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THERMOTOLERANT DESERT LIZARDS CHARACTERISTICALLY DIFFER IN TERMS OF HEAT-SHOCK SYSTEM REGULATION

O. G. ZATSEPINA[1,*], KH. A. ULMASOV[2], S. F. BERESTEN[1], V. B. MOLODTSOV[1], S. A. RYBTSOV[1] AND M. B. EVGEN'EV[3]

[1]*Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow 117984, Russia,* [2]*Institute of Physiology, Ashkhabad, Turkmenistan* and [3]*Institute of Cell Biophysics, Pushino, Russia*

*Present address: Laboratory of Genome Instability, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32 Vavilov Street, Moscow, 117984, Russia (e-mail: Evgenev@genome.eimb.rssi.ru)

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Summary

We compare the properties and activation of heat-shock transcription factor (HSF1) and the synthesis of a major family of heat-shock proteins (HSP70) in lizard species inhabiting ecological niches with strikingly different thermal parameters. Under normal non-heat-shock conditions, all desert-dwelling lizard species studied so far differ from a northern, non-desert species (*Lacerta vivipara*) in the electrophoretic mobility and content of proteins constitutively bound to the regulatory heat-shock elements in the heat-shock gene promoter. Under these conditions, levels of activated HSF1 and of both HSP70 mRNA and protein are higher in the desert species than in the non-desert species. Upon heat shock, HSF1 aggregates in all species studied, although in desert species HSF1 subsequently disaggregates more rapidly. Cells of the northern species have a lower thermal threshold for HSP expression than those of the desert species, which correlates with the relatively low constitutive level of HSPs and high basal content of HSF1 in their cells.

Key words: lizard, adaptation, thermoresistance, heat-shock protein, heat-shock factor.

Introduction

All organisms that have been studied to date respond to elevated temperatures and other stresses by expressing heat-shock proteins (HSPs) (Lindquist, 1986; Feder and Hofmann, 1998). In the absence of stress, these proteins and their constitutively expressed cognates play essential roles as molecular chaperones involved in the folding, assembly and translocation of many cellular proteins (Georgopoulos et al., 1996). The 70 kDa family of HSPs plays a key role in the cellular response to heat shock by maintaining the native state and proper folding of cellular proteins during physiological stress and by facilitating the restoration of cellular functions in higher eukaryotes (Morimoto et al., 1994; Morimoto, 1993).

The expression of HSP genes depends on the activation of heat-shock transcriptional factor (HSF1), which is constitutively present as a monomer. During activation, HSF1 monomers assemble into trimers, which then can bind specific motifs, known as heat-shock elements (HSEs), located in the promoter region of all HSP genes (Rabindran et al., 1993; Sarge et al., 1993). Such changes in HSF1 are readily detectable using the gel mobility-shift assay, in which HSF1 binding to a probe containing HSEs reduces the electrophoretic mobility of a probe. The expression of HSPs is also negatively regulated by a constitutive heat-shock-element-binding factor (CHBF) or Ku autoantigen, a heterodimer of 70 kDa (Ku-70) and 86 kDa (Ku-80) polypeptides (Liu et al., 1993; Kim et al., 1995; Li et al., 1995; Yang et al., 1996).

Most studies of the components of the heat-shock system have been carried out using cell lines or isolated tissues and organs (Liu et al., 1993; Kim et al., 1995; Shi et al., 1998). Although this approach has yielded much interesting and important data on the regulation of heat-shock genes, biological experiments exploring naturally occurring model systems that exhibit whole-body adaptation to extreme conditions (Bosch et al., 1988; Dietz and Somero, 1992; Krebs and Feder, 1997; Fader et al., 1994; Gehring and Wehner, 1995; Feder et al., 1996) are necessary to understand the evolutionary trends in the development of this character. In recent years, we have performed a systematic broad-scale analysis of the heat-shock response in different organisms inhabiting the dry sand deserts of Middle Asia. This analysis includes species of the unicellular parasite *Leishmania*, various species of silk worm and lizard, and several mammalian species (Evgen'ev et al., 1987; Lyashko et al., 1994; Ulmasov et al., 1992, 1988). In all these organisms, species adapted to high-temperature conditions are able to synthesize HSPs and many other cellular proteins at significantly higher temperatures than related forms from moderate climatic zones. Moreover, various desert thermophilic organisms are typically characterized by a significantly higher cellular content of

HSP70-like proteins at normal physiological temperatures compared with related species from Central and Northern parts of Europe (Gehring and Wehner, 1995; Lyashko et al., 1994; Ulmasov et al., 1992). To gain further insight into the evolution of heat-shock systems in related species differing in thermoresistance, we studied the characteristics of the heat-shock response at the molecular level in several lizard species living in environments with strikingly different average temperatures. Here, we demonstrate that the pattern of constitutive HSE-binding activity (CHBA) and HSF1 interaction observed in desert lizards is similar to that previously described in various thermoresistant mammalian cell cultures constitutively expressing HSP70 (Yang et al., 1996; Mosser et al., 1993).

Many studies, including our own previous work (Bosch et al., 1988; Fader et al., 1994; Gehring and Wehner, 1995; Ulmasov et al., 1992; Lyashko et al., 1994), have shown that species inhabiting hot environments constitutively express more HSP and have a higher thermal threshold for HSP induction than species inhabiting cooler environments (Feder and Hofmann, 1998). The regulatory basis for this difference, however, is poorly understood. Because both HSF1 and CHBA play important roles in regulating HSP expression in model eukaryote species in the laboratory, HSF1 and CHBA emerge as likely candidates to be involved in the process that allows ectothermic species living at diverse temperatures in nature to achieve differing patterns of HSP expression. For this reason, we have investigated HSF1 and CHBA in an array of lizard species from desert and temperate boreal habitats.

Materials and methods

Animals and heat-shock conditions

The following lizard species were used in experiments: the diurnal highly thermoresistant desert species *Phrynocephalus interscapularis* Lichtenstein (family Agamidae); two nocturnal desert species, *Crossobamon eversmanni* Weigmann and *Gymnodactylus caspius* Eischwald (family Gekkonidae), and one diurnal species, *Lacerta vivipara*, common in northern parts of Russia (family Lacertidae). Specimens of the latter species were caught in the suburbs of Moscow, while all other species were collected in a sand desert near Ashkhabad (Middle Asia). The choice of the species for molecular studies was based on a previous investigation in which we demonstrated a distinct correlation between the thermal characteristics of the ecological niche of a species and the level of HSP70 present in its cells under normal physiological conditions (Ulmasov et al., 1992) irrespective of phylogenetic position. *P. interscapularis* and *L. vivipara* exhibit maximal and minimal contents of HSP70, respectively, at normal temperatures, while the two nocturnal species occupy an intermediate position in this respect.

All the lizards used in this study were acclimated for at least 2 weeks at 25 °C before being used in an experiment. In addition, in one series of experiments, we used animals (*L. vivipara* and *P. interscapularis*) obtained from Moscow Zoo, where they had lived for several months. The experiments provided essentially the same results as those performed with lizards caught in the wild. The ecological niches of these species, animal maintenance, thermoresistance and heat-shock conditions are described elsewhere (Cherlin and Muzichenko, 1983).

Preparation of cell extracts and gel mobility-shift assay

Control or heat-shock-treated animals were decapitated, and then frozen and ground to a powder in liquid nitrogen. In the preliminary experiments, we worked with extracts prepared from the liver of the lizard species mentioned. However, since the results of gel mobility-shift analysis performed using liver extracts were similar to those when whole-body extracts were used (data not shown), we routinely used whole-body extracts to minimize wastage of valuable live material. Whole-body extracts were prepared according to Rabindran et al. (1991). Briefly, the frozen powder was suspended (1:5 w/v) in buffer (20 mmol l^{-1} Hepes, pH 7.9, 25 % v/v glycerol, 0.42 mol l^{-1} NaCl, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} EDTA, 0.5 mmol l^{-1} phenylmethylsulfonyl fluoride, PMSF, and 0.5 mmol l^{-1} dithiothreitol) and centrifuged at 100 000 ***g*** for 20 min. The supernatants were frozen in liquid nitrogen and stored at –70 °C. The protein concentration in the extracts was estimated using the modified Lowry method (Lowry et al., 1951; Ulmasov et al., 1992). Consensus HSE probe (Wu et al., 1988) was prepared by annealing partially complementary oligonucleotides (ATCCGAGCGCGCCTCGAATGTTCTA-GAA and CTCGCGCGGAGCTTACAAGATCTTTTCCA) in 10 mmol l^{-1} potassium phosphate buffer, pH 8.2, in the presence of 0.1 mol l^{-1} NaCl. Single-stranded termini were filled with Klenow polymerase and ^{32}P[ATP] (Sambrook et al., 1989). For the gel mobility-shift assay, whole-cell extracts containing 50 μg of protein were mixed with 0.5 ng of ^{32}P-labelled HSE in the binding buffer, as described previously (Mosser et al., 1993). The binding reaction mixture was incubated at room temperature (20 °C) for 20 min, and free probe was then separated from HSE–HSF1 complexes by electrophoresis through 5 % polyacrylamide gels (Mosser et al., 1993). The gels were dried and exposed to X-ray film (Kodak X-Omat) at –70 °C. All operations were performed at 4 °C except when indicated otherwise.

Ultraviolet cross-linking

A ^{32}P-labelled, bromodeoxyuridine (BRDU)-substituted HSE oligonucleotide was used in ultraviolet cross-linking experiments. Cell extracts (50 μg) were mixed with two volumes of the binding buffer and 1 ng of ^{32}P-labelled HSE–BRDU oligonucleotide. After standard gel electrophoresis through a 5 % polyacrylamide gel, both sides of the gel were irradiated in an ultraviolet crosslinker (Stratagene) for 5 min at 3 mW cm^{-2}. The gel was exposed to X-ray film (Kodak X-Omat). Bound probe–HSF1 complexes were cut from the gel and loaded onto a 10 % polyacrylamide gel for separation by SDS–PAGE. The gel was dried and exposed to X-ray film at –70 °C.

Protein labelling, gel electrophoresis and immunoblotting

In vivo labelling of liver proteins with [^{35}S]methionine, SDS–polyacrylamide gel electrophoresis and fluorography were carried out as described previously (Ulmasov et al., 1992). Polyclonal antibodies (PAbs) specific to human HSF1 (N180) were kindly provided by Dr Carl Wu (Bethesda NIH, MA, USA) and were used at a dilution of 1:1000 in western blotting. Monoclonal antibodies specific to human HSC70 (N27) were obtained from Stressgen Corp. and used at a dilution of 1:1500. The immune complexes were detected by chemoluminescence (ECL kit, Amersham) using the corresponding peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies as appropriate.

Preparation of RNA and northern hybridization

RNA was prepared by the standard method using 4 mol l^{-1} guanidine isothiocyanate (Chomezynsky and Sassi, 1987). The poly(A) mRNA fraction was isolated from total RNA by binding to oligo(dT) cellulose (Promega, 1991). Fractionation, blotting and northern hybridization with a *Xenopus laevis* HSP70-containing clone (pxL16P, a kind gift from Dr Meriam Bienz, Medical Research Council Laboratory, Cambridge, UK) were carried out as described elsewhere (Sambrook et al., 1989) with slight modifications. The hybridization took place overnight at 42 °C in 50 % formamide followed by two 20 min washes in 2×SSC (standard saline citrate), 0.2 % SDS at 42 °C, two 20 min washes in 1×SSC, 0.2 % SDS at 42 °C and one 20 min wash in 0.2×SSC, 0.2 % SDS at 50 °C.

Preparation of DNA and Southern blot analysis of genomic DNA

DNA from adult lizards (whole body) was isolated and digested with restriction enzymes. Southern analysis was carried out using standard procedures (Sambrook et al., 1989). Genomic blots were probed with *Xenopus laevis* HSP70-containing clone (pxL16P) under comparatively mild hybridization conditions (at 60 °C in 4×SSC) and subsequently repeatedly washed first in 2×SSC, 0.2 % SDS at room temperature and then at 60 °C in 0.5×SSC, 0.2 % SDS.

Results

Constitutive HSE-binding activity differs characteristically in lizard species of different origin

Phrynocephalus interscapularis and *Lacerta vivipara* represented the two extremes of thermoresistance over a spectrum of nine species compared in a study by Ulmasov et al. (1992). Fig. 1 shows the results of gel mobility-shift analysis of extracts from two desert species (*Gymnodactylus caspius* and *P. interscapularis*) and one northern species (*L. vivipara*). Extracts from all species exhibited specific gel mobility-shift complexes at their normal body temperatures (complexes I, II and III), which disappeared in the presence of excess unlabelled HSE (Fig. 2, lanes 1 and 16). Different complexes, however, are evident under normal non-heat-shock conditions in desert and northern forms (Fig. 1, lanes 1, 3, 7). Thus, all lizard species display a complex with a relatively high electrophoretic mobility (II), most prominently *L. vivipara.* Both *G. caspius* and *P. interscapularis*, however, constitutively display low-mobility bound HSE (I). The former complex may represent partial oligomerization of HSF1 at normal temperature. However, mobility did not increase when anti-HSF1 antibodies were added to such extracts (Fig. 2, lane 3). Interestingly, substantial amounts of complex III, representing HSF1 activated by temperature elevation, occur in both desert species at normal physiological temperatures (Fig. 1, lanes 1, 3). An obvious candidate for constitutive HSE-binding activity in lizards (complexes I and II) is Ku autoantigen (see Introduction). Results from ultraviolet cross-linking experiments are consistent with this supposition. Two components with molecular masses of approximately 70 kDa and 80 kDa, which resemble the masses of the Ku subunits described in mammals (Kim et al., 1995), bound labelled HSE (Fig. 3).

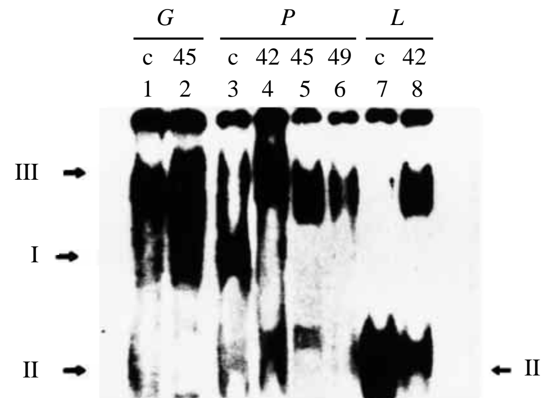

Fig. 1. Analysis of heat-shock element (HSE) binding activity in lizard species from different ecological niches. Gel mobility-shift analysis of whole-cell extracts from control (c) and heat-shocked animals. The extracts analyzed by gel mobility-shift were prepared from *Gymnodactylus caspius* (*G*) control animals (lane 1) and from individuals heated to 45 °C for 60 min (lane 2), from *Phrynocephalus interscapularis* (*P*) control animals (lane 3) and from individuals heated to 42, 45 or 49 °C for 60 min (lanes 4, 5 and 6, respectively), and from *Lacerta vivipara* control animals (lane 7) and from individuals heated to 42 °C for 60 min individuals (lane 8). The locations of the constitutive HSE-binding complexes (I and II) and the heat-shock-induced HSF1–HSE complex (III) are indicated by arrows. Equal amounts of total cellular proteins were loaded per lane. HSF1, heat-shock factor 1.

Inducible HSE-binding activity is similar in contrasting species, but the recovery from heat shock is much faster in southern species

After heat shock, a distinct complex of high molecular mass (III) was evident for all lizard species (Fig. 1, lanes 2, 4–6, 8). The complex is probably due to HSF1 binding because it disappears in the presence of a 200-fold excess of 'cold' HSE (Fig. 2, lanes 9, 16) and its mobility decreases

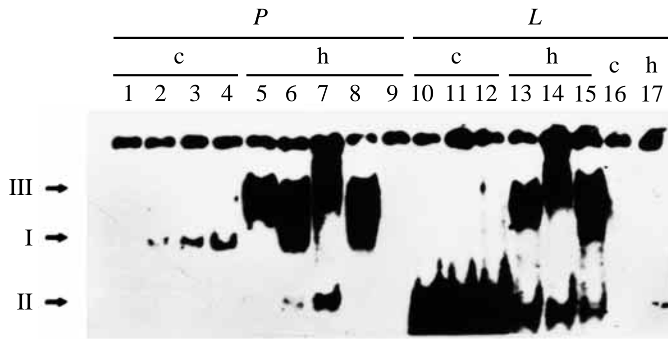

Fig. 2. Analysis of the specificity of heat-shock element (HSE) binding activity by competition experiments and serum-induced supershifts. Competition assays were performed by co-incubating cell extracts with a 200-fold excess of unlabelled HSE oligonucleotides ('cold HSE'). To allow the formation of the HSE–protein complexes in antiserum-induced supershift experiments, the cell extracts were incubated with either anti-HSF1 or anti-HSP70 serum after incubation with labelled HSE. *Phrynocephalus interscapularis* (*P*) (lanes 1–9): lane 1, control (c) plus cold HSE; lane 2, control; lane 3, control plus anti-HSF1 antibodies; lane 4, control plus anti-HSP70 antibodies; lane 5, animals heated to 42 °C for 60 min (h); lane 6, animals heated to 45 °C for 60 min; lane 7, animals heated to 45 °C for 60 min plus anti-HSF1 antibodies; lane 8, animals heated to 45 °C for 60 min plus anti-HSP70 antibodies; lane 9, animals heated to 45 °C for 60 min plus cold HSE. *Lacerta vivipara* (*L*) (lanes 10–17): lane 10, control; lane 11, control plus anti-HSF1 antibodies; lane 12, control plus anti-HSP70 antibodies; lane 13, animals heated to 42 °C for 60 min; lane 14, animals heated to 42 °C for 60 min plus anti-HSF1 antibodies; lane 15 animals heated to 42 °C for 60 min plus anti-HSP70 antibodies; lane 16, control plus cold HSE; lane 17, animals heated to 42 °C for 60 min plus cold HSE. The antiserum-induced supershift observed in lanes 7 and 14 clearly identifies band III as the HSF1–HSE complex. The free ^{32}P-labelled oligonucleotides migrated to the bottom of the gel. HSF1, heat-shock factor 1; HSP, heat-shock protein; I, II, constitutive HSE-binding complexes.

when anti-HSF1 polyclonal antibodies are added to the extracts (Fig. 2, lanes 7, 14). This supershift is probably due to the formation of additional immune complexes between antibodies and HSF1. Complex III apparently represents trimerized (i.e. activated) HSF1 bound to probe. In northern and southern species, complexes of the activated HSF1 with HSE have a similar mobility. In the southern *P. interscapularis*, however, these complexes persist at higher temperatures than in the northern *L. vivipara* (Fig. 1, lane 6). Indeed, temperatures higher than 42 °C usually killed *L. vivipara*. In contrast to the HS-induced active complexes (III) of HSF1–HSE, levels of the constitutive HSE-binding complexes (I and II) decreased with temperature elevation in all species. Interestingly, in *L. vivipara*, the level of complex II decreased significantly after heat shock but it did not disappear altogether as in desert lizards (Fig. 1, lane 8).

To investigate interspecific differences in the constitutive complexes, we performed competition experiments using crude cell extracts from control and heat-treated lizards.

Fig. 3. The mobility of the heat-shock element (HSE)-binding proteins. The proteins in the cell extracts of control *Phrynocephalus interscapularis* not subjected to heat shock were ultraviolet cross-linked to a ^{32}P-labelled bromodeoxyuridine-substituted HSE and developed by autoradiography. The molecular masses of the two main proteins constitutively bound to HSE are shown on the right.

Control extracts were incubated first with ^{32}P-labelled HSE for 15 min (allowing constitutively-bound complexes to form) and then with extract from heat-shocked animals. Addition of extract from heat-treated desert species (*P. interscapularis*) to control extracts from both lizard species (1:1 ratio) almost completely dissociated pre-formed constitutively-bound complexes (Fig. 4, lanes 4–7). In

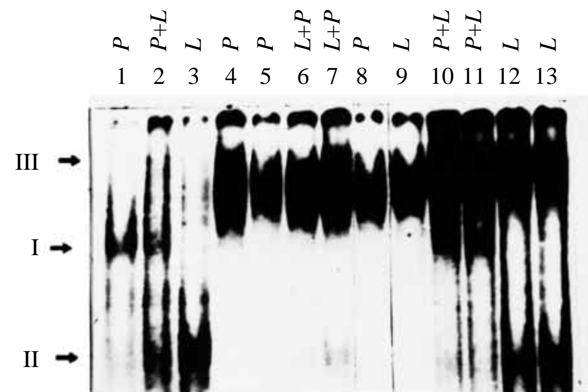

Fig. 4. *In vitro* competition between heat-shock factor 1 (HSF1) and constitutive heat-shock binding activity (CHBA) for heat-shock element (HSE) binding. After incubation of the cell extracts with labelled HSE to allow the formation of HSE–protein complexes, the samples were mixed with fixed amounts of cell extracts from other variants, incubated for an additional 20 min and subjected to gel mobility-shift analysis. Lane 1, *Phrynocephalus interscapularis* (*P*) control; lane 2, *P. interscapularis* control plus *Lacerta vivipara* (*L*) control (1:1 ratio); lane 3, *L. vivipara* control; lane 4, *P. interscapularis* control plus *P. interscapularis* heat-shocked at 45 °C for 60 min (1:1 ratio); lane 5, *P. interscapularis* control plus *P. interscapularis* heat-shocked at 45 °C for 60 min (2:1 ratio); lanes 6 and 7, *L. vivipara* control plus *P. interscapularis* heat-shocked at 45 °C for 60 min (1:1 ratio; lane 6) and (2:1 ratio, lane 7); lane 8, *P. interscapularis* heat-shocked at 45 °C for 60 min; lane 9, *L. vivipara* heat-shocked at 42 °C for 60 min; lanes 10 and 11, *P. interscapularis* control plus *L. vivipara* heat-shocked at 42 °C for 60 min (1:1 ratio; lane 10) and (2:1 ratio, lane 11); lanes 12 and 13, *L. vivipara* control plus *L. vivipara* heat-shocked at 42 °C for 60 min (1:1 ratio; lane 12) and (2:1 ratio; lane 13). Equal amounts of total cellular proteins were loaded per lane. I, II, constitutive HSE-binding complexes.; III, HSF1–HSE complex.

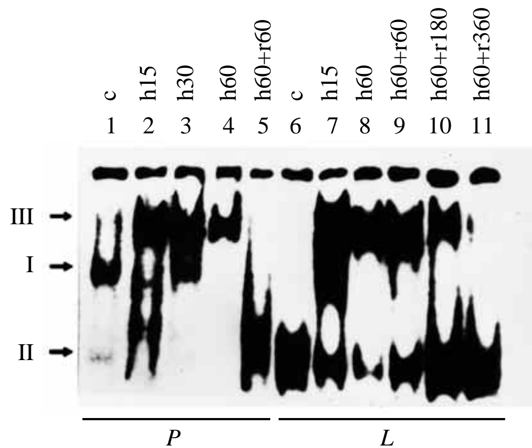

Fig. 5. Analysis of heat-shock element (HSE)-binding activity in lizards recovering from heat shock. Whole-cell extracts for gel mobility-shift analysis were prepared from the animals heat-shocked at 42 °C for 15 min, 30 min or 60 min and from individuals heat-shocked for 60 min and then returned to 25 °C for recovery periods of 60–360 min. Lanes 1 and 6, control *Phrynocephalus interscapularis* (*P*) (lane 1) and *Lacerta vivipara* (*L*) (lane 6) receiving no heat-shock treatment (c); lanes 2, 3 and 4, *P. interscapularis* heat-shocked (h) at 42 °C for 15, 30 and 60 min, respectively; lane 5, *P. interscapularis* heat-shocked at 42 °C for 60 min after 60 min of recovery (h+r); lanes 7 and 8, *L. vivipara* heat-shocked at 42 °C for 15 and 60 min, respectively; lanes 9, 10 and 11, *L. vivipara* heat-shocked at 42 °C for 60 min after 60, 180 and 360 min of recovery, respectively. The locations of the constitutive HSE-binding complexes (heat-shock binding activity, CHBA) and the heat-shock-induced HSF1–HSE complex are indicated at the left of the autoradiogram. HSF1, heat-shock factor 1. I, II, constitutive HSE-binding complexes.; III, HSF1–HSE complex.

contrast, extracts from heat-shocked *L. vivipara* cells (containing activated HSF1) did not eliminate constitutively-bound complexes in either species (Fig. 4, lanes 10–13), although they did compete for HSE binding. These results indicate that the HSF1 of the desert-dwelling species (*P. interscapularis*) has a higher binding affinity for HSE than the HSF1 of *L. vivipara*. We also performed recovery experiments (Fig. 5). After a similar heat shock, cells of *P. interscapularis* returned to their pre-existing pattern of constitutively-bound HSE complexes more rapidly (within an hour) than did cells from *L. vivipara*, which required at least 6 h (Fig. 4, lanes 5 and 11 respectively).

Constitutive expression of HSP70 and HSF1 genes in lizards

The cells of *L. vivipara* contain a higher content of constitutive HSE-binding factor II than the cells of either of the desert-dwelling species (Fig. 1), while the cells of the desert species contain significant amounts of HSF1 in activated form (complex III) even under normal physiological conditions. These differences correspond to a significantly higher (a three- to fivefold difference) HSP70 protein (Ulmasov et al., 1992) and HSP70 mRNA content in the desert species than in *L. vivipara* under non-heat-shock conditions. Fig. 6A shows the results of northern analysis of HSF1 and HSP70 mRNA in two contrasting species. It is evident that the most thermoresistant desert species (*P. interscapularis*) at normal and elevated temperatures (Fig. 6A, lanes 1, 3) is characterized by a significantly lower level of HSF1 mRNA than the thermosensitive form (*L. vivipara*) (Fig. 6A, lanes 2, 4). In contrast, a similar analysis performed using labelled HSP70 probe demonstrates that, in the cells of *P.*

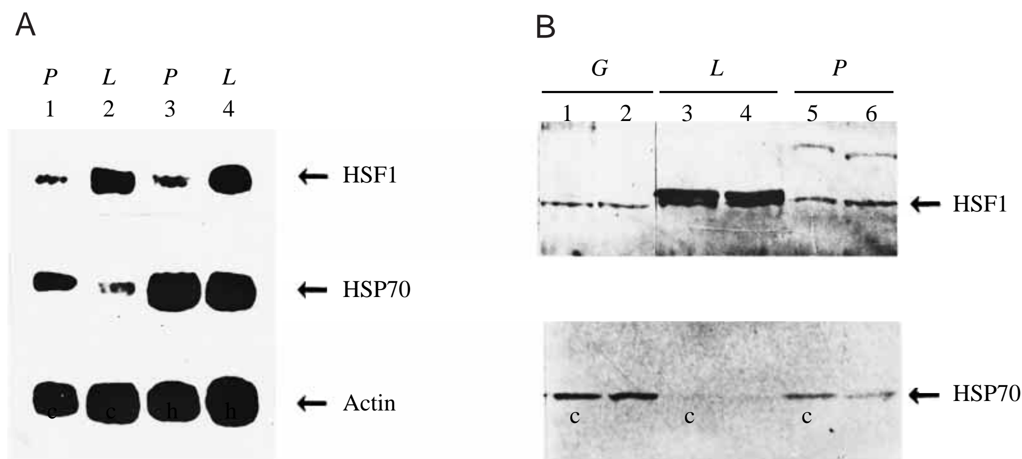

Fig. 6. Expression of HSP70 and HSF1 in different lizard species. (A) Northern analysis of RNA present in the cells of *Phrynocephalus interscapularis* (*P*) and *Lacerta vivipara* (*L*) at normal physiological temperature (c) (25 °C) (lanes 1, 2, respectively) and after heat-shock treatment (h) for 1 h at 42 °C (lanes 3, 4, respectively). Equal amounts of poly(A+) RNA were loaded per lane, size-fractionated on an agarose gel, transferred to ECL Hybond-N membrane (Amersham) and probed with lizard (*L. vivipara*) HSF1 gene and *Xenopus laevis* HSP gene, and reprobed with an αβ-actin gene. HSP70, HSF1 and actin are indicated by arrows. HSF1, heat-shock factor 1; HSP, heat-shock protein. (B) Western blot analysis of HSF1 and HSP70 levels in *Gymnodactilus caspius* (*G*) (lanes 1 and 2), *L. vivipara* (lanes 3 and 4) and *P. interscapularis* (lanes 5 and 6) at normal temperature and after heat shock. Lanes 1, 3 and 5, unheated control animals; lanes 2, 4 and 6, individuals treated to a 60 min heat shock at 42 °C. Total proteins (50 μg) were analyzed by SDS–PAGE and transferred to ECL Hybond-N membranes. The membranes were processed either with human polyclonal antibodies against HSF1 or with monoclonal antibodies against a constitutive member of human the HSP70 family. HSF1, heat-shock factor 1; HSP, heat-shock protein.

interscapularis, the constitutive level of HSP70 mRNA synthesis is significantly higher than in *L. vivipara* (Fig. 6A, lanes 1, 2). Both species show strong induction of HSP mRNA upon heat shock (Fig. 6A, lanes 3, 4).

Total HSF1 content, as determined by immunoblotting, differed among species (Fig. 6B). For *P. interscapularis* and *G. caspius*, a single band of immunoprecipitate corresponds to a 75 kDa HSF1 unit (Fig. 6B, lanes 1, 2, 5, 6). A band of similar molecular mass is present in *L. vivipara*, but a second band with a molecular mass of approximately 77 kDa is also evident (Fig. 6B, lanes 3, 4). Whether the two bands observed in *L. vivipara* represent different individual proteins encoded by different HSF1 genes or are post-translational modifications of the same polypeptide is unclear. No species showed an increase in the quantity of HSF1 at higher temperatures (Fig. 6B, lanes 2, 4, 6). Thus, the HSF1 genes in lizards, in contrast to the HSP genes, are apparently not induced by heat shock. Western analysis of the same blot using antibodies to constitutive human HSP70 proteins have shown that both desert species are characterized by significantly higher cellular levels of constitutive HSP70 family members at both temperatures than *L. vivipara.*

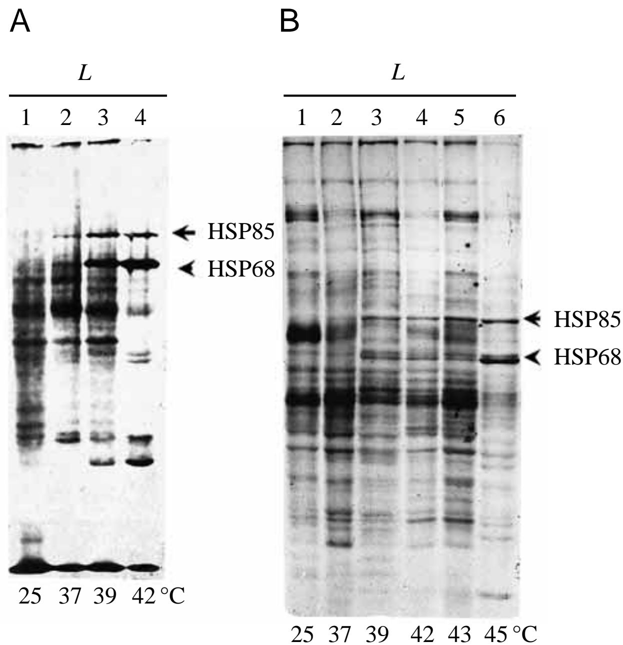

Fig. 7. The pattern of proteins synthesized in *Lacerta vivipara* (*L*) (A) and in *Crossobamon everesmanni* (*C*) (B) at different temperatures. Lane 1, 25 °C (controls); lane 2, 37 °C; lane 3, 39 °C; lane 4, 42 °C; lane 5, 43 °C; lane 6, 45 °C. The lizards of both species were heat-shocked at different temperatures for 1 h and labelled *in vivo* with [^{35}S]methionine for a further 1 h at 25 °C. The proteins were extracted from the liver and run on SDS–PAGE as described in Materials and methods. Equal amounts of protein were analyzed. Arrows indicate the positions of the major heat-shock proteins (HSP).

Different kinetics of HSP induction in lizards with different constitutive level of HSP70

The northern species *L. vivipara* and the nocturnal southern species *Crossobamon everesmanni* differ (by a factor of 2–3) in HSP70 content (Ulmasov et al., 1992) under normal conditions but do not differ strikingly in thermoresistance. Upon moderate heat shock (e.g. at 39 and 42 °C), the liver cells of *L. vivipara* synthesized much higher levels of HSPs than those of *C. everesmanni* (Fig. 7A,B). In the desert species, moreover, such moderate heat shock resulted in little suppression of the synthesis of other cellular proteins (Fig. 7B, lanes 2–5), which occurs only after severe heat shock (Fig. 7B, lane 6). This pattern of heat-shock response is typical of all desert lizards studied so far, including *P. interscapularis* (data not shown).

Lizards species with differing thermoresistance do not differ significantly in HSP70 gene copy number

Southern blot analysis using pertinent labelled clones as probes suggests that interspecific differences in HSP expression are not due to differing copy numbers of the corresponding genes. Genomic DNA from the two contrasting species hybridizes essentially identically to the HSP70 probe used, suggesting similar organization and the same copy number of these genes (Fig. 8).

Discussion

Heat-shock gene expression is tightly regulated, to ensure

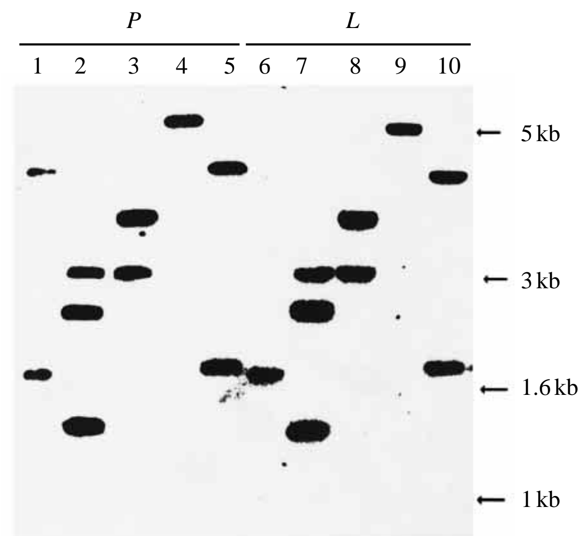

Fig. 8. Southern blot analysis of genomic DNA from *Phrynocephalus interscapularis* (*P*) (lanes 1–5) and *Lacerta vivipara* (*L*) (lanes 6–10). Total lizard DNA (10 μg) was digested with *Eco*RI (lanes 1 and 6), *Hin*dIII (lanes 2 and 7), *Pst*I (lanes 3 and 8), *Bam*HI (lanes 4 and 9) or *Eco*RV (lanes 5 and 10) and subjected to Southern blot analysis using *Xenopus laevis* HSP70-containing clone as a labelled probe. HSP, heat-shock protein.

that the response is proportional to the level of heat stress, and then repressed and terminated when normal physiological conditions recur (Lindquist, 1986). The lower and upper limits of the heat-shock response are genetically determined and evidently species-specific (Evgen'ev et al., 1987; Ulmasov et al., 1988; Gehring and Wehner, 1995; Feder and Hofmann, 1998). Experiments with yeast, *Drosophila melanogaster* and mammalian cells have provided evidence that HSF1 is the key regulatory protein in the heat-shock response (Wu, 1995) and that DNA-binding activity of mammalian HSF1 is partly regulated *in vivo* by HSP70 (Shi et al., 1998; Kim et al., 1995; Mosser et al., 1993). Experiments with extracts of control and heat-shocked mammalian cells demonstrated that the cells contain two different heat-shock element binding factors: in addition to HSF1, there appeared to be a constitutive HSE-binding factor (CHBF), which is identical to the Ku autoantigen (Liu et al., 1993; Kim et al., 1995; Mosser et al., 1993). Upon temperature elevation, the heat-induced decrease in CHBF–HSE binding activity correlates well with the increase in HSF1–HSE binding activity (Kim et al., 1995). During post-heat-shock recovery, HSF1–HSE binding activity decreases over time while CHBF–HSE binding activities recovers gradually (Kim et al., 1995). The constitutive expression of mammalian HSP70 genes and elevated expression of CHBF decreases HSF1 binding (Yang et al., 1996; Mosser et al., 1993) in the cell cultures. The inverse correlation between HSE binding activity of HSF1 and CHBF and their different interdependence on HSP70 level probably reflects the involvement of both proteins in the precise regulation of heat-shock gene expression, the former as a positive and the latter as a negative regulator (Yang et al., 1996).

All the data on the heat-shock system regulation mentioned above were accumulated when studying different cell cultures transfected with plasmids containing various heat-shock-related genes (HSP70, HSF1 or Ku 70) under the control of different promoters (Kim et al., 1995; Yang et al., 1996; Shi et al., 1998; Mosser et al., 1995). We still know very little, however, about the evolution of the heat-shock system at the molecular level that occurs in nature in organisms inhabiting strikingly different thermal environments. The absence of HSP synthesis induction in *Hydra oligactis* inhabiting deep cold water (Bosch et al., 1988) and the high constitutive level of HSP synthesis in various desert animals (Gehring and Wehner, 1995; Evgen'ev et al., 1987; Ulmasov et al., 1992) represent extreme examples of such evolution of thermoresistance. To understand the general trends and strategy in the evolution of the heat-shock gene system, we have focused our study on the peculiarities of the heat-shock response in several lizard species inhabiting drastically different environments and exhibiting various levels of thermoresistance (Ulmasov et al., 1992).

The major findings of the present study are as follows. First, the high content of HSP70 observed in desert species under normal non-heat-shock conditions is essentially regulated at the transcriptional level: (i) northern hybridization revealed a significantly higher content of HSP70 mRNA in the cells of desert forms under normal physiological conditions and (ii) desert forms have a detectable level of activated HSF1 bound to HSE (complex III) at normal physiological temperatures. Second, desert- and northern-dwelling animals differ characteristically in the quantity and state of HSF1 and CHBA both under normal and heat-shock conditions (since we have no data other than a size similarity proving that we are dealing with a lizard homologue of CHBF, we prefer to use the term 'CHBA' in our work): (i) northern and western blot analysis demonstrated significantly higher levels of HSF1 and the corresponding mRNA in the cells of northern thermosensitive species, (ii) gel mobility-shift experiments revealed less CHBA (a presumptive negative regulator of HSP genes) in extracts from desert animals under normal non-heat-shock conditions; (iii) during post-heat-shock recovery at 25 °C, activated HSF1 disappeared within 1 h and CHBA recovered with similar kinetics (1 h) in desert species, whereas northern species showed much slower HSF1 disappearance and CHBA recovery over approximately 6 h, and (iv) characteristically, a certain level of CHBA is present in extracts of northern species even after severe heat-shock treatment, whereas in desert forms CHBA binding after severe heat shock is completely substituted by HSF1 binding. This phenomenon is probably due to the higher affinity of desert lizard HSF1 to HSE, which was demonstrated in the present study in competition experiments. We assume that we are dealing with HSF1 in our studies; however, the existence of multiple forms of HSF in lizards is not excluded.

Generalizing from interspecific comparisons may be rather problematic (Garland and Adolph, 1994), particularly given that the species compared belong to phylogenetically distant groups. However, the distinctive behaviour of the HSPs (HSF1 and CHBA) described here was observed in all desert species studied irrespective of their phylogenetic affinities. Moreover, the correlations established closely resemble those demonstrated in cell cultures constitutively overexpressing HSP70 (Liu et al., 1993; Kim et al., 1995; Mosser et al., 1993). The differences in HSP70 level observed here cannot be attributed to a higher affinity of the antibodies used against desert lizard HSP70 because essentially the same pattern was previously demonstrated using different polyclonal antibodies raised against calf HSP73 (Ulmasov et al., 1992). One may speculate, therefore, that the constitutive expression of HSP70 in desert lizards, regulated as described above, represents an important physiological adaptation to the extreme conditions of hot and dry Middle Asia deserts and may occur universally.

In desert lizards, normal protein synthesis continues at temperatures up to 45 °C, whereas in northern lizards it is completely abolished at temperatures above 40 °C. The constitutive high HSP content of desert animals may facilitate life under extreme conditions without also requiring thermostable proteins. The presence of high constitutive levels of HSP in desert forms appears to shift the temperature threshold of HSP induction, which starts and finishes at temperatures 3–7 °C higher than in northern forms and occurs with slower kinetics. In contrast, low constitutive levels of

HSPs and relatively high levels of HSF1 in the cells of northern animals may ensure fast and intense synthesis of HSPs even after brief exposure to moderate heat shock. Further elevation of temperature (severe heat shock), easily tolerated by desert thermophilic forms, is usually lethal for northern animals. The northern forms probably never (or rarely) encounter such large thermal shifts in their normal environment and have not evolved a response to them. According to Southern analysis, differences in HSP70 protein levels in lizards from different ecological niches are not due to amplification of pertinent genes in desert forms, as occurs for antifreeze genes in some cold-resistant fish species (Hew et al., 1988). Whether thermophilic species in general differ characteristically in terms of structural and/or regulatory regions of HSP70-, HSF1- and CHBF-coding genes will be of great interest.

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