Variation in Heat Shock Proteins within Tropical and Desert Species of Poeciliid Fishes

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The 70-kilodalton heat shock protein (hsp70) family of molecular chaperones, which contains both stress-inducible and normally abundant constitutive members, is highly conserved across distantly related taxa. Analysis of this protein family in individuals from an outbred population of tropical topminnows, Poeciliopsis gracilis, showed that while constitutive hsp70 family members showed no variation in protein isoforms, inducibly synthesized hsp70 was polymorphic. Several species of Poeciliopsis adapted to desert environments exhibited lower levels of inducible hsp70 polymorphism than the tropical species, but constitutive forms were identical to those in P. gracilis, as they were in the congeneric species Gambusia affinis. These differences suggest that inducible and constitutive members of this family are under different evolutionary constraints and may indicate differences in their function within the cell. Also, northern desert species of Poeciliopsis synthesize a subset of the inducible hsp70 isoforms seen in tropical species. This distribution supports the theory that ancestral tropical fish migrated northward and colonized desert streams; the subsequent decrease in variation of inducible hsp70 may have been due to genetic drift or a consequence of adaptation to the desert environment. Higher levels of variability were found when the 30-kilodalton heat shock protein (hsp30) family was analyzed within different strains of two desert species of Poeciliopsis and also in wild-caught individuals of Gambusia affinis. In both cases the distribution of hsp30 isoform diversity was similar to that seen previously with allozyme polymorphisms.

Introduction

The genes encoding the 70-kDa and low molecular weight families of heat shock proteins (hsp's) exist in the vertebrate genome as small multigene families, implicating the processes of duplication and divergence in their evolution. Members of both protein families are thought to function as molecular chaperones, assisting in the folding and/or assembly of other proteins without becoming part of the final complex. The hsp70 family contains constitutively expressed homologs which are normally abundant and function as chaperones in the mitochondria, the endoplasmic reticulum (78-kilodalton glucose-regulated protein or grp78), and the nucleocytoplasmic compartment (heat shock cognate protein or hsc70). Evolutionary constraints on constitutive hsp70 homologs appear to be different in the different subcellular compartments; for example, endoplasmic reticulum-resident grp78 proteins from diverse species are more closely related to each other than to hsc70 from the same species. Stress-inducible hsp70 is evolutionarily most similar to hsc70, and it has been hypothesized that these two proteins make up an orthologous group, as do each of the organellar homologs (Boorstein et al. 1994).

The high degree of conservation between inducible hsp70 and constitutive hsc70 has also been thought to imply conservation of function (Boorstein et al. 1994). Both these proteins function in the nucleocytoplasmic compartment, interact transiently with newly synthesized proteins, and have peptide-stimulated ATPase activity. Co-localization of constitutive and inducible forms after stress also occurs. But the possibility has also been raised that mixed oligomers (probably dimers) of these proteins function differently than dimers of hsc70 alone (Brown et al. 1993). Inducible hsp70 and mixed dimers of hsp70 and hsc70 might provide a broader range of chaperoning activity necessary in the stressed cell, in order to deal with stress-damaged proteins in addition to nascent and unassembled proteins.

Previously we demonstrated biochemical diversity of inducible hsp's in closely related species of the desert topminnow Poeciliopsis (White et al. 1994). Four different complements of inducibly synthesized hsp70 isoforms are synthesized by inbred lines of six species, but
three of these complements have a major isoelectric variant in common. The hsp30 family in the same six lines is highly diverse; of 18 isoforms only a few are found in more than one species. Synthesis of these isoforms is highly reproducible (to the point of being diagnostic for each strain), and no precursor-product relationship has been detected among the isoforms of a particular hsp family, suggesting that each isoform is encoded by a separate gene. In contrast to stress-inducible members of the hsp70 family, constitutively expressed hsc70 and grp78 are electrophoretically identical in all six species. Taking biochemical diversity as an initial estimate of genetic diversity, the degree of variation among these closely related species suggests that even highly conserved proteins like stress-inducible hsp70 can evolve rapidly, while constitutive members of this protein family appear to be highly constrained evolutionarily.

As a continuation of our study of Poeciliopsis hps, we have assessed biochemical diversity of hsp70 and hsp30 within individual species. The presence of intraspecific diversity in hsp's allows for the possibility of rapid evolution in a changing environment: even isoforms which are selectively neutral in one environment may become advantageous in another. Maintenance of properly functioning hsp’s, which play an essential role in development as well as during periods of stress (Hightower and Nover 1991), would be critical to survival of a species confronted by environmental changes.

Material and Methods
Origin and Maintenance of Fish Stocks

Strains of sexually reproducing desert species of Poeciliopsis used in this study were established from fish collected from three river systems of northwestern Mexico between 1961 and 1987. They were maintained primarily by brother-sister matings from the time of capture. The tropical species P. gracilis was collected from a tributary of the Río Coatzacoalcos and maintained by random mating. Wild unisexual P. monacha-lucida strains were established from individual hybrid females collected from sites in the Río Fuerte between 1961 and 1973 as described in Vrijenhoek et al. (1978). Poeciliopsis monacha-lucida strains were also synthesized in the laboratory (Schultz 1973) by hybridization between P. lucida M61-31 males and virgin outbred females from the first laboratory generation of P. monacha strain S68-4 (hemiclone XXXVI S68-4) and S68-5 (hemiclone XXXVI S68-5). These hybrids were resynthesized, yielding strains Syn-4 and Syn-5, after more than 20 yr of inbreeding had rendered the two P. monacha stocks homozygous. All hybrid strains were maintained by continued mating to P. lucida M61-31 males. Table 1 provides a list of strains, arranged chronologically within each species by date of initial collection. Collection sites are shown in the map in figure 1.

Wild specimens of Gambusia affinis were collected in May 1993 from two sites in Nevada: Garrett Ranch in the Black Rock Desert and a site near Wabuska. These sites had been stocked at various times in the late 1930s (C. A. Stockwell, personal communication). Fish from each site were maintained in separate aquaria and assayed within 3 mo of collection.

The fish were maintained in greenhouse aquaria in the fish colony at the University of Connecticut as described previously (White et al. 1994).

Analysis of Heat Shock Proteins

Hsp's of Gambusia affinis and of the desert strains of Poeciliopsis were radiolabeled in cultured primary hepatocytes and analyzed by two-dimensional gel electrophoresis as described previously (White et al. 1994). Briefly, hepatocytes prepared from a single adult liver were split into two culture tubes and incubated for 2 d at 30°C. One culture was then heat shocked at 39.5°C for 1 h. Proteins were labeled with 35S-methionine for 1 h at 30°C for control or at 39.5°C for heat-shocked cultures. Proteins were solubilized in isoelectric focusing-sample buffer (9.5 M urea, 2% NP-40, 1.6% pH 5–7 amphotely, 0.4% pH 3.5–10 amphotely, 5% β-mercaptoethanol, 70 mM Tris-HCl pH 7.6) and separated by isoelectric focusing (IEF) under denaturing conditions in the first dimension and SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) in the second dimension. The IEF gels contained 120 μl pH 5–6 and 30 μl pH 3–10 amphotelys (Pharmalytes; Sigma Chemical Co.) per 3 ml of gel solution. The measured pH range of these first-dimension gels was 5.0–7.5; the marker protein ovalbumin (calculated isoelectric point [pI] 5.1) focused at pH 5.4 and carbonic anhydrase (calculated pI 7.0) focused at pH 7.5. Markers for denaturing IEF were purchased from Sigma.

Proteins extracted from the gill tissue of individual P. gracilis used in heat stress survival experiments (see below) were also analyzed. The gill tissue was dispersed in isoelectric focusing-sample buffer containing 1% sodium dodecyl sulfate, agitated briefly, and centrifuged at 10,000xg for 4 min; supernatants were harvested and stored at −70°C. In order to analyze P. gracilis hsp70 isoforms, the amphotely mixture in the IEF dimension was modified to establish a more basic pH gradient. A typical mixture was 100 μl pH 5–7, 40 μl pH 3.5–10, and 10 μl pH 6–8 amphotelys (LKB ampholines; Sigma Chemical Co.) per 3 ml gel solution.

Gambusia affinis hsp's were also labeled in vivo. Individual fish were injected intraperitoneally with 150 μCi of 35S-methionine and cysteine (Translabel, ICN Radiochemicals) and placed in a container with 250 ml
Table 1
Summary of Poeciliopsis Strains

<table>
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<th>Species/Hemiclone</th>
<th>Strain</th>
<th>No. of Lab Generations</th>
<th>Locality¹</th>
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* See fig. 1.
| Derived from wild-caught females.
| Derived from hybridizations performed in captivity.

tank water per fish. The container was placed in a water bath and the temperature was raised from 25° to 37°–38° at a constant rate of 0.15°C per minute. Under these conditions the fish were exposed to a gradual temperature increase over the first hour and then approximately 1 h at 37°–38°. Total labeling time was 3 h, after which proteins were extracted from gill tissue as for P. gracilis and analyzed on two-dimensional gels.

Radiolabeled proteins were visualized by fluorography (Laskey and Mills 1975). Unlabeled proteins were visualized by silver staining using the Sigma Silver Stain Kit, except that gels were placed in Reducer solution for 30 s and then rinsed briefly in water prior to incubation in silver solution. Under the conditions used for electrophoresis, polypeptides differing in isoelectric point (pI) due to a change in a single charged amino acid residue would be resolved in the isoelectric focusing dimension; differences of ~1 kDa or greater in molecular mass (M_r) would be resolved in the SDS-PAGE dimension. Identification of isoforms was based on relative migration on two-dimensional gels and confirmed by co-electrophoresis of two samples on a single gel. Isoforms from different extracts that comigrated on two-dimensional gels were considered identical. Hsp70 and hsp30 isoforms were designated with the numbering system used in White et al. (1994).

Mendelian Inheritance of hsp70 Isoforms

Arrangements of major inducible hsp70 isoforms in Poeciliopsis gracilis were inferred from the observed classes of genotypes assuming tandem arrangement of the respective genes. This resulted in a conservative estimate of linkage group diversity. Proposed linkage groups were designated with Roman numerals. Only major isoforms of inducible hsp70 (isoforms 0, 1 and 3; fig. 3) were included in this analysis because they accumulated to levels detectable with silver stain.

Heat Stress and Analysis of Survival

Analysis of adult survival and hsp70 variation in Poeciliopsis gracilis was conducted on lots of approximately 10 females from the second and third lab generations of stocks maintained by random breeding. Fish were acclimated prior to thermal stress for 1 wk at 30°C under a 14 h light:10 h dark photoperiod. Temperature stress was carried out by placing fish in a plastic container holding a volume of tank water equal to 250 ml per fish. This was aerated and allowed to cool to 25°C in a conventional water bath. The temperature was then raised at a constant rate of 0.15°C per minute to 41°C, a temperature well above that required to initiate hsp70 synthesis in other species and hemiclones of Poeciliopsis
hsp70, hsc70, and minutes of survival at 41°C were assessed using Spearman’s rank correlation (Sokal and Rohlf 1981). Where appropriate, trends in the data were indicated with lines or curves.

Results
Biochemical Diversity of hsp70

Incubation of Periclimnion lucida hepatocyte cultures at elevated temperature resulted in the induced synthesis of proteins of 100, 90, 70, 60, and 30 kDa (fig. 2). These size classes of heat shock proteins have been characterized previously in diverse organisms (Novel and Scharf 1991). In Periclimnion, multiple inducible isoforms of 30 and 70 kDa were synthesized; these clusters were shown previously to be two different families of related proteins whose members did not share precursor-product relationships (White et al. 1994). The hsp70 cluster contained the constitutively expressed family members hsc70 and the putative mitochondrial hsp70 (marked by arrows in fig. 2). Grp78, the hsp70 homolog resident in the endoplasmic reticulum, was synthesized inductively when analyzed by radiolabeling (fig. 2), but significant basal levels were also found when similar extracts were analyzed by silver stain (not shown). Four strictly heat-inducible isoforms were synthesized, but spot 3 was reproducibly synthesized at a much higher rate than spots 4, 5, and 7. The hsp30 cluster consisted of three major and three minor isoforms, none of which was synthesized in the control culture. Hsp patterns similar to that in figure 2 were highly reproducible among individuals of inbred strains of six desert species, but variation in hsp patterns was seen when these closely related species were compared with each other (White et al. 1994).

By extending this survey of hsp diversity in Periclimnion, we have now been able to demonstrate polymorphism of the major inducible isoform of hsp70 within an outbred population of the tropical species P. gracilis (fig. 3), as well as within different lines of the desert species P. occidentalis (fig. 4). In P. gracilis, three inducible isoforms of indistinguishable molecular mass but variable pl were observed (spots 0, 1, and 3; fig. 3; under the conditions of this experiment, minor isoforms did not accumulate to a high enough level to be detected). Co-electrophoresis of P. gracilis proteins and those from a desert species, P. viriosa (data not shown), demonstrated that spot 0 was unique to P. gracilis but that spots 1 and 3 comigrated with viriosa isoforms given the same number. Constitutively expressed hsc70 (spot c) and grp78 (spot d) were identical to those found in P. viriosa and five other desert species of Periclimnion (White et al. 1994). Similarly, the putative mitochondrial hsp70 in P. gracilis comigrated with that from P.
**Fig. 2.**—Two-dimensional pattern of proteins synthesized in control (A) and heat-shocked (B) cultures of *Poeciliopsis lucida* V85-9 hepatocytes. Cultures were labeled with 35S-methionine for 1 h at control or heat shock temperature as described in Methods. The measured pH range in the isoelectric focusing dimension was 5.0–7.5. Constitutively expressed proteins are indicated by arrows. Inducible members of the hsp70 family are numbered, and the 30-kDa heat shock protein family is bracketed.

*P. lucida* and was identical to that found in *P. prolifica* but distinct from that found in *P. monacha* and *P. viriosa*.

Comparison of electrophoretic mobilities of hsc70, grp78, and mitochondrial hsp70 relative to major inducible isoforms established the invariant migration of these constitutively expressed proteins in 47 females of *P. gracilis* (fig. 3A–F). These same individuals, however, possessed a variety of inducible hsp70 isoform complements (fig. 3B–F). All of the complements fell into one of five qualitative classes. These included isoform 1 only, isoform 3 only, isoforms 1 and 3, isoforms 0 and 1, and isoforms 0, 1, and 3 (table 2). Complements 0, 3, and 0 only were not observed. Inducible hsp70 isoforms appeared to be inherited in a Mendelian fashion in *P. gracilis*. Three hsp70 linkage groups were inferred from the five classes of observed isoform complements: linkage group I carries the gene-encoding isoform 1, linkage group II carries the gene-encoding isoform 3, and linkage group III carries the gene-encoding isoforms 0 and 1. The occurrence of isoform 3 as the single major isoform

**Fig. 3.**—Hsp70 patterns of individual outbred *Poeciliopsis gracilis*. Gill tissue from control (A) or heat-shocked (B–F) fish was solubilized, and proteins were run on two-dimensional gels, which were visualized by silver stain. Only the hsp70 region of the gels is shown. Lowercase letters indicate constitutively expressed proteins, including a, putative mitochondrial hsp70; b, an additional hsp70 family member expressed constitutively in gill tissue; c, hsc70; and d, grp78. Inducible hsp70 isoforms are numbered.
in homozygous lines of *P. monacha*, *P. lucida*, *P. prolifica*, and *P. occidentalis* was consistent with the proposed linkage groups; however, the combination of isoforms 1 and 3 in a homozygous line of *P. viriosa* (White et al. 1994) was not. This particular arrangement of isoforms may have gone undetected in our sample of *P. gracilis*, but it is also possible that the isoforms were rearranged in *P. viriosa*.

Hsp70 diversity in *P. occidentalis* was seen when two strains derived from different river systems were compared. In this experiment radiolabeled extracts of primary hepatocytes were assayed, and both major and minor hsp70 isoforms were visualized (fig. 4). All individuals of strain AV76-7 synthesized hsp70 isoforms identical to inbred *P. lucida* (compare fig. 4B and fig. 2B). However, fish of strain V87-15A synthesized isoform 1 (also found in *P. viriosa* and *P. gracilis*) in addition to isoform 3, and a more acidic form of the minor isoform, 7 (fig. 4A). The putative mitochondrial hsp70 (*unlabeled arrows*) and hsp30 clusters were also different between the two strains. Although strain V87-15A had been inbred for only five generations, the hsp70 pattern was identical in six fish, and the hsp30 pattern was identical in five of the six fish that were available for assay.

Biochemical Diversity of hsp30 in *Poeciliopsis lucida*

Hsp70 and hsp30 isoforms were analyzed in seven strains (see table 1) of *P. lucida*, four of which were inbred. Between 3 and 20 individuals were analyzed for each inbred strain; no variability in hsp isoforms was seen among individuals of a particular inbred strain. Analysis of individuals of noninbred strains was limited by the number of fish available. Hsp70 isoforms identical to those in figure 2 were synthesized by all fish sampled (not shown). However, four different hsp30 patterns were obtained from these same fish (fig. 5). The four

<table>
<thead>
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<th>Isoform Complement</th>
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<th>Mean Rankb</th>
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<tr>
<td>0,1,3</td>
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* Number of fish expressing a particular combination of major hsp70 isoforms.

* Raw data (sum of optical density of hsp70 isoforms or minutes of survival at 41°C) for individual fish were assigned ranks; the means of these ranks for each hsp70 genotype are listed. Analysis of ranks in the Kruskall-Wallis non-parametric ANOVA showed that neither hsp70 accumulation (*P* = 0.23) nor survival (*P* = 0.87) was significantly correlated with isoform complement.

**Fig. 4.**—Heat shock proteins synthesized in *Poeciliopsis occidentalis* strain V87-15A (*A*) and AV76-7 (*B*). The pH range across each panel was approximately 5.1–7.5. Isoforms of hsp70 (*upper cluster*) and hsp30 (*lower cluster*) are numbered; constitutively expressed proteins are labeled with arrows. Isoforms which co-electrophoresed with those of other *Poeciliopsis* species were given the same numbers (see fig. 1 and White et al. 1994).
patterns contained identical constitutive proteins used as markers (arrows in fig. 5) but differed in which subset of the six inducible isoforms was present. Some of these inducible protein isoforms also differed in intensity among the four patterns. Only isoform 11 was synthesized at a high rate in all four patterns, while isoform 14 was always synthesized at a lower rate. Isoform 2 was a major component only in pattern A, isoform 6/8? (which was probably two isoforms which did not resolve well from each other) was a major component of patterns C and D, and isoform 7 was a major component of patterns B and D. These isoforms were also found at low levels in the other patterns. Isoform 12 was found only in pattern A.

Patterns A and B were characteristic of both inbred and noninbred strains and were therefore the products of genomes that were homozygous at hsp30 loci; patterns C and D were found only in individual fish of the noninbred stock V87-10 (table 3) and may represent heterozygous patterns. The other two noninbred stocks showed no variation in hsp30 among individuals, but our ability to detect such variation was limited by the small sample size. We have demonstrated hsp30 variation among and within different stocks, but this represents only a minimum estimate of the variation present within the *P. lucida* population.

**Hsp30 Diversity in Poeciliopsis monacha**

In addition to analysis of hsp's synthesized by inbred strains of *Poeciliopsis monacha*, we were able to assay the contribution of haploid “wild” monacha genomes to the hsp's synthesized by *P. monacha-lucida* hemizygotes. Successful hybridization in the wild between a *P. monacha* female and a *P. lucida* male generates *P.*

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**Table 3**

**Hsp30 Patterns in Poeciliopsis lucida**

<table>
<thead>
<tr>
<th>Arroyo</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<td>San Pedro</td>
<td>V87-10 a,b&lt;sup&gt;a&lt;/sup&gt;</td>
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</table>

<sup>a</sup> Lowercase letters indicate individual fish from noninbred strains.

<sup>b</sup> Bold type indicates inbred strains which are homozygous (Schultz and Schultz 1984); no variation in hsp30 pattern was seen among individuals (n ≥ 3) for these strains.
monacha-lucida) hybrid females. Each of the hybrid females contains a randomly assorted haploid monacha genome which will then be passed clonally to all offspring due to a unique method of reproduction termed hybri-dogenesis (Schultz 1961, 1969). This entails loss of the paternal lucida genome during premeiotic divisions of oogenesis in the hybrid, thus precluding any recombination between maternal and paternal genomes. Upon fertilization of the hybrid female by a P. lucida male, the clonally derived monacha genome and a new paternal genome are incorporated into the zygote. Thus, individuals derived from a single hybrid female share identical maternal genetic material but unique paternal genomes in the wild. In the lab, hybrid females are mated to a homozygous strain of P. lucida, standardizing the paternal genome. Each female becomes the progenitor of a strain of genetically identical individuals, and strains established from different females contain different collections of monacha alleles but a common paternal genome. Analysis of hsp30 expression in parental inbred strains of P. monacha (fig. 6C) and P. lucida (fig. 5A) and hybrids synthesized in the lab from them (P. monacha-lucida strains Syn-4 and Syn-5) showed that maternal and paternal hsp30 isoforms were co-expressed in the hybrid (shown in fig. 6D). Hsp70 isoforms were also co-expressed (data not shown). Thus the contribution of the monacha genome to the hsp pattern of wild hemi clones mated to inbred P. lucida M61-31 could be deduced by subtracting isoforms synthesized by the paternal strain. This is indicated in figure 6 by circling only those isoforms not seen in the P. lucida pattern in figure 5A.

Identical hsp70 isoforms were synthesized by all hemiclones and the two inbred strains of P. monacha that were assayed (data not shown; see White et al. 1994). However, biochemical diversity was observed in the hsp30 cluster (fig. 6; summarized in table 4). Nine monacha-lucida hemiclones synthesized either pattern A or pattern B. Isoforms in these patterns included those synthesized by the paternal P. lucida genome, including isoforms 2, 6, 11, 12, 14, and constitutively expressed spot a. The isoforms attributable to the maternal monacha genome included 1, 4, 5, 9, and constitutively expressed spot c. The difference between these two patterns was the presence or absence of isoform 5. Pattern C was synthesized by both inbred P. monacha strains tested; in this pattern all the protein isoforms were the products of monacha genes. The monacha contribution to hsp30s of hemiclones synthesized from the inbred P. monacha strains and inbred P. lucida M61-31 was identical to the hsp30s of the maternal strain (compare fig. 6C and D).

Hsp Diversity in Gambusia affinis

Hsp patterns in Gambusia affinis, which is conspecific with Poeciliopsis, were also analyzed. Twenty-two wild-caught individuals from two geographically separated populations of Gambusia synthesized identical isoforms of hsp70 (fig. 7E): the major inducible isoform (isoform 3) and constitutively expressed hsc70 and grp78 were identical to those in P. lucida. When the hsp30 patterns of 18 of these fish were assayed, a variety of hsp30 patterns were found (fig. 7A–D). Fish from both sites synthesized pattern C (isoforms 1, 2, 3, and 4). In this limited sampling, patterns A and B were synthesized.

![Figure 6](image-url) - Hsp30 isoforms synthesized in Poeciliopsis monacha strains and P. monacha-lucida hemiclones. The hsp30 region of fluorographs is shown as in fig. 5. Constitutively expressed proteins are marked by arrows. Isoforms which were electrophoretically identical to P. lucida isoforms in fig. 5 were given the same number designations. Circled numbers indicate isoforms attributable to the P. monacha genome. Table 4 lists the P. monacha and P. monacha-lucida strains for which these hsp30 patterns were characteristic.
only by fish from the Wabuska site, and pattern D was synthesized only by Garrett Ranch fish.

Survival of *Poeciliopsis gracilis* under Severe Stress

Individual *Poeciliopsis gracilis* females possessing distinct isofrom complements did not differ in resistance to an experimentally induced severe heat stress (41°C after ramping at 0.15°C per minute; table 2). Also, no differences were observed among isofrom complements in total accumulation of hsp70: complements with three isofroms accumulated as much total hsp70 in response to stress as those expressing single isofroms (table 2). Implicit in these results is the nonsignificant effect of number of isofroms or relative pl of isofrom complement on survival or total hsp70 accumulation. The four genotypes scorable as heterozygotes (1,3 and 0,1,3) and homozygotes (3 only and 1 only) were not significantly different in duration of stress resistance or total hsp70 accumulation and were not different from the group consisting of a combination of homozygotes and heterozygotes (0,1).

A significant, nonlinear relationship between amount of hsp70 (measured as optical density) and minutes of survival at 41°C was observed, regardless of which hsp70 isofroms were expressed, but there was considerable variation in this association (fig. 8A). Abundance of hsc70 was not associated with resistance to a 41°C stress, but levels of hsc70 were highly correlated with increased amounts of hsp70 (fig. 8B). In addition, the relationship between minutes of survival and the sum of the amounts of hsp70 and hsc70 was weaker than that observed between survival and hsp70 alone (data not shown).

**Discussion**

Diversity of hsp70 within Species of *Poeciliopsis*

Although the hsp70 family is the most highly conserved of the hsp families across distantly related taxa (Boorstein et al. 1994), a comparison of closely related species of *Poeciliopsis* demonstrated biochemical diversity in inducible members of this family. The constitutively expressed members hsc70 and grp78 were electrophoretically identical in six desert species (White et al. 1994), as well as in the tropical species *P. gracilis* (fig. 3) and *P. fasciata* (not shown), and a confamilial species, *Gambusia affinis* (fig. 7E). Anderson et al. (1982) made a similar observation when comparing animal species including mammals, birds, and reptiles: hsc70 (designated MSN5) was electrophoretically identical in all species, but inducible hsp70s differed in pl and/or Mr. The lack of diversity in hsc70 and grp78, and the fact that viable mutants for these proteins have not been isolated, underscores their essential role (hsc70 in the nucleocytoplasmic compartment and grp78 in the endoplasmic reticulum) under nonstressful conditions and supports the idea that these proteins are under strong evolutionary constraints (Boorstein et al. 1994). These constraints might include conservation of binding sites for ATP/ADP and unfolded proteins as well as sites for interaction with other hsc70 molecules or accessory proteins involved in modulating hsc70 activity (Hightower et al. 1994).

In contrast to hsc70, the major isofrom of inducible hsp70 was polymorphic. Two of the isofroms (1 and 3) found in *P. gracilis* (fig. 3) were also synthesized by inbred lines of various desert species (White et al. 1994); isofrom 0, which had the most basic pl, was unique to *P. gracilis*. Much less variation in hsp70 was seen in desert species. Analysis of multiple strains of *P. monacha* and *P. lucida* showed no hsp70 variation within either species (fig. 2; White et al. 1994). All these *monacha* and *lucida* strains originated from fish collected in different tributaries of the Río del Fuerte (fig. 1, inset). However, one of two strains of *P. occidentalis* synthesized isofrom 1 in addition to isofrom 3; this strain also

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*a* Italic type indicates strains developed from *P. monacha-lucida* hybrids collected in the wild.

*b* Regular type indicates *P. monacha-lucida* hybrid strains synthesized in the laboratory (Schultz 1973).

*c* Bold type indicates inbred strains of *P. monacha* which are homozygous as judged by allozyme analysis (Vrijenhoek et al. 1978) and by histocompatibility analysis (Angus and Schultz 1979; Schultz 1989).
had an altered mobility of a minor isoform (fig. 4). These strains originated from two rivers (Río de la Concepción and Río Matape; see fig. 1) separated at their mouths by ~200 mi; the hsp70 variation between them may represent differences in the progenitor populations maintained by physical separation of the two populations. Since only one strain from each river was available for study, it is also possible that hsp70 diversity was present within each population.

*Poeciliopsis occidentalis* strain V87-15A was only five generations inbred and thus not homozygous; however, the six fish available for study had identical hsp70 patterns, and only one synthesized a different hsp30 complement (data not shown; the pattern contained two additional isoforms compared to the common pattern in fig. 4A). Rapid development of homozygosity at histocompatibility loci upon inbreeding of platyfish has been attributed to limited diversity in the founder population. One such population was so homozygous that tissue grafts were accepted among wild individuals (Kallman 1964). *Poeciliopsis occidentalis* strain V87-15A, although not extensively inbred in the laboratory, may also have developed considerable homozygosity in the wild before capture. The low degree of hsp70 variation in northern species, in contrast to the diversity seen in *P. gracilis*, may be a consequence of the desert environment. Fish populations in desert streams are exposed to large fluctuations in temperature, both seasonally and in the course of a single day (Bulger and Schultz 1979). Their habitats often become fragmented during dry seasons, and population bottlenecks and extinction/recolonization events have had profound effects on the genetic makeup of isolated populations (Vrijenhoek 1994). Under these conditions fixation of alternative alleles in different populations would be expected.

Hsp70 isoform 3 was also synthesized in the con-familial species *Gambusia affinis* (fig. 7E) collected from two sites in Nevada. Both these sites were stocked with fish from a population at Fallon, Nevada, in the late 1930s. A 25%-40% decrease in allelic diversity at enzyme loci was seen when these populations were compared to a “reconstructed” population thought to be similar to the ancestral population initially used for translocations in the 1920s; all losses were of relatively rare alleles (C. A. Stockwell and M. Mulvey, unpublished manuscript). This may indicate that isoform 3 represents a common allele in *Gambusia*; it also appears to be the most common allele in *Poeciliopsis*, where it is found.
in six of the eight species that have been analyzed (White et al. 1994; this paper). The presence of isofrm 3 in Gambusia suggests that this protein was ancestral to isoforms 1 and 0 and that the process of duplication and divergence gave rise to additional isoelectric variants after the split between Gambusia and Poeciliopsis. The alternative is that P. gracilis maintains an ancestral polymorphism which has subsequently been lost in both Gambusia and other species of Poeciliopsis.

The major hsp70 isoforms found in desert species of Poeciliopsis made up a subset of the isoforms found in tropical species. P. lucida and P. occidentalis (this paper) as well as P. monacha, P. viriosa, and P. prolifica (White et al. 1994) synthesized isoforms also found in P. gracilis (isoforms 3 and 1). Another desert species, P. latidens, synthesized isoform 2 (White et al. 1994). This isoform, which had a PI intermediate between isoforms 1 and 3, was synthesized in a different tropical species, P. fasciata (data not shown). Poeciliopsis as well as other Poeciilidae are thought to have arisen in the tropics and then radiated northward (Rosen and Bailey 1963); the distribution of hsp70 isoforms in desert and tropical species of Poeciliopsis is consistent with this hypothesis. As ancestral fish, which were adapted to the warm, thermally stable tropical environment, moved northward and colonized desert streams, they would have encountered fluctuating temperatures, including cooler temperatures than those experienced in the tropics. Adaptation to this environment may have involved changes in isofrm complements, as well as regulatory changes to provide for rapid turnover of hsp70, which is thought to be deleterious when expressed under non-stress conditions (Feder et al. 1992). The preponderance of isofrm 3 in desert species may indicate that it is a member of a repertoire of proteins needed for successful colonization of the desert environment, or because it is linked to another such protein; the short half-life of isofrm 3 (C. E. Norris, unpublished data) may also be important for survival in a desert environment.

Hsp30 Diversity in Poeciliopsis monacha and Poeciliopsis lucida of the Río del Fuerte

The geographical distribution of hsp30 diversity in Poeciliopsis monacha separates the fishes of the Arroyo Jaguari from those found in the rest of the Río Fuerte (table 4). This grouping parallels extensive work on allozyme frequencies in both hemiclonal (Vrijenhoek et al. 1978) and sexual (Vrijenhoek 1979) monacha genomes. Hemiclonal monacha genomes sampled from the Jaguari over a 15-yr period were found to be of only two allozyme haplotypes, each linked with a specific histocompatibility locus (Angus and Schultz 1979) and mitochondrial DNA haplotype (Quattro et al. 1992). These haplotypes represented a subset of the diversity found in the sexually reproducing monacha population at this site and were not found elsewhere in the Fuerte, while haplotypes found in the other three tributaries were not found in the Jaguari. This is similar to the hsp30 isoform distribution: patterns B and C were found only in Jaguari fishes, while pattern A was common to the other three tributaries and was not found in the Jaguari. Isolation of the Jaguari population from the rest of the Fuerte is thought to account for these differences; hybridization events occurring in headwaters of the Jaguari where populations of P. monacha and P. lucida overlap would fix monacha genomes unique to this locale. The barriers restricting monacha gene flow would presumably also restrict the spread of the resulting hemiclones to the rest of the Río Fuerte.
Poeciliopsis lucida collected from the Río Fuerte exhibited lower levels of allozyme polymorphism than P. monacha in the same sites (Leslie 1979). Again, the laguari fishes exhibited differential traits when compared to the rest of the Fuerte. Differences in caudal fin ray number (Angus and Schultz 1983) and the monomorphic nature of the allozyme complement of P. lucida collected from the headwaters of the Laguari over many years (R. C. Vrijenhoek, personal communication) indicated isolation of the Laguari lucida population. However, heterozygosity in histocompatibility loci was seen when tissue graft analysis was performed on this population (E. Fielding, personal communication). The hsp30 complements of strains of P. lucida from the laguari (patterns A and B, fig. 5) were not unique to that site; each pattern was also found in at least two other sites (table 3). Pattern A showed reduced levels of isoform 7, and both A and B showed reduced expression of isoform 6 relative to some of the other patterns. A and B were patterns from homozygous fish; thus it appears that stable, heritable regulatory changes can also govern expression of these proteins and contribute to diversity. It is important to note that inbred and partially inbred strains of P. lucida were analyzed, as opposed to the monacha genomes assayed mainly in hemiclonal form. In both cases a minimum estimate of the variability present in wild populations was obtained, but missing sites in the two sites at 7 of 11 loci, while allelic differences were detected at the remaining four loci (C. A. Stockwell and M. Mulvey, unpublished manuscript). The maintenance of diversity of hsp30 isoforms in these populations demonstrates that hsp diversity among the individuals of a single species is not restricted to Poeciliopsis.

Hsp30 Diversity in Gambusia affinis

Although no variation in hsp70 complements in wild-caught specimens of Gambusia affinis were seen, several distinct hsp30 patterns were found. The distribution of hsp30 patterns in fish from the Wabushka and Garrett Ranch sites was consistent with data on allozyme complements: identical alleles were found at the two sites at 7 of 11 loci, while allelic differences were detected at the remaining four loci (C. A. Stockwell and M. Mulvey, unpublished manuscript). The maintenance of diversity of hsp70 isoforms in these populations demonstrates that hsp diversity among the individuals of a single species is not restricted to Poeciliopsis.

Hsp70 and Survival of Heat Stress

A previous comparison of hsp70 sequences from divergent organisms indicated that the C-terminal peptide-binding domain of this protein has evolved more rapidly than the N-terminal ATPase domain (Milarski and Morimoto 1989). Thus it is likely that variation among P. gracilis hsp70 isoforms resides mainly in the peptide-binding domain. This type of variation could alter the range of unfolded proteins recognized by hsp70 in its role as a molecular chaperone: for example, two isoforms of yeast hsp70 sharing 97% amino acid sequence identity have different affinities for clathrin, a specific protein substrate (Gao et al. 1991). Hsp70 isoforms may also differ in stability and/or affinity for accessory proteins.

Thermal resistance in P. gracilis, which survived up to 50 min at 41°C after a constant 0.15°C per minute increase in temperature, was higher than in even the most resistant northern strains of Poeciliopsis, which survived approximately 15 min at 41°C (P. J. dilorio, unpublished manuscript). However, individual P. gracilis differing in hsp70 isoform complement did not differ in survival (table 2). This test, which involved a constant rate of temperature increase to lethal temperatures, is more realistic than critical thermal maxima measurements in which organisms are transferred directly to high temperatures. Our heating regimen mimics the thermal exposure of desert species that become trapped in shallow pools during the dry season and cannot escape the near-lethal temperatures, which can rise from 30°C to 40°C over 3 h. However, there are additional heating regimens that need to be tested, including ones that mimic diurnal cycles and have lower maximum temperatures. This may be particularly relevant to analysis of hsp70, since hsp70 induction temperatures are more closely associated with physiological optima than temperature extremes in P. lucida, P. monacha, and two wild hemiclones (P. J. dilorio, unpublished manuscript). Hsp70 may play an important role during moderate heat stress, raising the possibility that different P. gracilis genotypes could be differentially adapted to these sublethal temperatures. Also, niche partitioning has been observed in closely related clonal genotypes and gonochoristic species of Poeciliopsis in northern Mexico (Schenk and Vrijenhoek 1986; Schultz 1977); different hsp70 genotypes of P. gracilis may spatially subdivide thermal niches in the Río Coatzacoalcos in a similar manner.

Resistance to thermal stress is only one test of the importance of hsp70 isoform variation to survival. These proteins play a number of biological roles and respond to a variety of environmental stressors (Lindquist and Craig 1988). Variation in hsp70 may be maintained in response to selective forces such as wound repair (Brown et al. 1989) and viral infection (Collins and Hightower 1982). Variation in hsp70 could also be maintained if oligomerization of this protein into dimers and higher-order complexes is critical to its function (Elguindi et al. 1993; Hightower et al. 1994).
While the kinds of isoforms expressed did not contribute to increased stress resistance under the conditions of our heat stress regimen, or to differences in accumulation of hsp70, the quantity of hsp70 accumulated was associated with increased time of survival at 41°C (fig. 8A). Cells which overexpress hsp’s in response to stress or express them at high constitutive levels do become thermotolerant (Angelidis et al. 1991; Li et al. 1983; Mosser et al. 1986), but a thermotolerant state can also be acquired independently of hsp synthesis (Hall 1983; Smith and Yaffé 1991). Increased expression of hsp70 may contribute to an increase in stress resistance; alternatively, an individual with increased thermal resistance due to an unknown factor would synthesize hsp’s over a longer period of time and thus accumulate higher levels.

The considerable variation observed in the relationship between hsp70 abundance and thermotolerance was probably due to a number of factors. First, the relationship between the abundance of these proteins and a phenotype as complex as acquired stress resistance is likely to be indirect. The contribution of hsp70 to stress resistance would be mediated by its interactions with proteins which might vary in outbred populations (Stephanou and Alahiotis 1986). Measures of abundance alone, without accounting for variation at other loci, may be an inadequate measure of this relationship. Hsp70 may be viewed best as a marker for the magnitude of the heat shock response, contributing to acquired thermotolerance along with other proteins such as the small hsp’s and hsp100. Second, kinetics of hsp70 accumulation vary with the physiological status of cells (C. E. Norris, unpublished data), and presumably also with the physiological status of organisms. At the least, physiology may place an upper limit on stress resistance, regardless of the level to which hsp’s have accumulated.

Abundance of hsc70 was not correlated with increased stress resistance but was tightly associated with high levels of hsp70 (fig. 8B). It is possible that high levels of both proteins are simply the result of fish possessing faster or slower rates of protein synthesis. However, this explanation is unsatisfactory because higher rates of protein synthesis would result in the accumulation of higher levels of damaged proteins during stress, since nascent and unassembled proteins are among the most thermolabile. Rather, hsp70 and hsc70 may be performing different roles in the development of thermal resistance. In particular, hsc70 may be important in early stages of stress, repairing or targeting damaged proteins for degradation before they accumulate to toxic levels. Additionally, hsc70 may play an important role in chaperoning newly made hsp70 and may mediate the magnitude of the inducible response (P. J. diFiorio, unpublished manuscript).

Conclusions

Our finding of biochemical diversity in inducible hsp70, in contrast to the highly conserved nature of hsc70 and grp78, suggests that inducible and constitutive members of this protein family are under different evolutionary constraints. An evolutionary scenario that could explain the maintenance of hsp70 polymorphism in Poeciliopsis gracilis, and the limited variation in the desert species, is suggested by the inducible nature of hsp70 expression. De novo synthesis of hsp’s is a threshold phenomenon. The intensity of selection on these proteins likely depends on the frequency with which the environment reaches that threshold and the degree to which the environment exceeds it. During periods between stress events, functional constraints may be relaxed, and quiescent genes could accumulate mutations. Periodic selection events might then cull out deleterious mutations, either driving the population to homozygosity or leaving behind those mutations with neutral effects on stress resistance. Alternatively, maintenance of isoform variation could be due to selective advantage manifested during development or in resistance to stress.

The hsp70 patterns seen in Poeciliopsis and Gambusia suggest that isoform 3 existed in a common tropica ancestor of these two genera. Further duplication and divergence of the hsp70 genes in Poeciliopsis after its split from Gambusia apparently gave rise to additional isoforms. Fish which subsequently colonized norther desert habitats maintained an ancestral ability to survive high temperatures, as proposed for Cyprinodon species (Heath et al. 1993); however, adaptation to the fluctuating desert environment may have resulted in some loss of hsp diversity. Fixation of alternate alleles in populations subjected to frequent bottlenecks during periods of drought would also decrease diversity in northerm species of Poeciliopsis.

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