fish. *Comp. Biochem. Physiol. A* **125**, 229-238.

Cocca, E., Ratnayakelecamwasam, M., Parker, S. K., Camardella, L., Ciaramella, M., DiPrisco, G. and Detrich, H. W. (1997). Do the hemoglobinless icefishes have globin genes? *Comp. Biochem. Physiol. A* **118**, 1027-1030.

DeVries, A. L. (1988). The role of antifreeze glycopeptides and peptides in the freezing avoidance of Antarctic fishes. *Comp. Biochem. Physiol. B* **90**, 611-621.

Eastman, J. T. (1988). Lipid storage systems and the biology of two neutrally buoyant Antarctic notothenioid fishes. *Comp. Biochem. Physiol. B* **90**, 529-537.

Eastman, J. T. (1993). *Antarctic Fish Biology: Evolution in a Unique Environment*. New York: Academic Press.

Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol*. **61**, 243-282.

Fink, A. (1999). Chaperone-mediated protein folding. *Physiol. Rev*. **79**, 425-449.

Gellner, K., Praetzel, G. and Bosch, T. C. (1992). Cloning and expression of a heat-inducible hsp70 gene in two species of *Hydra* which differ in their stress response. *Eur. J. Biochem*. **210**, 683-691.

Gon, O. and Heemstra, P. C., ed. (1990). *Fishes of the Southern Ocean*. Grahamstown: J. L. B. Smith Institute of Ichthyology.

Gordon, S., Bharadwaj, S., Hnatov, A., Ali, A. and Ovsenek, N. (1997). Distinct stress-inducible and developmentally regulated heat shock transcription factors in *Xenopus* oocytes. *Dev. Biol.* **181**, 47-63.

Hartl, F. U. (1996). Molecular chaperones in cellular protein folding. *Nature* **381**, 571-580.

Hartl, F. U. and Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* **29**, 1852-1858.

Heikkila, J. J., Schultz, G. A., Iatrou, K. and Gedamu, L. (1982). Expression of a set of fish genes after heat or metal ion exposure. *J. Biol. Chem*. **257**, 12000-12005.

Hightower, L. E. (1980). Cultured animal cells exposed to amino acid analogues or purumycin rapidly synthesize several polypeptides. *J. Cell. Physiol*. **102**, 407-424.

Hofmann, G. E., Buckley, B. A., Airaksinen, S., Keen, J. E. and Somero, G. N. (2000). Heat-shock protein expression is absent in the Antarctic fish *Trematomus bernacchii* (Family Nototheniidae). *J. Exp. Biol*. **203**, 2331-2339.

Hung, J.-J., Cheng, T.-J., Chang, M. D.-T., Chen, K.-D., Huang, H.-L. and Lai, Y.-K. (1998). Involvement of heat shock elements and basal transcription elements in the differential induction of the 70-kDa heat shock protein and its cognate by cadmium chloride in 9L rat brain tumor cells. *J. Cell. Biochem*. **71**, 21-35.

Hunt, B. M., Hoefling, K. and Cheng, C.-H. C. (2003). Annual warming in seawater temperatures in McMurdo Sound in relationship to endogenous ice in notothenioid fish. *Antarc. Sci.* **15**, 333-338.

Kim, D., Kim, S.-H. and Li, G. C. (1999). Proteasome inhibitors MG132 and lactacystin hyperphosphorylate HSF1 and induce hsp70 and hsp27 expression. *Biochem. Biophys. Res. Comm*. **254**, 264-268.

LaTerza, A. L., Miceli, C. and Luporine, P. (2001). Divergence between two Antarctic species of the ciliate *Euplotes*, *E. focardii* and *E. nobilii*, in the expression of heat-shock protein 70 genes. *Mol. Ecol*. **10**, 1061-1067.

LaTerza, A. L., Miceli, C. and Luporine, P. (2004). The gene for the heat-shock protein 70 of *Euplotes focardii*, an Antarctic psychrophilic ciliate. *Ant. Sci*. **16**, 23-28.

Lai, Y.-K., Shen, C.-H., Cheng, T.-J., Hou, M.-C. and Lee, W.-C. (1993). Enhanced phosphorylation of a 65 kDa stress protein is associated with induction of stress protein in 9L rat brain tumor cells. *J. Cell Physiol*. **149**, 66-76.

Lee, D.-H. and Goldberg, A. L. (1998). Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol*. **8**, 397-403.

Liao, V. H.-C. and Freedman, J. H. (1998). Cadmium-regulated genes from the nematode *Caenorhabditis elegans*: identification and cloning of new cadmium-responsive genes by differential display. *J. Biol. Chem*. **237**, 31962-31970.

Lindquist, S. (1986). The heat shock response. *Annu. Rev. Biochem.* **55**, 1151-1191.

Londraville, R. L. and Sidell, B. D. (1990). Ultrastructure of aerobic muscle in Antarctic fishes may contribute to maintenance of diffusive fluxes. *J. Exp. Biol*. **150**, 205-220.

Manca, D., Ricard, A. C., Trottier, B. and Chevalier, G. (1991). Studies on lipid-peroxidation in rat-tissues following administration of low and moderate doses of cadmium chloride. *Toxicology* **67**, 303-323.

Maresca, B., Patriarcha, E., Goldenberg, C. and Sacco, M. (1988). Heat shock and cold adaptation in Antarctic fishes: a molecular approach. *Comp. Biochem. Physiol. B* **90**, 614-623.

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

Ovelgonne, J. H., Souren, J. E., Wiegant, F. A. and Van Wijk, R. (1995). Relationship between cadmium-induced expression of heat shock genes, inhibition of protein synthesis and cell death. *Toxicology* **99**, 19-30.

Parsell, D. A. and Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* **27**, 437-439.

Pelham, H. R. B. (1982). A regulatory upstream promoter element in the *Drosophila* hsp70 heat shock gene. *Cell* **30**, 517-528.

Pirkkala, L., Nykanen, P. and Sistonen, L. (2001). Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB J*. **15**, 1118-1131.

Place, S. P., Zippay, M. L. and Hofmann, G. E. (2004). Constitutive roles for inducible genes: evidence for the alteration in expression of the inducible hsp70 gene in Antarctic notothenioid fishes. *Am. J. Physiol.* **287**, R429-R436.

Sidell, B. D., Crockett, E. L. and Driedzic, W. R. (1995). Antarctic fish tissues preferentially catabolize monoenoic fatty acids. *J. Exp. Zool*. **271**, 73-81.

Sidell, B. D., Vayda, M. E., Small, D. J., Moylan, T. J., Londraville, R. L., Yuan, M. L., Rodnick, K. J., Eppley, Z. A. and Costello, L. (1997). Variable expression of myoglobin among the hemoglobinless Antarctic icefishes. *Proc. Natl. Acad. Sci. USA* **94**, 3420-3424.

Somero, G. N. and DeVries, A. L. (1967). Temperature tolerance of some Antarctic fishes. *Science* **156**, 257-258.

Somero, G. N., Fields, P. A., Hofmann, G. E., Weinstein, R. B. and Kawall, H. (1998). Cold adaptation and stenothermy in Antarctic notothenioid fishes: what has been gained and what has been lost? In *Fishes of Antarctica. A Biological Overview* (ed. G. DiPrisco, E. Pisano and A. Clarke), pp. 97-109. Berlin: Springer-Verlag.

Tomanek, L. and Somero, G. N. (2002). Interspecific- and acclimation-induced variation in levels of heat-shock proteins 70 (hsp70) and 90 (hsp90) and heat-shock transcription factor-1 (HSF1) in congeneric marine snails (genus *Tegula*): implications for regulation of *hsp* gene expression. *J. Exp. Biol.* **205**, 677-685.

Vijayan, M. M., Pereira, C., Forsyth, R. B., Kennedy, C. J. and Iwama, G. K. (1997). Handling stress does not affect the expression of hepatic heat shock protein 70 and conjugation heat-shock-cognate hsc71 gene from rainbow trout. *Eur. J. Biochem*. **204**, 893-900.

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

Xiao, H. and Lis, J. T. (1988). Germline transformation used to define key features of heat-shock response elements. *Science* **239**, 1139-1142