EVIDENCE FOR THE INVOLVEMENT OF HSP60 AND C-FOS GENES IN OSMOREGULATION IN *Fundulus heteroclitus*.

Ian G. Welsford, Susan. L. Whittemore and Andrew Mountcastle

Department of Biology, Keene State College, Keene, NH 03442

The ability to respond rapidly and effectively to environmental stressors (i.e. extremes in temperature, salinity, pH, pollutants) is requisite for normal physiology in all organisms. The growing consensus within biology is that animals and plants utilize a specific set of genes to protect themselves against such stressors. The best characterized of these are the genes which control the heat shock proteins (HSPs) which have been found in humans, fish, fungi, insects, bacteria and higher plants. Upon exposure to environmental insult, HSP genes are activated, resulting in the production of HSPs which serve both to protect the cells of the organism from damage and maintain function during exposure to the stress (e.g. Neuman, D., *et al.* Biol. Zent.bl. 108: 1-156, 1989.)

Apart from HSPs, organisms may utilize a number of different genetic "systems" to respond to environmental stressors. One example has been reported in mammalian brain, where alterations in osmolality, temperature, pH and oxygen partial pressure result in the activation of both the HSP system and the "immediate early gene" (IEG) response system including c-Fos and c-Jun (e.g. Herdegen, T., Neuroscientist 2(3): 153-161, 1996). However, little is known about how the IEG and HSP systems may interact in regulating and coordinating the stress response. In fact, there exists very little information on the precise role played by IEGs in the stress responses of animals. We have initiated experiments designed to address the possible role the HSPs and IEGs may play in osmoregulation in the killifish, *Fundulus heteroclitus*, as a model for how such systems may interact in coordinating appropriate responses to environmental insult.

Fundulus which had been collected locally and adapted to freshwater for approximately 2 months were used in the present study. Fish were transferred from freshwater (approximately 150 mOsm kg H_2O^{-1}) to full strength seawater (approximately 1025 mOsm kg H_2O^{-1}) and RNA was extracted after 24, 48 and 72 h of exposure. Control animals were transferred into a new tank of freshwater and samples were obtained 24, 48 and 72 h after transfer. Total RNA and genomic DNA were isolated from gill and brain using TriReagent protocol (Molecular Biosystems Inc.). Briefly, tissue was extracted on ice from cold-anesthetized fish and ground to homogeneity in baked, DEPC-treated glass homogenizers containing TriReagent, a mix of phenol and guanidine isothiocyanate. Bromochloropropane (BCP) was added to the homogenate and the mixture was spun at 10,000 X g for 20 min at 4^0 C. The aqueous phase containing the RNA was transferred to a new tube and isopropanol was added to precipitate the RNA. The precipitant was centrifuged at 12,000 X g for 30 min at 4^0C and the resulting pellet was washed twice in 70% ethanol. The pellet was suspended in DEPC treated water and yield was quantified using fluorescent spectroscopy. Degradation was assessed qualitatively by running agarose formaldehyde gels on the samples. DNA was isolated from the organic phase of the extract by first precipitating with 100% ethanol, spinning at 7,500 X g for 10 min at 4^0C, then washing the pellet with 0.1 M sodium citrate in 10% ethanol. The pellet was then washed in 70% ethanol, air dried and dissolved in 8 mM NaOH. Concentration was determined by fluorescent absorbance (i.e. OD_{260}). RNA yields were normalized to a percentage of DNA yield as an additional internal qualitative check for differential RNA degradation between treatment groups. RT-PCR was performed on RNA using an Access RT-PCR kit from Promega Inc. RT-PCR was run using a degenerate sequence downstream primer for first strand synthesis followed by a degenerate upstream primer for amplification. Primers for HSP60 were designed to produce a 345 bp product while those

for c-Fos were designed to produce a 389 bp product. Primers were synthesized by Ransom Hill BioScience Inc. The thermal cycle protocol followed was 45 min at 48° C for reverse transcriptase followed by 94° C for 2 min to inactivate the AMV Reverse Transcriptase, then 45 cycles of 94°C for 30 s (denaturation), 59° C for 1 min (annealing temperature; determined empirically based on Tm of primer), and 68°C for 2 min. Amplification was followed by a final extension step of 7 min at 68°C. Samples were run on 1.6% TBE agarose gels using blunt end PCR molecular weight markers from BioRad Inc. Photographs of gels were scanned with an analog densitometer (Hoeffer Instr.)

Both the gill tissue and the brain tissue of *Fundulus* exposed to seawater gave significantly greater PCR reaction product with the HSP60 primers than did the time matched controls (Fig. 1). There was no detectable difference between the PCR product of 24h, 48h or 72 h exposure groups (Fig. 1). For all groups, the reaction products were calculated by linear regression to be approximately 343 bp, close to the predicted 345 bp. For the c-Fos primers, the brain tissue, of *Fundulus* exposed to seawater gave significantly greater PCR reaction product than did the brain tissue from time matched controls (Fig. 1). Again, there were no detectable differences in reaction product between the 24h, 48h or 72h exposure groups. The size of the reaction product was calculated to be approximately

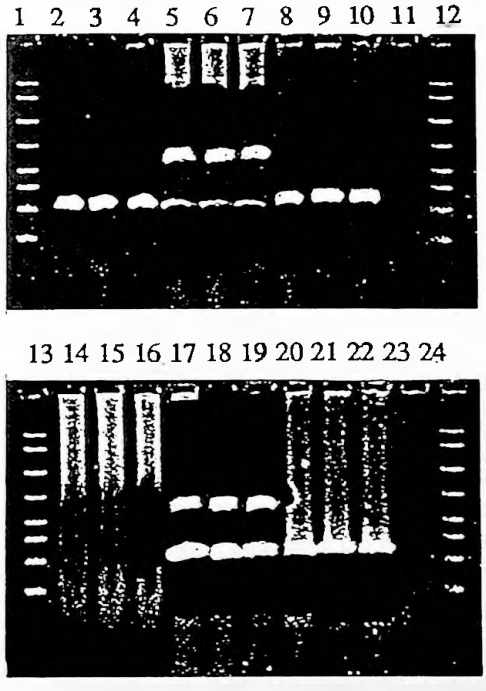

Fig. 1. Gel electrophoresis of PCR run on RNA from gill tissue and brain tissue extracted from *Fundulus*. Lanes 1, 12, 13 and 24 are MW standards, Lanes 2-4 are gills from *Fundulus* exposed to seawater for 24, 48 and 72 h respectively using the HSP60 primers, Lanes 5, 6 are time matched (24 and 48 h) controls for gill with HSP 60 primers, Lane 7 is time matched controls for brain (24 h) with HSP60 primers, Lanes 8-10 are brain extracted from *Fundulus* exposed to seawater for 24, 48 and 72h respectively with HSP 60 primers, Lane 11 is a negative (no RNA control), Lanes 14-16 are time matched controls (24, 48, 72h respectively) for gills with c-Fos primers, Lanes 17-19 are brain time matched controls (24, 48, 72h respectively) with c-FOS primers, Lanes 20-22 are brain extracted from *Fundulus* exposed to seawater for 24, 48 and 72h respectively with c-FOS primers, and Lane 23 is a negative control (no primers). Note that only 24h brain HSP 60 time matched control is shown due to lack of space on the gel, but, as was the case with other controls, the results from 48 and 72h were very similar.