Biochemical and Biophysical Research Communications **259,** 15–20 (1999)
Article ID bbrc.1999.0729, available online at http://www.idealibrary.com on IDEAL®

HSF1 Activation Occurs at Different Temperatures in Somatic and Male Germ Cells in the Poikilotherm Rainbow Trout

Pascale Le Goff[1] and Denis Michel
UPRES-A 6026 CNRS, Université de Rennes 1, Campus de Beaulieu, 35042 Rennes Cedex, France

Received April 19, 1999

The heat shock factor 1 (HSF1) is the central actor of the response to hyperthermia in eukaryotic cells. In mammals, male germ cells are an exception among all cellular populations for their HSF1 activation occurs at low temperature. This feature was believed to be specific of homeotherms whose testicular compartment is located outside the main body cavity, where temperature is lower. In the present study, we show that in the poikilotherm rainbow trout, the maximal heat shock response of male germ cells, that are located in the same body compartment than the other organs, occurs also at a significantly lower temperature (22°C) than for somatic cells (28°C), regardless of culture conditions before heat shock. In addition, the acquisition of the HSE-binding activity of HSF1 upon heat shock is not associated with the classical hsp70 mRNA accumulation. Taken together, these results strongly suggest the existence of a particular mode of heat shock response that could be specific of male germ cells but not restricted to homeotherms.

Key Words: **heat shock factor; male, germ cell; temperature; trout.**

All cellular activities critically depend on the unique three-dimensional structure of proteins. The acquisition of the correct protein folding is determined in part by the amino-acid sequence of the proteins, but often requires the assistance of protein chaperones (1). Under protein-damaging conditions, these protein chaperones are also necessary for preventing protein aggregation, refolding misfolded proteins in ATP-dependent mechanisms, and driving irreversibly denatured proteins to degradation machineries (2). The originally discovered and one of the most potent protein-damaging treatment is hyperthermia, that leads to protein aggregation by exposing normally buried hydrophobic protein domains.

In this respect, many protein chaperones capable of preventing heat-denaturation of proteins and called heat shock proteins (HSP), strongly accumulate after heat shock, due to an increased transcription rate of their genes, mediated by the ubiquitous transcription factor heat shock factor 1 (HSF1). Several related HSF have been identified but HSF1 has the unique ability to bind to the heat shock DNA elements (HSE) (3) in a heat shock-dependent manner (4, 5). The temperature at which HSF1 is activated is finely tuned to the heat sensitivity of the cellular protein machineries and generally fixed several degrees above the normal living temperature. In plants and poikilotherm animals, this set point strikingly depends on the environmental temperature, and may be for example as low as 5°C for an alga from antarctic (6). For homeotherm animals who have acquired a relative independence of the external temperature, it was admitted that the threshold temperature for HSF1 activation is unique for all cellular populations. However, it has thereafter been shown in mammals that a particular cell type, namely male germ cells, are an exception to this rule, since their temperature of HSF1 activation is only 37°C, that is to say more than 4°C below that observed in somatic cells. This finding is likely to explain why mammalian testes are generally located outside the main body cavity (7). However, this altered heat shock response is not a mere consequence of the presence of germ cells at lower temperature in the scrotal compartment, since the heat shock set point for somatic testicular cells remains the same as for somatic cell of the organism (41°C) (8).

To determine whether the lower HSF1 set point observed in male germ cells is specific of mammals or a general feature in the animal kingdom, we have compared the temperature of HSF1 activation in somatic

[1] Corresponding author. CNRS UPRES-A 6026, Bat.13, Campus de Beaulieu, 35042 Rennes Cedex, France. Fax: 33 2 99 28 67 94. E-mail: pascale.le-goff@univ-rennes1.fr.

0006-291X/99 $30.00

and male germ cells from a poikilotherm animal, whose cells are all present at the same temperature. For this purpose, we studied the rainbow trout (*Onchorhynchus mykiss*) as a model system in which the testis compartment sits at the same temperature than the other tissue of the body. Moreover, this species can live only in a limited temperature range, from 4°C to about 18°C (stenotherm). We show that in this animal, the HSF1 activation temperature is also clearly different between male germ cells and somatic cells. Moreover, the male germ cell specific HSF1 activation at very low temperature is not associated with an increased transcription of the hsp70 gene. This first observation obtained with a poikilotherm species, allows to propose that the lower set point of HSF1 activation in germ cells is a unique property of this cell type, that is not restricted to homeotherm species.

MATERIALS AND METHODS

Experimental animals. Male rainbow trouts, weighing 800 g in average, were supplied by an experimental fish farm (Sizun, Finistère, France) and kept in circulating fresh water tanks between 12-14°C. Trout were anaesthetised with 3% 2-phenoxyethanol and killed by a blow to the head before liver perfusion or testis removal.

Cell culture and heat shocks. The trout embryonic fibroblastic cell line STE (Steelhead Trout Embryo) was grown at 18°C in 75 cm^2 flasks in Dulbecco's modified Eagle's and Ham's F-12 nutrient mixture with L-glutamine (1:1), 15 mM Hepes, 15 mM N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid, 12 mM $NaHCO_3$, 1% (v/v) antibiotics (penicillin, streptomycin and amphotericin B, Sigma, St Quentin Fallavier, France) and supplemented with 10% foetal bovine serum. Hepatocytes were obtained after dissociation with collagenase A (Bœhringer) as previously described (9). 1-2 $\times$ 10^7 hepatocytes were seeded in 5 ml of the medium described above but supplemented with 2% Ultroser SF (Biosepra) and cultured under gyratory shaking at 55 rpm at 18°C (Novotron, INFORS AG, Switzerland) to form aggregates. Hepatocyte functions are perfectly preserved under these culture conditions (9). Male germ cells were obtained by mechanical dissociation, exactly as previously described (10), of testes at mid spermatogenesis, with spermatogonia to spermatozoa i.e. stage IV-V according to a classification adapted from Billard and Escaffre (11). At all stages of the dissociation process, testes or cells were maintained around 12°C. For the heat shock experiments, flasks for STE cells or tubes containing hepatocyte aggregates or germ cells were submerged for one hour in a water bath at the indicated temperatures. When a recovery period was necessary, cells were put back into the incubator for half an hour at 18°C for STE cells and 12°C for germ cells. STE cells were harvested by scraping and centrifugation at 2000 rpm at 4°C for 5 min or by centrifugation of hepatocytes or germ cells. Cell pellets were immediately frozen and kept at −80°C.

Protein extracts and gel mobility shift assays. Whole cell extracts were prepared as described in (12), from frozen pellet by adding 5 volumes of extraction solution, 10 mM Hepes pH 7.9, 0.4 M NaCl, 0.1 mM EGTA, 0.5 mM dithiothreithol (DTT), 5% glycerol, 0.5 mM PMSF, vortexing and repetitive pipetting until the suspension appeared homogeneous. The use of a Teflon glass homogeniser was necessary to complete germ cell disruption. Homogenates were spun at 25000 g at 4°C for 5 min. Supernatants were collected, protein concentrations were measured, adjusted with extraction buffer at 1 $\mu g/\mu l$ for STE cells and hepatocytes or at 0.5 $\mu g/\mu l$ for germ cells, and kept at −80°C. For gel shift analyses 10 μl of whole cell extract were incubated 30 min at 4°C in the presence of 12.5 mM Tris-HCl pH 7.8, 62.5 mM NaCl, 1.25 mM EDTA, 0.625 mM DTT, 6.25% glycerol, 1 μg of poly dI/dC, 10 μg of BSA and about 5 ng of ^{32}P labeled HSE (60000 cpm) in a final volume of 20 μl. The binding reactions were performed at 4°C for 30 min and loaded on a 4% polyacrylamide gel in 8.9 mM Tris base, 8.9 mM boric acid, 0.2 mM EDTA, pH:8. Complex were separated during 2 hours at 220 volts. Gels were dried, exposed overnight to a X-ray film (Kodak X-omat). For supershift or competition experiments 1 μl of anti-mouse HSF1 monoclonal antibody (kindly provided by Dr. Richard I. Morimoto, Northwestern university, Evanston, Illinois) or the indicated amount of unlabeled wild type or mutated HSE was mixed prior to adding the labeled HSE. The wild type HSE was the double stranded DNA fragment 5′-tcgagcGAAtgTTCtaGAAac-3′ with XhoI hanging extremities to allow the labeling with the Klenow fragment of the DNA polymerase I. Mutated HSE was the double stranded fragment, 5′-cGAAtgGGAtaGAAa, devoid of the central TTC trinucleotide.

Reverse transcriptase (RT)-PCR analyses. RNAs were extracted from control or heat shock cells after a recovery period using the TRIZOL reagent (Gibco BRL). RNAs were reverse transcribed into cDNA using random primers as primers. hsp70 and βactin cDNAs were measured by RT-PCR using the following oligonucleotides as primers: 5′-ggACATCAgCCAgAACAAgC-3′; 5′-gTgTAgAAgTCgATgCCCTC-3′ (132 bp amplified fragment) for hsp70 and 5′-TTgCTgATCCACATCTgCTg-3′; 5′-gACAggATgCAgAAggAgAT-3′ (around 150 bp amplified fragment) for βactin, in the presence of deoxynucleotides at 50 μm final concentration. The amplification reactions were carried out using 25 cycles: 95°C 30s; 61°C 30s; 72°C 40s, the PCR products were analysed on 3% agarose gel in the presence of ethidium bromide. The absence of contaminating DNA was verified by PCR on non reverse transcribed RNAs.

RESULTS

Heat-induction of the HSE binding activity in trout cells. Previous heat shock experiments using trout cells have only measured the accumulation of hsp transcripts, and have defined that the maximal response is obtained after 28°C heat shock (13). We wanted to study the heat shock response at an upstream level, by determining the heat shock temperature leading to maximal HSE binding activity in trout cells. For this purpose, nuclear extracts from two different trout somatic cell types: an embryonic cell line (STE) and hepatocytes, were tested for their HSE binding activity in electrophoretic mobility analyses, using a labeled oligonucleotide probe containing the consensus HSE binding site. Extracts were prepared from cells grown at 18°C or after 1 hour incubation period at different temperatures. In the two cell types, a weak HSE binding activity was observed in control extracts, which was strongly enhanced after heat shock (Figures 1A and 2A). In hepatocytes as well as in STE cells, the maximal HSE-binding activity was observed after a 28°C heat shock. Since the retarded complex observed in STE cells and hepatocytes were identical, we used only the STE cells as a source of somatic cells in the following experiments.

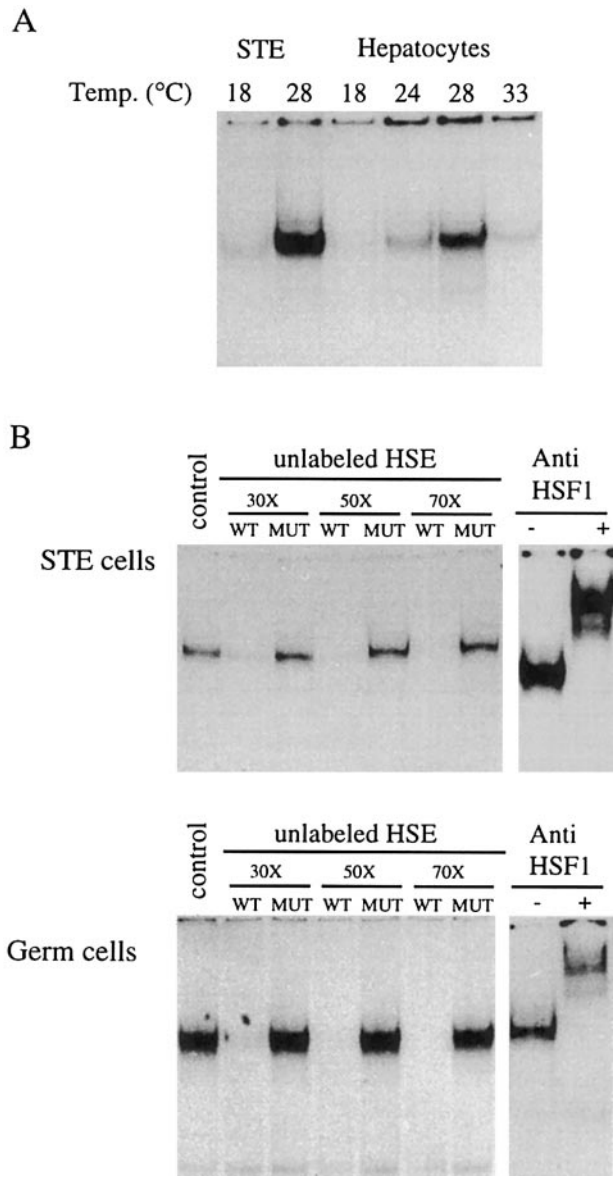

FIG. 1. Heat induction of a HSF1 binding activity in STE cells, hepatocytes and male germ cells. (A) Gel mobility shift analysis of extract prepared from STE cells and hepatocytes grown at 18°C or after a one hour heat shock at the indicated temperatures. (B) Gel mobility shift analysis of extract prepared from STE cells or male germ cells after a one hour heat shock at 28°C. Extracts were incubated with the labeled oligonucleotide probe in absence (control), or presence of a 30- 50- or 70-fold excess of unlabeled competitors corresponding to wild type (WT) or mutated (MUT) HSE. For supershift experiments (right panels), a monoclonal antibody directed against mouse HSF1 was added (+) or not (−), to the HSE-HSF binding reaction.

Different thresholds for the activation of STE and germ cell HSE binding activities. HSE gel shift analyses using nuclear extracts from either STE cells or freshly dissociated germ cells obtained from mature males, were conducted in parallel. Cells were incubated for one hour at different incubation temperatures prior to extracts preparation and gel shift analyses. As shown in Figure 2, HSE-binding activity was barely detectable at 18°C in STE cells, increased progressively to peak at 28°C and then decreased. In contrast, the maximal HSE-binding activity was observed at a significantly lower temperature in germ cells. A low level of binding was observed from 12°C to 18°C, a maximum level at 22°C and decreasing levels from 25°C to 33°C (Figure 2C). It is worth noticing that the mechanical dissociation process of the cells did not modify the HSE binding activity, since the same band shift pattern was observed when using extracts prepared from the whole testis from trouts living at 12°C or freshly

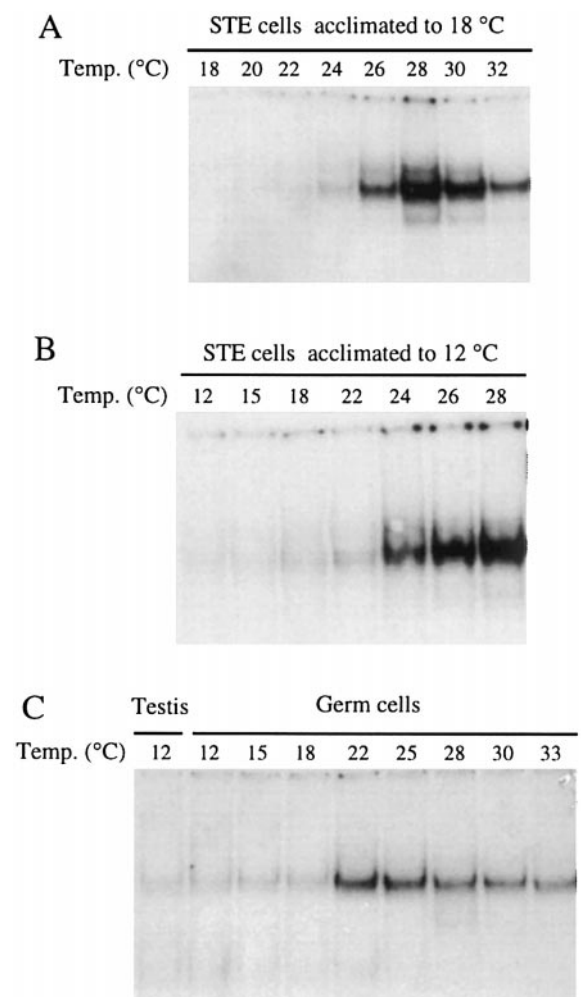

FIG. 2. HSF1 activation temperature profile in STE and male germ cells. Gel mobility shift analysis of extracts prepared from STE cells grown at 18°C (A) or 12°C (B) and from freshly dissociated male germ cells (C) after one hour incubation period at the indicated temperature. The temperature of maximal HSE binding activity is obtained at approximately 6°C lower temperature in germ cells as compared with STE cells.

dissociated germ cells cultured at 12°C (Figure 2C, lanes 1 and 2).

Molecular composition of the HSE complex. The striking difference of HSE binding temperature activation observed between somatic and germ cells, might reflect either different thresholds for the activation of the same transcription factor, or the use of different HSE-binding factors in the different cell types. Hence, we wanted then to verify the identity of the protein components from somatic and germ cells, responsible for the HSE complex formation. We have first examined the effects of oligonucleotide competitors added in the binding reactions with nuclear extracts from STE cells and freshly dissociated male germ cells. As shown in Figure 1B, the retarded complex was specific to the HSE oligonucleotide, since it disappeared in the presence of an excess of wild type competitor but not of mutated HSE where the successive nGAAn blocks are not arranged in opposite orientations (see Materials and Methods). This specificity for the canonical HSE indicates that the HSE complex does contain a typical HSF. Furthermore, co-incubation of the band shift reactions with an anti-mouse HSF1 antibody resulted in the formation of a slower migrating ternary complex, when starting from germ cell as well as STE cell extracts, indicating that the factor involved in HSE complexes is likely to be the same in both cell types. Since anti-HSF antibodies are currently used in mammals to discriminate the different HSFs (8), the cross reactivity of the anti HSF1 antibody with the trout HSF is a strong indication that it is closely related to mammalian HSF1. In addition, considering that HSF1 is the only HSF whose DNA binding activity is strictly dependent on heat shock, the possibility that the trout HSF visualised in this study may correspond to an immunologically related HSF other than HSF1 is improbable. Finally, the absence of residual normally migrating complex in supershift lanes, strongly suggests that the HSF DNA binding activity present in the two trout cell types is mainly, if not exclusively, composed of trout HSF1.

Acclimation to a lower temperature does not affect the threshold temperature for HSF1 DNA binding activity. Since the fishes used for the preparation of germ cells were kept at 12°C, one may hypothesise that the different thresholds observed for the two cell types are a consequence of the different starting temperature: 12°C for germ cells versus 18°C for STE cells. To test this possibility, HSF1 binding activity was examined in STE cells grown at 12°C. Figure 2 (panels A and B) reveals that maximal HSF1 binding activity was not affected by lowering the previous incubation temperature. If STE cells are acclimated to a lower temperature, one may observe HSE complex formation at 24°C, but the maximal heat shock response still occurs at 28°C. In addition, at the temperature responsible for the maximum HSF1 DNA binding in germ cells (22°C), the DNA binding activity observed was as low whether the STE cells were cultured at 12°C or 18°C. These results clearly show that in our conditions, the temperature set point for maximal HSF1 activation is an intrinsic property of each cell type which does not depend on culture conditions.

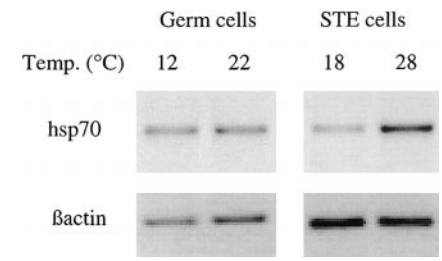

FIG. 3. Accumulation of hsp70 mRNAs in response to heat shock. Male germ cells and STE cells were heat shocked for one hour at the temperature leading to maximal HSE binding activity: 22°C or 28°C respectively and hsp70 and β-actin mRNA were detected by RT-PCR 30 min after temperature shift down. RT-PCR products were analyzed on a 3% agarose gel in presence of ethidium bromide.

Accumulation of hsp70 mRNA in STE and germ cells after heat shock. In mammalian cells, heat activation of HSF1 generally mediates the increased expression of hsp70 gene (4). To examine this point in trout cells, RNA was prepared from STE and germ cells, heat shocked for one hour at 28°C or 22°C respectively and then shifted back to 18°C or 12°C for 30 min. RNA were then reverse transcribed using random primers, hsp70, and βactin cDNAs were measured by PCR. As shown in Figure 3, hsp70 mRNA level increased after heat shock in STE cells, but remained constant in germ cells in despite of HSF1 activation.

DISCUSSION

The results presented here reveal surprising differences between cell populations from a poikilotherm animal upon heat shock. The HSF1 activity that we measured in trout somatic cells are in agreement with previous reports. The heat shock temperature inducing maximal HSF1 binding activity in primary cultured hepatocytes as well as in STE cells is 28°C. In these cells, the activation of HSF1 was accompanied by a classical increase of hsp70 mRNA. Accordingly, Mosser et al. (13) have shown that synthesis of heat shock proteins in trout fibroblasts was maximal at 28°C. In addition, we found that the temperature activation profile of HSF1 remained unchanged when the growth temperature of STE cells was reduced from 18°C to 12°C, in agreement with two previous studies on other poikilotherms. Hepa-

tocytes prepared from channel catfish *Ictalurus punctatus* acclimatised to 7°C or 25°C, exhibited a maximum HSP synthesis around 40°C (14). Likewise, varying the growth temperature of *Drosophila* cells between 19°C and 26°C had little effect on the pattern of HSP synthesis (15). On the contrary, a study from Abravaya et al (16) indicates that the level of activated HSF1 was dramatically increased in Hela cells, when grown at 35°C instead of 37°C prior to a 42°C heat shock. It is thus tempting to propose that in poikilotherm organisms whose cells are commonly exposed to temperature fluctuations, the heat shock response is not as tightly linked to their growth temperature as seen in homeotherms.

More surprisingly, we found that the temperature inducing maximal HSF1 DNA binding activity is 22°C in freshly isolated male germ cells, that is 6°C below the activation temperature of HSF1 in somatic cells. This is the second report indicating a reduced HSF1 activation temperature in male germ cells. HSF1 activation was previously shown to occur at low temperature in mouse male germ cells, when compared to somatic cells (7). Indeed, HSF1 activation occurs at 37°C in mouse germ cells, instead of 42°C for non testicular as well as testicular mouse somatic cells (8). The present data clearly indicates that male germ cells isolated in the testis at a lower temperature than the one observed for somatic cells in other tissues, possess a high heat sensitivity which is not restricted to mammalian species as shown here in the trout. Therefore, the observation of a lower threshold temperature of heat shock activation in mammalian male germ cells is probably not a consequence of the lower growth temperature of these cells within the scrotal compartment, but probably more a general property of animal male germ cells.

The biological signification of the HSF1 activation at low temperature in germ cells is puzzling. Since damaged proteins have been suggested to be the triggering signal of HSF1 activation (4, 17), one may hypothesise that this lower set point for HSF1 activation may be due to a particular thermosensitivity of certain male germ cell specific protein components. However, this explanation is not in agreement with the other observation presented in this report, that the transcription of the protein chaperone hsp70 gene does not increase after HSF1 activation in trout germ cells. On the contrary, a classical hsp70 induction upon heat shock, is observed in trout somatic cells. Such a lack of correspondence between DNA binding induction of HSF1 and hsp70 expression in germ cells may appear surprising since these two molecular events are classically associated (18). In fact, several cases of uncoupling between HSF1 activation and HSP expression have recently been reported. For example, a lack of induction of hsp70 after heat shock has also been observed in mouse testicular cells (19). Conversely, HSP are expressed at high level in 293 cells in absence of any HSE-HSF binding activity (20). Interestingly, beside male germ cells, a disconnection between the HSE binding activity and the transcriptional activity of HSF1 has also been demonstrated in *Xenopus* oocytes (21).

From a physiological point of view, these observations are of particular interest in the reproduction context. Indeed, male fertility is known for a long time to decline after a rise in temperature. In this respect, an increase of the scrotal temperature was used as a method of human male contraception by ancient romans. A particular sentitivity of male reproductive functions has also been established in rainbow trout (22, 23). It has been proposed that this phenomenon could be linked to a particular heat sensitivity of male germ cells. This assumption was then strongly strengthened at the molecular level by the recent observation that in mouse, HSF1 activation occurs in male germ cells at a temperature dramatically lower than in somatic cells (8). The present report tends to support the idea that the heat shock response at lower temperature is a general feature of male germ cells whose origin remains to be elucidated.

ACKNOWLEDGMENTS

This work was funded by Education Nationale (DRED), Centre National de la Recherche Scientifique (CNRS) and supported by the Association pour la Recherche contre le Cancer (ARC). We are grateful to Maurice Loir for his advice in the preparation of male germ cells and Dr. Richard Morimoto for his generous gift of the anti mouse HSF1 monoclonal antibody. We thank Yolande Cubadda for critical reading of the manuscript.

REFERENCES

1. Georgopoulos, C., and Welch, W. J. (1993) *Ann. Rev. Cell. Biol.* **9,** 601–634.
2. Gottesman, S., Wickner, S., and Maurizi, M. R. (1997) *Genes Dev.* **11,** 815–823.
3. Amin, J., Ananthan, J., and Voellmy, R. (1988) *Mol. Cell. Biol.* **8,** 3761–3769.
4. Morimoto, R. I. (1993) *Science* **259,** 1409–1410.
5. Mager, W. H., and De Kruijff, A. J. J. (1995) *Microb. Rev.* **59,** 506–531.
6. Vayda, M. E., and Yuan, M. (1994) *Plant. Mol. Biol.* **24,** 229–233.
7. Sarge, K. D., Bray, A. E., and Goodson, M. L. (1995) *Science* **374,** 126.
8. Sarge, K. D. (1995) *J. Biol. Chem.* **270,** 18745–18748.
9. Flouriot, G., Vaillant, C., Salbert, G., Pelissero, C., Guiraud, J. M., and Valotaire, Y. (1993) *J. Cell. Sci.* **105,** 407–416.
10. Loir, M., and Sourdaine, P. (1995) *Hochachka PW and Mommsen TP, Biochemistry and Molecular Biology of Fishes* **3,** 249–272.
11. Billard, B., and Escaffre, A. M. (1975) *Bull. Fr. Piscic.* **256,** 111–116.

12. Zimarino, V., and Wu, C. (1987) *Science* **327,** 727–730.
13. Mosser, D. D., Heikkila, J. J., and Bols, N. C. (1986) *J. Cell. Physiol.* **128,** 432–440.
14. Koban, M., Graham, G., and Prosser, C. L. (1987) *Physiol. Zool.* **60,** 290–296.
15. Linquist, S. (1980) *Dev. Biol.* **77,** 463–479.
16. Abravaya, K., Phillips, B., and Morimoto, R. I. (1991) *Genes. Dev.* **5,** 2117–2127.
17. Baler, R., Welch, W. J., and Voellmy, R. (1992) *J. Cell. Biol.* **117,** 1151–1159.
18. Mosser, D. D., Theodorakis, N. G., and Morimoto, R. I. (1988) *Mol. Cell. Biol.* **8,** 4736–4744.
19. Mezquita, B., Mezquita, C., and Mezquita, J. (1998) *FEBS Lett.* **436,** 382–386.
20. Victor, M., and Benecke, B. J. (1998) *Mol. Biol. Rep.* **25,** 135–141.
21. Bharadwaj, S., Hnatov, A., Ali, A., and Ovsenek, N. (1998) *Biochim. Biophys. Acta* **1402,** 79–85.
22. Billard, R., and Breton, B. (1977) *Cah. Lab. Montereau* **5,** 5–24.
23. Manning, N. J., and Kime, D. E. (1985) *Gen. Comp. Endocrinol.* **57,** 377–382.