Variation in Heat-Shock Proteins among Species of Desert Fishes (Poeciliidae, Poeciliopsis)

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Analysis of the heat-shock proteins (hsps) of six closely related species of Poeciliopsis demonstrated the existence of biochemical diversity in the hsp100, hsp70, hsp60, and hsp30 protein families among species. Each species expressed five to seven hsp70-related isoforms. Constitutive 70-kD isoforms were identical among species, but four different patterns of heat-inducible isoforms were seen in these six species. Members of the hsp70 family of molecular chaperones are included among the most highly conserved proteins known, and the possibility of variation in hsp70 among closely related species has rarely been addressed. The hsp30 family is known to be less conserved than the hsp70 family, and, as expected, the Poeciliopsis hsp30 patterns showed more variation. Most of the hsp30 isoforms characteristic of a particular species were unique to that species. Hsp100 and hsp60 were identical in five of the species, but alternate isoforms were found in P. monacha. The small size and limited geographical distribution of the P. monacha population have probably contributed to the uniqueness of the monacha pattern. Two of the species were shown to acquire thermotolerance, the ability to withstand normally lethal temperatures when subjected to a gradual temperature increase. Rapid-heating protocols commonly used to establish critical thermal maxima of organisms do not include this inducible component of thermoresistance and therefore do not adequately assess an organism’s capacity to withstand thermal stress.

Introduction

When the heat-shock responses of widely divergent organisms such as bacteria and mammals are compared, the nearly universal induction of heat-shock protein (hsp) synthesis in response to thermal or chemical stress and the degree of conservation of hsps (especially the 70-kD family) at the level of nucleotide sequence and protein function have resulted in an emphasis on the conserved nature of the heat-shock response (Nover and Scharf 1991). Few studies have been designed to assess hsp variability and its possible significance. We have compared hsp isoforms in six closely related species of desert topminnow, genus Poeciliopsis, as an initial step in the analysis of hsp diversity. This type of comparison has been proposed as a means of detecting minor yet selectively significant protein differences (Graves and Somero 1982), since related species should share a high degree of conservation in many genes. In addition, these fish commonly experience temperature extremes (4°C–40°C) that include temperatures close to their thermal maximum and can be exposed to a 22°C temperature change in the course of a single day (Bulger and Schultz 1979, 1982).

Increased diversity of hsps as well as accumulation of larger amounts of hsps may contribute to survival in thermally stressful environments. Differences in hsp expression have been found in closely related organisms which occupy different environmental niches. One species of hydra, Hydra oligactis, is highly sensitive to heat shock and apparently unable to synthesize hsps following stress. Another species, H. attenuata, synthesizes an hsp60-related protein and survives short-term heating. These differences were correlated with field data showing exclusion of H. oligactis from shallow pond environments and its rapid population decline after an increase in surface-water temperature (Bosch et al. 1988, 1991). Similar results were found for two species of Collisella limpets which occupy different intertidal zones; the species which occupies the more stressful high intertidal is more tolerant to acute heat shock and also synthesizes a more diverse complement of hsps (Sanders et al. 1991).

Insight into the role played by several hsp families in cells during normal growth and under conditions of stress has come from biochemical analysis of individual hsps. Several hsp families have been identified as molecular chaperones (reviewed in Ang et al. 1991). Chap-
Chaperones associate transiently with unfolded polypeptides, preventing premature folding of nascent polypeptide chains or aggregation of unfolded proteins, thus facilitating proper folding and/or assembly of these polypeptides (Ellis 1990). Different chaperones are found in different subcellular compartments and cooperate in the translocation of proteins across intracellular membranes, a process which requires the translocated polypeptide to be maintained in an unfolded, extended configuration until it reaches the proper compartment. Constitutively expressed forms of hsp60, hsp70, and hsp90 perform this function in unstressed cells (Hendrick and Hartl 1993; Georgopoulos and Welch, accepted). Small hsps, in the 20–30-kD range, have also been shown to function as molecular chaperones in vitro (Jakob et al. 1993).

There is evidence that heat and other stressors cause the accumulation of damaged proteins, resulting in the induction of additional hsp synthesis (Edington et al. 1989; Hightower 1991). Induced hsps could also function as chaperones, facilitating the refolding of damaged proteins or targeting them for degradation. Thus hsps would be a critical component of cellular defenses against stress-induced proteotoxicity, a term applied to the deleterious effects of proteins damaged by chemical and physical stressors on cells (Hightower 1991).

Our initial approach to exploring diversity in hsps has been to examine biochemical variability of hsp isoforms on two-dimensional protein gels. This allows relatively rapid screening of multiple isoform families, compared with the time required to clone and sequence all the members of a multigene family. However, unlike direct sequence comparisons, characterization of protein isoforms as primary gene products is not conclusive, since it is based on indirect evidence such as the absence of detectable posttranslational modifications and the lack of a precursor-product relationship between related isoforms. Comparison of proteins does offer the advantage of characterizing only functional genes through analysis of their protein products and allows determination of the relative levels of expression of different multigene family members.

**Material and Methods**

**Origin and Maintenance of Experimental Fish Stocks**

The fishes used in this study were collected from four river systems of northwestern Mexico between 1961 and 1983. Stock designation numbers such as M61-31 provide the initial of the last name of the collector, the year the collection was made, and the station number. Collection sites and ranges for each species are shown in figure 1. More specific locality and collector information is to be found in the report by Schultz (1989).

The species used in this study were as follows: *Poeciliopsis monacha* S68-4 and S68-5, *P. lucida* M61-31, *P. viriosa* M65-23, *P. latidens* SV73-7, *P. occidentalis* AV76-7, and *P. prolifica* SS83-51. These sexually reproducing species were maintained primarily by brother-sister matings from the time of capture and, except for *P. prolifica*, were homozygous as judged by allozyme analysis (Vrijenhoek et al. 1978) and by histocompatibility analysis (Angus and Schultz 1979; Schultz 1989). *Poeciliopsis prolifica* has been inbred for five generations, but its degree of homozygosity has not been tested.

The fish were reared in greenhouses where an average temperature of 27 ± 5°C was maintained with thermostatically controlled vents, fans, and heaters, except on rare occasions when outside temperature extremes overwhelm the system and drive the range a few degrees in either direction. The fish were fed twice a day on a high-protein diet designed for trout fry and received supplements of frozen brine shrimp, brine shrimp nauplii, and live *Daphnia* approximately twice a week.

**Thermal Stress in Whole Fish**

Three days before specimens of inbred *P. lucida* or *P. monacha* were heat stressed, they were removed from the ambient temperatures of the aquarium facility to a holding tank maintained at ~24°C in an air-conditioned room. They were held without food for 24 h prior to treatment. At the beginning of the trial, two to eight fish were placed in a plastic aquarium with 4 liters of water from their holding tank. This container was then immersed in a water bath at the same temperature as the holding tank. The temperature was increased either rapidly or gradually to ~40°C, with constant monitoring of the temperature. Incubation at 40°C was continued until the fish could no longer maintain equilibrium. At this point the temperature was decreased gradually by the addition of cool water to the tank. Any surviving fish were returned to the holding tank; final survival figures were taken from the number of fish alive after a 24-h recovery period.

**Cell Culture**

Primary hepatocytes were prepared as follows: Livers were removed and rinsed in phosphate-buffered saline. The tissue was minced and subjected to three 10–15-min rounds of trypsinization (0.05% trypsin and 0.5 mM ethylenediaminetetraacetic acid in a phosphate-buffered saline) at room temperature, with gentle stirring. Cells released by trypsinization were pooled in calf serum and then were washed in minimal essential medium (Eagle’s minimal essential medium [MEM] containing Earle’s salts; GIBCO-BRL Life Technologies) containing 5% calf serum and 5% fetal bovine serum. Cells were resuspended in the same medium at ~2 × 10^6 cells/ml and were plated in either 35-mm culture...
FIG. 1.—Geographical distribution of six species of *Poeciliopsis* in the river systems of northwestern Mexico. The range of each species is marked with boldface lines and is labeled with the name of the species. Numbered sites indicate actual collection sites of fish used to establish inbred strains: 1 = Rio Concepcion, *P. occidentalis*; 2 = Rio Mayo, *P. prolifica*; 3 = Rio del Fuerte, *P. monacha*, *P. lucida*, and *P. latidens*; and 4 = Rio Mocorito, *P. viriosa*. An enlargement of site 3 in the Rio del Fuerte drainage is shown in the inset. Numbered sites in the inset panel indicate collection sites of progenitor fish of *P. lucida* and *P. monacha-lucida* strain M65-24 (1); *P. latidens* and *P. monacha-lucida* strains SV73-7s and SV73-7v (2); *P. monacha* strain S68-4 and *P. monacha-lucida* strains T70-3 and S68-4 (3); *P. monacha* strain S68-5 (4); and *P. monacha-lucida* strains M61-35 and M65-26 (5). The cross-hatched area is a man-made lake; point “X” indicates the position of the dam.

Dishes (Falcon; Becton Dickinson) or tissue culture tubes (Corning). Culture vessels were precoated either with Cell-tak (Collaborative Research) or with poly-L-lysine (Sigma Chemical), to aid in cell attachment. Cultures were incubated at 30°C in a humidified 5% CO₂ atmosphere for 2–3 d before use.

The PLHC-1 cell line was isolated in 1985 from a dimethylbenz(a) anthracene induced hepatocellular carcinoma of *P. lucida* (Schultz and Schultz 1985; Hightower and Renfro 1988). The cells show a hepatocyte-like morphology and retain some liver-specific functions, such as inducibility of P450-dependent monooxygenase activity (Babich et al. 1991; Hahn et al. 1993). Cultures were routinely passaged once a week by gentle trypsinization followed by a 1:4 or 1:5 split. The same medium and incubation conditions described for primary hepatocytes were used. Experiments were performed on confluent monolayers.

Radioisotopic Labeling and Gel Electrophoresis

Proteins for two-dimensional analysis were labeled with ³⁵S-methionine (Translabel; ICN) at 100 μCi/ml in MEM containing 2%–10% of the normal concentration of methionine and 2% dialyzed calf serum. Control cultures were labeled for 1 h at 30°C; heat-shocked cultures were transferred to an incubator at heat-shock temperature (38°C–40°C) for 1 h and then were labeled during the second hour, at heat-shock temperature. Cultures were washed with 70 mM Tris pH 7.6, and then solubilized in isoelectric focusing–sample buffer (9.5 M
urea, 2% NP-40, 1.6% pH 5–7 ampholites, 0.4% pH 3.5–10 ampholites [LKB], 5% β-mercaptoethanol, and 70 mM Tris pH 7.6). The pH of this buffer is critical; at a lower pH, protease activity results in the breakdown of the 70-kD hsp. Samples were treated with 40 μg RNase A/ml for 10 min at room temperature and were stored at -70°C. Isoelectric focusing was performed as described by O'Farrell (1975), with the following modifications: (a) The gels contained a mixture of ampholites (Pharmalytes; Sigma Chemical) optimized for the separation of 70-kD and 30-kD hsp isoforms, including the separation of grp78 into two spots. A typical mixture was 120 μl pH 5–6, 20 μl pH 4.5–5.4, and 10 μl pH 3–10 Pharmalytes/3 ml of gel solution; this generated a gradient range of ~pH 5–6. The proper mixture was determined empirically for each batch of Pharmalytes. (b) Electrode buffers were 25 mM H3PO4 and 50 mM NaOH. (c) Gels were run at 800 V for >17 h (Duncan and Hershey 1984). The second dimension was sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 9% acrylamide slabs. Gels were processed for fluorography and were exposed to preflashed X-ray film (X-Omat AR; Kodak) at -70°C (Laskey and Mills 1975).

Pulse-chase analysis of hsps was performed with cultures of PLHC-1 cells. The hsps were radiolabeled with 35S-methionine (300 μCi/ml in methionine-free medium) for 5 min at 39°C after a 1-h heat shock at 39°C. One culture was lysed immediately after the labeling period. In the other cultures the labeling medium was replaced with medium containing 25 times the normal concentration of methionine. These cultures were lysed after 0.5-h and 1-h chase periods for analysis on two-dimensional gels.

Phosphorylated proteins were labeled with 32P-orthophosphate (carrier free; Du Pont) at 200 μCi/ml in phosphate-free medium. Cultures were rinsed three times with phosphate-free medium and then incubated for a total of 5 h in 32PO4-containing medium. Control cultures were incubated at 30°C; heat-shocked cultures were incubated with label for 3 h at 30°C and then shifted to 39°C for 2 h. Cultures were lysed in isoelectric focusing–sample buffer supplemented with 0.5% SDS, 10 mM NaF (phosphatase inhibitor), and 1 mM phenylmethylsulfonyl fluoride (protease inhibitor) and were run on two-dimensional gels in parallel with 35S-methionine-labeled samples.

A modification of the method of Cleveland et al. (1977) was used for partial digest peptide mapping. Cultures of PLHC-1 cells were labeled with 35S-methionine at 200 μCi/ml for 2 h at 40°C, followed by 3 h at 30°C. Labeled extracts were separated on two-dimensional gels, and the major 30-kD stress protein isoforms were excised from the stained, dried gels. Detergent-limited proteolytic digestion of proteins followed by separation of peptide fragments on 15% SDS-PAGE gels was performed as described elsewhere (Hightower 1980).

Densitometry and Isoform Quantitation

Scanning and analysis of fluorographic images were performed using a Molecular Dynamics computing densitometer (model 300A). Careful analysis of the linearity of the film response to different levels of radioactive protein was done prior to image quantitation. This included both calibration of film prefllashing to eliminate underrepresentation of minor spots (Laskey and Mills 1975) and calculation of film-saturation levels to avoid underrepresentation of intensely labeled spots. Linearity of integrated spot volumes was checked by running serial dilutions of a highly labeled extract on two-dimensional gels and processing for fluorography. Integrated spot volumes calculated by the densitometer were then compared with actual fold dilutions of the samples. Contour plots were generated from computer scan images by using the software provided by Molecular Dynamics.

Immunoblot Analysis

Extracts from control cultures or from cultures which had been heat shocked for several hours were run on two-dimensional gels, and the proteins were electroblotted onto nitrocellulose (Schleicher and Schuell) or polyvinylidene fluoride (Millipore) membranes. The membranes were blocked and then incubated with primary antibody. A monoclonal antibody (7.10) originally raised against Drosophila hsp70 (Kurtz et al. 1986) was used to identify hsp70 family members. For identification of hsp60 isoforms, blots were incubated with a polyclonal antibody raised against moth hsp60 (StressGen Biotechnologies). Antibody binding was visualized in one of two ways. The first technique involved sequential incubations with (a) a biotinylated secondary antibody, (b) avidin-conjugated alkaline phosphatase (Vectastain ABC-AP; Vector Labs), and (c) alkaline phosphatase Substrate Kit II (Vector Labs). The second technique used an alkaline phosphatase–linked secondary antibody (Sigma Chemical) visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Both techniques detected Poeciliopsis hsp family members, but the avidin-biotin system also gave a strong nonspecific (i.e., independent of incubation with primary antibody) signal with several non-hsps.

Results

Induction of Hsp Synthesis in Poeciliopsis

Incubation of hepatocyte cultures from six species of Poeciliopsis at elevated temperatures induced the
synthesis of proteins which migrated with approximate molecular masses of 100 kD, 90 kD, 70 kD, 60 kD, and 30 kD. Typical two-dimensional patterns of heat-induced polypeptides synthesized in cultures from two of these species (P. monacha and P. viriosa) are shown in figure 2. The electrophoretic variants seen in various hsp families on these fluorographs represent polypeptides which differ in isoelectric point (pI) and/or molecular mass (Mr). Missense mutations which cause either changes in charged amino acids or changes in posttranslational addition of charged groups to the polypeptide chain would result in an altered pI. Insertions, deletions, or nonsense mutations which change polypeptide chain length by ≥10 amino acids (which would change Mr by ~1 kD) would result in detectable changes in migration in the second dimension. Smaller size changes and changes in uncharged amino acids probably would not be detected. Therefore this technique gives a conservative estimate of the extent of biochemical diversity.

The five size classes of stress proteins synthesized in Poeciliopsis cell cultures are marked in figure 2. The two members of the hsp100 family are marked by arrowheads, as is the hsp90 family. Both these hsp families were identified on the basis of their molecular weight, pI, and inducibility by heat. Five species of Poeciliopsis including P. viriosa have identical isofoms of hsp100, but the hsp100s of P. monacha have more basic ps. The 70-kD region is marked by rectangles and includes the constitutively expressed heat-shock cognate (hsc70), present in both Co (control) and HS (heat-shock) panels, and a number of heat-induced isofoms. Grp78 is the most acidic spot and migrates identically in all species tested, as does hsc70. Depending on the species, three to five additional heat-induced isofoms were also synthesized. Several of these isofoms were synthesized by more than one species. The position of the 60-kD stress protein is indicated by an arrowhead. Hsp60 was identified by immunoblot analysis (data not shown); like hsp100, it migrates identically in all species except P. monacha. The 30-kD region, also marked by rectangles, shows a greater biochemical diversity than do the other stress-protein families. Up to 14 different heat-induced 30-kD polypeptides are synthesized among the six species studied; only two or three isofoms are synthesized in more than one species.

Biochemical Diversity in Hsp Families

Detailed qualitative comparisons of the 70-kD (fig. 3) and 30-kD (fig. 4) hsp families in six species of Poeciliopsis were made, in order to assess biochemical diversity among species. Radioisotopic labeling and gel analysis of hsp5 were performed a minimum of three times for each species. The 70-kD and 30-kD regions (as defined by the rectangles in fig. 2) of representative fluorograms were scanned, and the optical density images were converted into contour images. Electrophoretically identical polypeptides were assigned the same isoform number in the hsp patterns of each of the six species. Electrophoretic identity was defined as comigration of protein spots during two-dimensional gel analysis of a mixture of 35S-methionine-labeled proteins of two species.

The 70-kD patterns (fig. 3) show that two of the 70-kD family members (grp78 and hsc70) migrate identically in all six species. Differences in the intensity of the constitutively expressed hsc70 spot is due to variable inhibition of protein synthesis observed over the range of heat-shock temperatures used in different experiments. Synthesis of inducible hsp70 is more resistant to this inhibition than are constitutively expressed proteins.

Fig. 2.—Comparison of hsp isoforms synthesized in two species of Poeciliopsis. Cultures of primary hepatocytes were labeled with 35S-methionine for 1 h either at the control temperature of 30°C (Co) or during the second hour of a 2-h heat shock at 39°C (HS). After the labeling, proteins were solubilized and separated on two-dimensional gels. The first-dimension isoelectric focusing (IEF) gels resolved polypeptides with ps of pH 5–6. The second dimension was SDS-PAGE on 9% slab gels. Fluorograms of the dried gels are shown. The 70-kD and 30-kD regions in each pattern have been outlined, and at the 100-kD, 90-kD, and 60-kD hsp5 are indicated by arrowheads. The position of each of these hsp families is indicated by Mr, numbers, in kilodaltons, on the right of the fluorograms.
FIG. 3.—Schematic representation of 70-kD hsp-family isoform patterns: comparison among six species of Poeciliopsis. The 70-kD region of fluorograms such as those in fig. 2 was scanned, and the images were converted into contour plots. Spots with the same pI have been aligned vertically; major heat-inducible isoforms have been numbered. Hsp70 = clusters of inducible hsps; Hsc70 = constitutively expressed 70-kD heat-shock cognate protein; and Grp78 = 78-kD glucose-regulated protein. The "V" marks the position of a constitutively synthesized 70-kD protein used as a marker spot for aligning patterns.

such as hsc70 (Lindquist 1980, 1981). Three of the six species—*P. occidentalis, P. lucida*, and *P. prolifica*—have identical patterns of hsp70 isoforms (spots 3–5 and 7). *Poeciliopsis monacha* and *P. viriosa* differ from the first three species, in the position of one of the minor isoforms; they contain spot 6 instead of spot 7. Also, *P. viriosa* has an additional highly induced isoform with the most basic pI of the hsp70 cluster. *Poeciliopsis latidens* has the most unusual pattern; it is the only species which does not synthesize spot 3 as the major hsp70 isoform. Minor spots which are marked by lines but which are not numbered probably resulted from incomplete focusing of more abundant radioactively labeled spots in the first-dimension gels.

In general, low-\(M_c\) hsps from diverse organisms show much less conservation at the nucleotide and amino acid sequence levels than does the 70-kD family (Nover and Scharf 1991). The 30-kD family of the same six species of *Poeciliopsis* exhibited a high degree of bi-
FIG. 5.—Reproducibility of hsp70-family isoform patterns. Multiple independent primary cultures of Poeciliopsis monacha (A) and P. lucida (B) were labeled at heat-shock temperature with 35S-methionine, and the proteins were analyzed on two-dimensional gels as in fig. 2. The 70-kD regions were scanned, and individual spot volumes were obtained by integration. Each spot volume was then expressed as a percent of the total hsp70-family volume (including hsc and grp). Spot numbers are as defined in fig. 3. Error bars indicate one standard deviation from the mean for the P. monacha (n = 5) and P. lucida (n = 4) hsp70 families.

In the absence of an antibody specific for the 30-kD hsp family, the three major 30-kD isoforms of P. lucida were identified as related proteins by partial digest patterns, since the data were collected over a period of several years.

Identification of Isoforms as Related Members of Protein Families

Further characterization of the 70-kD isoforms was performed by immunoblot analysis (fig. 6) using a monoclonal antibody (7.10) which recognizes a highly conserved epitope found on most members of the 70-kD family in many different organisms (Kurtz et al. 1986). When proteins from control and heat-shocked P. lucida cells were compared (fig. 6A), high levels of grp78 and hsc70 were found in the control cells, with grp78 occurring as a pair of spots differing in pI. The more acidic spot is a posttranslationally modified form of grp78 (Carlsson and Lazarides 1983; L. E. Hightower, unpublished results) which undergoes reversible loss of the modifying group on heat shock. The heat-shocked P. lucida sample showed levels of hsc70 and grp78 similar to those in the control and, in addition, showed hsp70 spots 3 and 5. The relative abundances of the various isoforms seen on these blots were similar to those detected on silver-stained gels run in parallel with the immunoblotted gels; much less of spot 5 than of spot 3 accumulated during the prolonged heat shock. Spot 7 accumulated to a slightly lower level (by silver stain) than did spot 5, and it seemed to have a decreased affinity for the antibody. It was visualized on blots in which the development step was prolonged (data not shown). Levels of various hsp70 isoforms visualized by immunoblot differed from those seen in labeling experiments (figs. 2–5), in part because of different heat-shock regimens. Labeling experiments visualized only the proteins synthesized during the 1-h labeling period, while immunoblot experiments analyzed proteins which accumulated during a 4-h heat shock.

Heat-shocked extracts from P. viriosa and P. latidens (fig. 6B) were also analyzed by immunoblotting with monoclonal 7.10. These species were tested because their isoform patterns included major spots different from spot 3 (see fig. 3). In P. viriosa, both spot 1 and spot 3 accumulated to high levels, whereas spot 5 was present in a much lower amount. Spot 6 in P. viriosa behaved like spot 7 in P. lucida; it accumulated to slightly lower levels than did spot 5, as assayed by silver stain, and it was difficult to visualize with the antibody. In the P. latidens sample, the only heat-inducible isoform which reacted with the antibody was spot 2. Spot 7 was not visible on silver-stained gels of this extract; it may have been synthesized at lower levels in P. latidens than in P. lucida.
shock of this cell line are identical to those synthesized in primary hepatocytes of *P. lucida*, but the rate of protein synthesis is much higher in the cell line. Therefore, PLHC-1 cell cultures were used when high levels of radioisotopic incorporation into protein were required. Spots 11, 12, and 2 as identified in figure 4 are labeled “a,” “b,” and “c,” respectively, in figure 7. Under the conditions of proteolytic digestion used in this experiment, related proteins would be expected to produce similar patterns. Digestion with *S. aureus* V8 protease yielded virtually identical patterns for all three isoforms. Digestion with chymotrypsin gave similar patterns for spots a and c but generated a unique peptide from spot b, indicating that the chymotrypsin cleavage sites are not as conserved as the V8 cleavage sites in the hsp30 family. Thus, these isoforms constitute a family of closely related but not identical proteins.

Further evidence that the hsp isoforms of *P. lucida* are products of a family of related genes is shown in a

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**Fig. 6.**—Immunoblot analysis of hsp70 family. *A*, Primary cultures of *Poeziopsis lucida* were heat shocked at 38°C for 3–4 h and then were either returned to 30°C for 2 h (HS) or left at 30°C for the duration of the experiment (CON). Proteins were electroblotted onto membranes after separation on two-dimensional gels. The primary antibody was monoclonal antibody 7.10. Spots which react specifically with the primary antibody are indicated by arrowheads and are identified by comparison with silver-stained and radiolabeled patterns. Spot numbers are as defined in fig. 3. Only the upper half of each gel is shown, and the orientation of the isoelectric focusing dimension in these blots is reversed relative to that in figs. 2 and 3. *B*, Analysis of hsp70 family in *P. latidens* and *P. viriosa*. Only blots of heat-shocked samples are shown.

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**Fig. 7.**—Partial digest peptide mapping of the three major 30-kD hsp-family isoforms of *Poeziopsis lucida*. PLHC-1 cells were labeled with 35S-methionine for 5 h during a 2-h heat-shock and 3-h recovery. The labeled proteins were separated on two-dimensional gels. The hsp30 region of one of these gels is shown at the top of the figure, with the isoforms which were subsequently excised for mapping (a–c). Analysis of partial digest products is shown in the lower panels. The “NO PROTEASE” panel shows the migration of eluted proteins in the 15% mapping gel in the absence of enzymatic digestion. Digestion with 0.5 mg of *Staphylococcus aureus* V8 protease (V8 PROTEASE) and with 0.1 mg of chymotrypsin (CHYMO) resulted in the patterns shown in the other two panels.
pulse-chase experiment (fig. 8). Proteins were radioactively labeled in heat-shocked PLHC-1 cells. Under conditions used for the chase, incorporation of $^{35}$S-methionine into protein after the 5-min labeling period (the "pulse") is terminated by the addition of excess unlabeled methionine, and the fate of polypeptides synthesized during the pulse can be followed during the chase period. Posttranslational modification causing pI and/or $M_r$ shifts of polypeptides synthesized during the pulse would result in both the loss of label from the precursor spot and accumulation of label in the modified spot during the chase period. Analysis of 70-kD isoform patterns after 0-, 0.5-, or 1-h chase periods showed slight increases in spot 5 and spot 7 during the chase, but without corresponding decreases in other hsp70-related spots. This indicates that no precursor-product relationships exist among the hsp70 family members, and it supports the hypothesis that isoforms are the products of individual genes. The major *P. lucida* 30-kD isoforms also showed no evidence of protein processing during the chase period (data not shown).

Posttranslational phosphorylation of small hsp7, which results in acidic shifts in pl, has been characterized in other systems. For example, in human cells multiple isoforms of hsp30 were shown to arise from a single primary translation product through differential phosphorylation (Cretien and Landry 1988). Proteins labeled in *P. lucida* (PLHC-1 cells) with either $^{32}$PO$_4$ or $^{35}$S-methionine were analyzed in parallel on two-dimensional gels (fig. 9). Although both grp78 and hsp90 were clearly labeled with radioactive phosphate, there was no evidence of phosphorylation of the 30-kD proteins. This experiment was also performed with primary cultures of *P. viriosa, P. occidentalis, and P. latidens*, with the same result, indicating that posttranslational phosphorylation does not contribute to the diversity of hsp30 isoforms found in *Poeciliopsis* and making it more likely that they represent the products of individual genes.

![Figure 8](image)

**Fig. 8.**—Pulse-chase analysis of hsp70 family in *Poeciliopsis lucida*. Cultures of PLHC-1 cells were heat shocked at 39°C for 1 h and then were pulse labeled with $^{35}$S-methionine for 5 min at 39°C. One culture was lysed at the end of the labeling period (Pulse); the other cultures were rinsed and incubated at 39°C in medium containing 25 times the normal concentration of methionine for 0.5 h (0.5 h Chase) or 1 h (1 h Chase), prior to lysis. Labeled proteins were separated on two-dimensional gels. Fluorograms of the gels were scanned, and individual protein spot volumes were obtained by integration. Hsp70 isoform volumes were normalized to the sum of the volumes of two proteins whose amounts did not change during the experiment; error bars indicate one standard deviation from the mean ($n = 2$). Isoform spot numbers are as defined in fig. 3.
Effect of Preheating on Thermal Resistance in Whole Fish

The acquisition of thermotolerance, in which cells develop a transient resistance to normally lethal temperatures after a period of several hours of exposure to elevated but sublethal temperature, has been correlated with the synthesis of hsp90 in cell cultures (Nover 1991a). In organismal studies the thermal resistance of a species is defined by its critical thermal maximum (the 50% survival temperature). This parameter is often determined by using rapid rates of heating which preclude analysis of the ability of intact organisms to acquire the type of thermotolerance which has been studied extensively in cell culture. The ability of *Poeciliopsis* to acquire thermal resistance was tested in two species, *P. lucida* and *P. monacha*, by comparing survival of fish subjected to various heat-stress regimens (fig. 10). Neither species could survive an extended period at 40°C when the temperature was raised rapidly (fig. 10, filled symbols). Under these conditions, which are comparable to those employed in the determination of critical thermal maxima, the ∼25-min interval used to increase from the threshold hsp-induction temperature of 34°C to 40°C allowed very little accumulation of hsps (P. diliorio and R. J. Schultz, unpublished data). Fish lost equilibrium after <0.5 h, for *P. monacha* (fig. 10B), or <1 h, for *P. lucida* (fig. 10A), after the temperature reached 40°C, and mortality was close to 100% in both species. In contrast, a temperature treatment which prolonged the length of time at 34°C–38°C to ∼100 min before elevation to normally lethal temperatures of 40°C–41°C resulted in extended survival times (fig. 10, unfilled symbols). This regimen allowed substantial accumulation of hsps in both cultured cells and intact fish (authors' unpublished data). Under these conditions, individuals of *P. monacha* (fig. 10B) lost equilibrium only after 1 full hour at 40°C, after which 95% of the fish survived when allowed to recover at normal temperatures. *Poeciliopsis lucida* (fig. 10A) was able to cope with >1 h at temperatures ≤41°C, after which 79% of the fish were able to recover. Thus, thermotolerance was acquired by the intact animal under conditions which allowed substantial accumulation of hsps. In other studies (Schultz et al. 1993; P. diliorio,
personal communication), preconditioning heat treatments extended survival times at temperatures in the low end of the lethal range and increased the maximum tolerated temperature \( \sim 1^\circ C \).

**Discussion**

**Variation of Hsps among Species of *Poeciliopsis***

By performing an extensive analysis of hsps in *Poeciliopsis*, we have been able to document not only the existence of multiple-isoform families, but also differences in the isoforms expressed by individual species. The number of isoforms seen in the inducible hsp70 and 30-kD clusters in *Poeciliopsis* is similar to that seen in poikilotherms such as *Drosophila* (Palter et al. 1986; Rollet and Best-Belpomme 1986) and *Xenopus* (Darasch et al. 1988). In contrast, homeothermic species often synthesize a single form of inducible hsp70 (Anderson et al. 1982). However, the need for molecular chaperones in multiple subcellular compartments (where they function as part of the protein-folding and translocation pathway in unstressed cells) maintains a certain degree of diversity in the hsp70 family. Compartment-specific isoforms are found in the endoplasmic reticulum (grp78), mitochondria, and cytoplasm/nucleus (hsc/hsp70) (Nover et al. 1991). Variability in the hsp30 family in homeotherms is often generated by phosphorylation of a primary translation product, resulting in the appearance of multiple isoforms (Cretien and Landry 1988). Tissue-specific forms may contribute to diversity in both poikilotherms (Koban et al. 1991) and homeotherms (Zakeri et al. 1990). However, to date, tissue-specific isoforms have not been found in *Poeciliopsis* liver, gill, muscle, brain, or skin epithelial tissue (P. dilorio and R. J. Schultz, unpublished data). The greater diversity in hsp families of poikilotherms such as *Poeciliopsis* may be advantageous in coping with environmental temperature changes unbuffered by homeothermic mechanisms.

In the six species of *Poeciliopsis* studied here, hsc70 and grp78 exhibit no isoform variation and, by inference, are highly conserved genetically. This suggests that changes in polypeptide recognition and chaperone activity by these constitutive family members, which function in unstressed cells, have not occurred during speciation. Individual species synthesize two to five inducible hsp70 isoforms which differ mainly in pl. During evolution of these species, changes in the amino acid sequence of cellular proteins with which hsp70 interacts during stress may have accumulated. Random mutation of hsp70 genes may have generated isoforms with an enhanced ability to interact with these altered proteins. Alternatively, this variability may represent divergence due to reduced functional constraint on the inducible members of the hsp70 family compared with the constitutive members. Consistent with these possibilities, nonlinear relationships among evolutionary rates were found for several protein domains of the yeast hsp70 family (Hughes 1993).

Each of the six species synthesizes two to six hsp30 isoforms, which differ in both pl and molecular weight (fig. 4). Isoelectric variants are not the result of protein phosphorylation (fig. 9). Although the function of small hsps is less well understood than that of the hsp70 family, current evidence indicates that they also interact with other proteins (Miron et al. 1988, 1991; Jakob et al. 1993) and perhaps with other cellular components such as mRNA (reviewed in Nover and Scharf 1991). The diversity of hsp30 isoforms among species of *Poeciliopsis* may offer a selective advantage but could also be due to fewer selective constraints on hsp30 than on other hsps.

Some similarities in the patterns of hsp70 isoforms in closely related species, as determined by classical taxonomic methods, were found. *Poeciliopsis lucida*, *P. occidentalis*, and *P. prolifica* have identical hsp70 patterns and are closely grouped taxonomically (Miller 1960). Also, *P. latidens* is the most divergent among the six species studied and is also the only species whose major hsp70 isoform is not spot 3. However, the hsp70 patterns of the closely related species *P. monacha* and *P. viriosa* (which produce fertile F_1 hybrid offspring) are different: the monacha isoforms constitute a subset of the viriosa pattern, in that no spot 1 is synthesized.

*Poeciliopsis monacha* also synthesizes unique forms of both hsp60 and hsp100, compared with the other five species. The distinctive nature of hsp patterns in *P. monacha* may be related to its geographical distribution. It is known as a recluse species (Miller 1960), on the basis of both its limitation to headwater habitats and its semi-isolation from other fishes. The steep-sided walls of these headwater arroyos provide shaded, rocky pools of clear, spring-fed water. *Poeciliopsis monacha* living at site 4 (fig. 1, inset) is less exposed to the high temperatures experienced by the five other species of *Poeciliopsis* in their adjacent downstream habitats (e.g., site 3; fig. 1, inset). A combination of (a) the different selective pressures found in the *P. monacha* habitat and (b) fluctuations in population size may have resulted in fixation of alternative alleles for hsp60 and hsp100. The highly homozygous nature of *P. monacha* at site 4 and *P. lucida* at site 3 of the Arroyo Jaguari (fig. 1, inset), as well as the differential fixation of other traits in the fishes of the Jaguari compared with the rest of the Rio Fuerte (Angus and Schultz 1979; Keegan-Rogers and Schultz 1985; Vrijenhoek 1992), supports the view that selection and/or drift are especially active at this locality.
Possible Significance of Variability in Hsps

In *Poeciliopsis*, the biochemical data presented here support the idea that each isoform is the product of a unique gene (figs. 8 and 9), leading to the prediction that the family of hsp70-related genes in *Poeciliopsis* would contain six or seven members per haploid genome. Evidence from Southern blot analysis with a probe specific for inducible hsp70 sequences indicates that the genes exist in a small multigene family (T. Li, S. M. Leung, and L. E. Hightower, unpublished data) similar to that seen in other organisms (Nover 1991b). The existence of stress genes as multigene families in the genome suggests that evolution of these genes includes gene-duplication events. Multiple gene copies may allow for the appearance of divergent isoforms while maintaining normal levels of the unmutated protein (Li 1983).

Although functional diversity has not been demonstrated yet for the *Poeciliopsis* isoforms, two isoelectric variants of yeast hsp70, the SSA1 and SSA2 proteins, have been shown to differ in activity (Gao et al. 1991). These proteins were isolated and tested for their ability to uncoat clathrin-coated vesicles (Chappell et al. 1986), an assay based on the polypeptide-stimulated ATPase activity of hsp70. The SSA2 protein is strongly stimulated by clathrin, and the SSA1 protein is not. These two proteins differ in only 3% of their amino acid sequence and, on two-dimensional gels, appear as partially separable isoforms. Changes in certain amino acids in the highly conserved N-terminal ATPase domain of hsp70 might alter the half-lives of complexes between this chaperone and its protein substrates. The less-conserved C-terminal domain is involved in binding to unfolded proteins and to conformationally flexible regions of folded proteins such as clathrin (Morimoto 1991). Changes in the sequence of the peptide-binding domain may accompany changes in the nature of the binding sites on unfolded polypeptides and on flexible regions of proteins with which a particular 70-kD isoform interacts.

Conclusion

The six species of *Poeciliopsis* analyzed herein are found in environments which undergo significant changes in temperature, not only seasonally but often within a single day. The diversity of hsps demonstrated here, in an organism which experiences rapid daily changes over a broad range of temperatures, is consistent with a homeostatic view of the stress response in which hsps contribute to phenotypic plasticity in an organism’s response to thermal stress. The possibility that the presence of one or more particular isoforms may correlate with higher levels of thermal resistance can now be studied in the whole organism by using a heating protocol which allows both the accumulation of hsps and the acquisition of thermotolerance.

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